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NUCLEAR MAGNETIC RESONANCE STUDY OF ANTIGEN-ANTIBODY
COMPLEXES, INCLUDING SEQUENCE SPECIFIC ASSIGNMENTS AND
STRUCTURAL ANALYSIS OF NEUROPHYSIN AS AN ANTIGEN MODEL

by

ELISAR JAMIL BARBAR

A dissertation submitted in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY
in
ENVIRONMENTAL SCIENCES AND RESOURCES:
CHEMISTRY

Portland State University
1993

TO THE OFFICE OF GRADUATE STUDIES

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AN ABSTRACT OF THE DISSERTATION OF Elisar Jamil Barbar for the Doctor of
Philosophy in Environmental Sciences and Resources: Chemistry presented May 07, 1993.

Title: Nuclear Magnetic Resonance Study of Antigen-Antibody Complexes, Including
Sequence Specific Assignments and Structural Analysis of Neurophysin as an
Antigen Model.

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The interaction between molecules is essential in a wide range of biological
processes. A detailed knowledge of these interactions is necessary for understanding these
processes. Among the systems that involve important interactions is the immune system.

NMR spectroscopy has a large number of spectral parameters that were used in this work to study antibody-antigen interactions. These same parameters were also used to begin a structural analysis of a medium-sized protein, neurophysin, that has important interactions with neurohormones, and served here as a model antigen.

A set of ligands differing in size and charge was designed and used to probe the binding site of anti-phosphocholine antibodies. These ligands ranged from small organic species to medium sized proteins. Amino acids, peptides and proteins were homogeneously linked to phenyl phosphocholine and analyzed by NMR techniques. Transferred nuclear Overhauser effect measurements were used to determine the conformation of bound ligands. The conformational change observed in some ligands was explained as either due to the antibody selecting one conformation that already exists, or the antibody binding inducing a conformational change. Titration data and detailed NMR analysis showed a more rigid M3C65 antibody fragment upon binding. In summary, with eight examples of ligands and four examples of antibodies studied by NMR, a spectrum of effects was seen, including a lock-and-key model and limited local induced fit. The contribution of the carrier molecule to antibody binding was in restricting the conformation of the ligand. Bigger ligands that are expected to be more immunogenic, showed less binding avidity as determined by immunological assays. Fluorinated ligands were synthesized to determine the kinetics of binding using ^{19}F NMR spectra. Higher concentration of a fragment of the antibody M3C65 was analyzed to determine assignments of some residues in the combining site of the antibody.

High resolution NMR techniques were used to assign resonances in neurophysin. The physiological role of neurophysin includes hormone storage and stabilization of oxytocin and vasopressin against proteolytic degradation within the posterior pituitary. Neurophysin is a 10 KD protein that dimerizes at high concentrations needed for NMR studies. An organic cosolvent was used to lower the dimerization constant, and hence

increase the spectral resolution. This permitted sequence-specific assignments that were then used to identify residues in the neurophysin-hormone binding site. Chemical shift differences and conformational changes were observed for the residues glutamate 47 and leucine 50. The 3_{10} helix was further stabilized towards a more ideal helix upon hormone-analog peptide binding. Some of the residues contributing to the monomer-monomer interface were also assigned. Dimerization induced chemical shift differences and conformational changes were observed for phenylalanine 35, threonine 38, and alanine 69. Tyrosine 49 and phenylalanine 22 were affected but to a lesser extent. One characteristic of neurophysin in all studied cases was dynamic equilibrium between different folding states.

To my beloved parents, Helen and Jamil Barbar
who showed me a perfect example of perseverance, honesty, generosity, sacrifice and
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ABBREVIATIONS AND DEFINITIONS

NMR, nuclear magnetic resonance; **2D**, two-dimensional;

NOE, nuclear Overhauser effect (through space interactions);

TRNOE, transferred NOE;

COSY, 2D J correlated spectroscopy (through bond interactions);

TOCSY, 2D total correlation spectroscopy;

ROESY, rotating frame Overhauser effect spectroscopy; |

PC, phosphocholine;

BSA, bovine serum albumin;

CDR, complementarity determining region;

VH, variable region gene - heavy chain;

VL, variable region gene - light chain;

Fv, antibody fragment that is made of the variable region of the light chain and the variable region of the heavy chain and has one combining site for an antigen;

SFv, single chain Fv;

Fab, antibody fragment that is made of the F_v, the light chain constant region and the first constant region of the heavy chain;

Immunogen, a molecule that generates immune response;

Hapten, a small molecule attached to macromolecules before immunization;

Epitope, the specific portion of the antigen that binds to the antibody.

PC-KLH, phosphocholine-keyhole limpet hemocyanin, the oxygen carrying pigment of this marine gastropod;

ELISA, Enzyme-linked immunosorbent assay |

CHAPTER I

GENERAL INTRODUCTION

The interaction between small molecules and large molecules is essential in a wide range of biological processes. The small molecules may be drugs, environmental chemicals, hormones, and inhibitors. The large molecules include proteins such as enzymes, transport proteins, and antibodies. A detailed knowledge of these interactions is necessary for the understanding of biological systems. Among the systems that involve important interactions is the topic of this document, the immune system. NMR spectroscopy has a large number of spectral parameters that can be used in understanding chemical and biological systems. NMR spectroscopy was used in this research to study antibody-antigen interactions, as well as to begin a structural analysis of a medium-sized protein, neurophysin, that has important interactions with neurohormones, and serves here as a model antigen.

ANTIBODY FUNCTION AND STRUCTURE

Antibody Function

Specific immune responses are normally stimulated when an individual is exposed to foreign substances called antigens. One type of specific immune response is humoral immunity, which is mediated by B lymphocytes. This type of lymphocytes respond to antigens by developing into antibody-producing cells. Antibodies (immunoglobulins) are protein molecules in the blood that are responsible for specific recognition and subsequent elimination of antigens. Humoral immunity is the principle defense mechanism against extracellular microbes and their secreted toxins. This protects the host by a variety of mechanisms (Abbas et al., 1991). Some of these mechanisms are discussed below. One

mechanism is virus neutralization. Antibodies made against viral antigens bind to the virus particles and thus prevent them from infecting target cells. Some antibodies do not directly neutralize virus infectivity, but inhibit the enzyme that is coded by the viral genome and thus terminate the infection (Air et al., 1989). An example of such an enzyme is influenza virus neuraminidase that allows virus to penetrate and infect the cell. Another mechanism is opsonization, in which antibody molecules bind to and coat antigenic particles, and so enhance the efficiency of phagocytosis. Phagocytosis occurs when the antigen-antibody complex binds to the Fc receptors on the phagocyte. Fc receptors are specific for the Fc portion of the antibody molecules. The third mechanism is lysis of cells. In this mechanism, an antibody binds to antigens on target cells. The antigen-antibody complex on the target cells activates the complement system which generates its lytic activity, resulting in lysis of the cells .

The other type of immune lymphocytes is the T cell, which produces another class of antigen receptor molecules. T cell receptors do not circulate in the bloodstream but remain cell-bound, hence T cell mediated immunity is referred to as “cellular immunity”. T cells destroy tissue cells that are infected with viruses, tumor cells, etc. T cell receptors have structural similarities to antibodies. Thus B and T lymphocytes express membrane receptors that distinguish between foreign antigens. The first part of the dissertation will focus exclusively on the antigen-binding sites of antibodies.

Structural Background

The structure of the antibody and its fragments are illustrated in Figure 1. Antibodies are multivalent molecules made up of light chains 220 amino acid residues long, and heavy chains 450-575 amino acids long. The light chains are made of two immunoglobulin domains: an N-terminal domain that varies from one antibody to another, and a relatively constant C-terminal domain. The heavy chains contain a variable N-

terminal domain and three or four constant domains. The antibody fragment containing the variable, V, domains of both a heavy chain and a light chain is called the Fv, and maintains its antigen binding properties after being cleaved from the remainder of the protein. The fragment that contains the entire light chain and the N-terminal variable and one constant domain of the heavy chain is termed the Fab. In all antibody molecules, each light chain is attached to a heavy chain, and each heavy chain pairs with another heavy chain, by covalent interactions in the form of disulfide bonds and by hydrophobic interactions.

Within the V-region for each chain, there are segments that show extreme variability of amino acid composition. These are termed hypervariable regions. There are three hypervariable regions in each chain, held in place by the more conserved framework regions or FRs. The FRs consist of polypeptides that are arranged in β -pleated sheets connected by disulfide bonds. The hypervariable regions fold to form the combining site of the antibody (Wu and Kabat, 1970) as shown in Figure 2. These six hypervariable segments are referred to as the complementarity determining regions, or CDRs. These regions are each about 10-20 amino acids long. They contribute to the formation of a cavity in which the antigen binds. The antibody does not require the use of all six CDRs to bind effectively with the antigen, and the FR residues might contribute to binding as shown from the crystal structures of neuraminidase-antibody complexes (Tulip et al., 1989). As will be discussed later, the hypervariability of the CDRs arises both from inherited germline diversity (gene usage) and somatic mutation (point mutations).

Features of the Immune Response

The immune response exhibits three characteristic features: specificity, diversity, and maturation. The reason for the specificity for distinct protein and polysaccharide antigens is the complementarity of the antibody combining site structure to that of the

antigenic determinant. The lymphocyte repertoire, the total number of antigenic specificity, is extremely large. At least 10^9 different antigenic determinants can in principle be distinguished (Abbas et al., 1991). This number is a reflection of all the B cell clones capable of immunoglobulin (Ig) synthesis and secretion in response to antigenic stimulus.

B lymphocytes change from a stem cell in the bone marrow to a mature cell capable of antibody secretion after exposure to antigen. Once the mature B cells are activated by antigen, they proliferate and differentiate to secrete antibodies. Proliferation is the expansion of the clones of lymphocytes specific for the stimulating antigen. Other activated B cells differentiate to become memory B cells that survive for prolonged periods. The stimulation of memory B cells by antigen leads to the secondary immune response. Stimulated memory B cells produce antibodies that bind antigens with generally higher affinities than unstimulated B cells (Berek and Milstein, 1987). Figure 3 is a schematic diagram adapted from Campbell (1991) that shows maturation of the immune response.

Antibody-Antigen Interactions

The components of the free energy involved in antibody-antigen interactions include hydrophobic, electrostatic, van der Waals, and hydrogen bonding forces. The topography of the charged residues and the steric fit in antigen-antibody complexes are crucial for successful binding. Hydrophobic stabilization is regarded as the energy gained from transferring an amino acid side chain from water to a nonpolar solvent. The entropically favored removal of the bound water molecules thus stabilizes the polar side-chain positions of the free antigen. The energy due to the hydrophobic effect is directly proportional to the solvent-accessible surface of the side chains (Chothia, 1974). As an example, one square angstrom of some buried residues corresponds to 104.5 J of

hydrophobic stabilization (Novotny et al., 1989). In forming electrostatic interactions and hydrogen bonds, the interactions that surface atoms have with water are replaced with protein-protein interactions.

The entropic contributions upon binding involve changes in solvent entropy, loss of bond configurational entropy due to the formation of a compact structure, and the decrease in molecular translational entropy when the complex is formed (Novotny et al., 1989). There is loss of side-chain conformational entropy at the interface surface or region as the side-chains are forced to adopt the single conformation required in the complex. Also as the complex is formed, the system becomes more ordered and leads to a loss in translational-rotational entropy.

The reason for the specificity of an antibody for its antigen has attracted much attention. According to the lock-and-key hypothesis, proposed by Emil Fisher in 1894, the specificity arises from geometrically complementary shapes as a key fits in a lock. The induced-fit mechanism proposes a flexible initial interaction, that induces a conformational change in the antibody molecules upon antigen binding (Karush, 1952). To distinguish between these alternatives, the conformation of the free antibody should be compared to its conformation in the bound complex. The conformation of the variable light and heavy chains (VL-VH) dimer is largely based on noncovalent interaction of hydrophobic residues and hydrogen bonding especially in the β -sheet geometry, as shown in Figure 2. This conformation is found to be conserved among immunoglobulins (Novotny and Haber, 1985; Chothia et al., 1985). Analysis of crystallographic data of six structures of the bound and free combining sites showed no structural differences, indicating that the interfacial residues and the β -barrel are preserved (Novotny et al., 1989). Since antigen and antibody proteins are stable in solution, large conformational changes during complex formation are unlikely, and antibodies do not need to unfold in order to bind (Getzoff et al., 1987). However, both antibody and antigen may undergo local side-chain adjustments

such as rotations of aromatic rings and displacements in the hypervariable regions to improve the affinity and reduce any gaps at the interface. These subtle changes may be important for complex stabilization.

In most antibody-antigen reactions, the dissociation rate is very important in determining the affinity, because it can vary over 8-9 orders of magnitude and is sensitive to environmental conditions such as pH and temperature (Campbell, 1984). Detection of interaction is dependent on concentration and assay conditions.

Hapten-Carrier Conjugation as Antigens

The portion of the antigen that comes into contact with the antibody constitutes an antigenic determinant or epitope of the antigen. Regions on unfolded or denatured proteins that bind antibodies are not considered to be epitopes. Most of the epitopes characterized by X-ray crystallography are made up of several short amino acid sequences close together in the three-dimensional structure, but may be widely separated in the primary sequence. Each epitope contains between 15 and 22 residues on the antigen which contacts a similar number of residues on the antibody (Laver et al., 1990). The contact residues of the antibody and the antigen contribute to binding and must be complementary to each other.

In order to produce antibodies against amino acid- and small peptide-phosphocholine (PC) analogs, it is necessary to couple them to protein carriers to enhance their immunogenicity (Butler and Beiser, 1973). Several factors govern the kind of linkage obtained, including protein concentration, ratio of coupling agent to protein, ionic strength, and pH (VanRegenmortel et al., 1988). Keyhole-limpet hemocyanin (KLH) is a very common carrier protein because it is very immunogenic. In addition to KLH, PC has been conjugated to ovalbumin, bovine serum albumin and Limulus hemocyanin (Feeney

and Mosier, 1984; Wicker et al, 1983; Huang et al., 1988). In all these couplings, the type of the carrier protein is often tacitly considered to be irrelevant.

ENVIRONMENTAL SIGNIFICANCE

The immune system in acting as a shield against disease is affected by environmental chemicals. Exposure to these environmental toxins can result in increased susceptibility to disease as will be shown below. At the same time the properties of the immune response can be exploited to be used as detoxifying agents, drug analogs, and catalysts.

Immunotoxicity Of Selected Environmental Chemicals

Advances in civilization have resulted in large number of synthetic chemicals in the environment. Environmental chemicals and drugs can affect the immune system at extremely low levels of exposure. Some of the types of undesirable effects of exposure to immunotoxicants are immunosuppression, allergy, and autoimmunity. Immunosuppression results when chemicals depress the immune response, and hence lower the natural resistance of the individual to disease. Allergic sensitization is caused when chemicals bind with host proteins and elicit an immune response. Autoimmunity is caused when chemical damage to certain organs or tissues alters the structure of the tissue, and causes production of antibodies against body proteins.

Several chemicals have been shown to cause immunosuppression. Benzene can be processed to produce hydroquinone that concentrates in the bone marrow and blocks the final stages of B cell differentiation (King et al., 1987). The suppression of B cell differentiation may lead to carcinogenesis. Studies in benzene-exposed rabbits showed increased susceptibility to tuberculosis and pneumonia, as well as reduced antibody response to bacterial antigens. Similar antibody suppression was observed after exposure

to halogenated aromatic hydrocarbons (Kerkvliet, 1984), in which both B cell and T cell mediated immune responses were altered. Organophosphate insecticides and pesticides (e.g., methyl parathion) were shown to be immunosuppressive in certain animal species by reducing humoral responses (Street, 1981).

Airborne pollutants such as airborne nickel and cadmium species were shown to alter alveolar macrophage function and depress primary humoral immunity (Graham et al., 1979). Inhalation exposure to gaseous pollutants such as ozone, asbestos, oxidant gases, and tobacco smoke has been shown to produce reduced susceptibility to bacterial challenge due to impairment of phagocytic, enzymatic, and bactericidal activity in alveolar macrophages (Gardner, 1984). Systemic metal exposure may also affect the immune response and suppress host resistance to infectious agents and tumors (Koller, 1979). Some examples of this include lead and cadmium, which appear to inhibit the development of antibody-producing cells, and thus reduce serum antibody titers. This effect may be due to interference with antigen presentation to lymphocytes (Blakley et al., 1980). Exposure to organic or inorganic mercury results in significantly depressed primary antibody responses (Koller et al., 1977). Exposure to methyl mercury at very low concentration resulted in suppression of antibody titers in rabbits due to reduction in primary and secondary antibody production. Exposure to nickel alters resistance to viruses and bacteria (Adkins et al., 1979) and suppresses macrophage activity, hence it decreases resistance to tumor cell formation. The mechanism for immunosuppression caused by metals could also involve changes in DNA which would be reflected in changes in the antibody synthesis.

Antibodies As Chemical Agents

The specificity of antibodies may be used for a variety of practical applications. Antibodies can be produced against drugs to act as artificial receptors. If the antigen is

linked to a drug molecule, antibodies raised against this antigen can mimic the binding site of the receptor for the drug. Using monoclonal antibodies as artificial receptors is very useful in the discovery of compounds for pharmaceutical and related purposes (Cook and Drayer, 1988). Antibodies can be used as catalysts capable of carrying out many different transformations when raised against transition state analogs (Schultz, 1988). Another practical potential application for antibodies is the removal of toxins from the environment. Antibodies were successfully produced in transgenic tobacco plants by transfecting plants with either the heavy-chain or the light chain genes. These plants were then crossed to give progeny that expressed complete antibody molecules. Since antibodies can be raised against toxins, it might be feasible to express anti-toxin antibodies in trees that could both trap and degrade environmental toxins (Mayforth and Quintans, 1990).

Our understanding of the molecular basis of antibody specificity may enable the future tailoring of antibodies with improved functions for uses as drugs, diagnostic reagents, and toxin traps. Moreover, because exposure to chemicals alters the immune system, a better understanding of the immune response helps to make the study of immunotoxicity useful in the routine safety evaluations of chemicals and drugs under development.

OVERVIEW OF 3D PROTEIN STRUCTURE DETERMINATION

NMR spectroscopy is the leading technique for obtaining structural and dynamic information about proteins in solution (Wüthrich, 1986). NMR is the only approach, besides diffraction techniques, that is available for detailed protein structure determination. NMR offers the additional opportunity to study dynamic aspects of antibody-antigen complexes. In addition to combining site structure determination, NMR solution-state studies can give reliable binding data (Goetz and Richards, 1977). Most of the

immunochemical techniques for measuring binding constants involve solid phase binding assays. Binding a protein to a solid phase may cause denaturation, and hence loss in enzymatic activity (Berkowitz and Webert, 1981). This would lead to selection of antibodies which bind to epitopes not available in the native protein. To test whether antibodies truly recognize the native form of the protein, it is advantageous to test the antibodies at least for binding by a solution state method such as NMR, that keeps the original form of the protein.

NMR analysis may be compared with crystallography methods for determining the structure in solution state. In a recent review of NMR structure determination in solution (Wagner et al., 1992), the difference between, and contributions of both techniques were clearly demonstrated. Each cross peak in a 2D NOESY NMR spectrum contains direct information on one particular distance within the protein, while each individual peak in a diffraction pattern contains information on the whole structure. To interpret an individual peak in a crystal structure, the rest of the peaks should be taken into account. Another difference between these methods is in the actual structure of the sample. The result of crystallographic refinements is presented by one structure while the result of distance geometry calculations from NMR is represented by an ensemble of structures derived from restraints such as interproton distances, torsion angle restraints of backbone, side chain torsion angle restraints, and stereospecific assignments. Torsion angle restraints are available from coupling constants. The reason for this ensemble of structures is the mobility of proteins in solution that makes NMR data more complicated and diverse.

NMR provides valuable checks on the accuracy of the crystallographic results. Some of the advantages of NMR are first, the structures are free of artifacts that result from crystallization, especially if the surface residues are perturbed by intermolecular contacts in the crystal. These surface residues are important because they are the sites of interaction with ligands and proteins. Second, NMR is the only method for determining

the structures of the many proteins that do not crystallize. A third advantage is the information given by NMR on estimating the time scales of intramolecular motions and in characterization of the conformation of bound ligands, as will be discussed in more detail.

NMR has a number of limitations. It is difficult to apply detailed NMR analysis to proteins that aggregate at a concentration below 1 mM. Linewidth depends on rotational correlation time (the reciprocal of the rate of tumbling in solution) which increases with the molecular weight. Therefore, solution structure determination using homonuclear NMR techniques is restricted to small proteins of a maximum of about 100 amino acid residues. Around this limit the extensive spectral overlap due to the large number of resonances would be so severe that assignments would be ambiguous, and so there would be no basis for a structure.

OVERVIEW OF THIS WORK

NMR spectroscopy was used to study the structure and conformations of a set of ligands, starting from small organic molecules, and ending with a medium-sized protein. NMR was then used to study the interaction of these ligands or antigen models with antibodies to understand the structural changes in the antigen and antibody upon binding. Antibodies and fragments were generated from the mouse immune response against PC-KLH, where KLH is the protein carrier conjugated to phosphocholine (PC). Studying the structural interactions between PC-protein conjugates and monoclonal antibodies gives information on conformational changes of antibodies upon immune maturation to memory. The small molecule models for PC-KLH, differing in size and charge, were designed to evaluate the influence of antibody binding on PC-based haptens. Working with chemically altered ligands as probes gave insight into the antibody structure, specificity, diversity and kinetics.

Antibody-hapten complexes are imperfect models of antibody-antigen complexes because a hapten only partially fills the antibody's binding site. Since antibodies were generated to PC-protein, then it is possible that neither p-nitrophenylphosphocholine (NPPC) nor N-(2,4-dinitrophenyl)-p-aminophenylphosphocholine (DPPC), the haptens initially used to probe the combining site of anti-PC-KLH antibodies (Bruderer et al., 1989), can adequately reflect the immunizing form of PC in PC-KLH. PC conjugates of amino acids, peptides and proteins were therefore synthesized to determine the epitope(s) recognized by the antibodies and the contribution of the carrier. The binding of these PC analogs to antibodies was studied by both enzyme linked immunosorbent assay (ELISA) and NMR. The conformations of the free and antibody-bound ligands were then characterized by NMR experiments. Comparing the conformation of the free ligand to the bound ligand gave information about the structure of the antibody binding pocket and the effect of the antibody on ligand stabilization.

Neurophysin (NP) is one of the proteins that was coupled to PC to be used as an antigen model. To determine the conformational changes induced by binding, the conformation of the free protein should be known first. NMR was used to assign residues on NP that might be part of the epitope. NP also is an interesting protein to study because it aggregates at high concentration and binds to hormones. These two properties were also investigated by NMR. Although in practice a separate study, the self-interaction of NP is relevant to its interactions with the antibodies because NP dimerization is accompanied by substantial tertiary structural changes which might be affected during binding to the antibodies.

Figure 1. The structure of various antibody fragments. The antigen binding sites are formed by the folding of the variable light and heavy domains (the black area). This Figure is adapted from Campbell, 1991.

Figure 2. Folding of the variable and constant domains of the light chain. The arrows represent the polypeptide arranged in β pleated sheets. The bars represent intrachain disulfide bonds. The three shaded regions are the CDR loops of the variable region which are brought together to form the antigen binding site. The numbers indicate the position of the amino acid residues in the sequence (Edmundson et al., 1975).

Figure 3. Maturation of the immune response. Low affinity antibodies are produced by first encounter with antigen. After somatic mutation, antibodies with higher affinities are produced (Campbell, 1991).

CHAPTER II

EXPERIMENTAL METHODS AND MATERIALS

PROTEIN ISOLATION

Neurophysin (NP) was isolated from pituitaries by a combination and modification of literature preparations (Whittaker and Allewell, 1984; Breslow et al., 1971; Hollenberg and Hope, 1968). The starting material was bovine pituitaries, which are commercially available from Pel-Freeze Biologicals as a tissue culture medium. Isolation of the crude NP-hormone complex was done by extracting the ground pituitaries (18 g) in 600 mL of 0.1 N HCl to minimize proteolysis. The mixture of the acid and pituitaries was ground with a blender and incubated at 4°C for 20 hr.; the final pH was 1.6. Insoluble material was removed by centrifugation at 15,000 rpm for 20 min at 0°C on a SORVAL RC2-B refrigerated centrifuge. The insoluble material was re-extracted in 300 mL 0.1 N HCl for another 20 hr. at 4°C. The final pH was 1.62. Supernatants were combined, then neutralized with approximately 60 mL of cold 2 N NaOH. Care was taken not to raise the pH above 7.0 to reduce proteolytic activity. The cloudy solution was centrifuged at 15,000 rpm for 30 min. The supernatant was decanted, and the pH adjusted to 3.90 followed by addition of 10 g of finely ground NaCl for every 100 mL solution to cause precipitation of the protein, at 0°C (Hollenberg and Hope, 1968). Precipitation was allowed to proceed overnight at 4°C, and then the suspension was centrifuged at 15,000 rpm for 30 min. The sediment, which was the crude protein-hormone complex, was dispersed in 300 mL of water and dialyzed with Spectrapor membrane tubing (MW cutoff = 6 KD) against water to remove the salt (3 × 18 L). The residual precipitate was dissolved by the addition of several drops of 1 N acetic acid, and the solution was then lyophilized. The salt-free protein-hormone complex weighed 1.5 g.

This was dissolved in 1 N formic acid, and separated from the hormones by chromatography on a (5 × 30 cm) Sephadex G-75 column, which has a useful working range of 3,000- 70,000 D. The separation was conducted at room temperature, with 1 N formic acid as eluting solvent (Breslow et al., 1971). The column was connected to an ISCO UA-5 absorbance/ fluorescence detector with model 1133 Multiplexer-Expander. The flow was regulated with a WIZ peristaltic pump, and the fractions were collected with a Retriever IV fraction collector. From the chromatograms shown in Figure 4, three peaks were resolved; the middle peak is the crude NP. The yield was 700 mg of crude neurophysin after lyophilization.

The NP was resolved at room temperature on a column of DEAE-Sephadex A-50, a weak anion exchanger, (600 mL column volume). The presence of several aspartic and glutamic acid side chains results in a low isoelectric point pI of 4.3 and 4.7 for NP-I and NP-II, respectively (Pliska et al., 1972). The column was pre-equilibrated with pH 7.90, 60 mM Tris-HCl buffer. Elution was performed with the same buffer with a linear 0-0.4 M NaCl concentration gradient, at 12 mL per hour (Whittaker and Allewell, 1984). Four types of NP were obtained. The yield of the first peak after ion exchange, NP-II, was 245 mg. The last peak, NP-I gave 275 mg yield. In between, there were two other kinds of NPs: NP-B and NP-C that were present in smaller amounts. After dialysis against 0.1 N formic acid (4 × 4L), the purified proteins were lyophilized and stored at -20°C.

NMR SPECTROSCOPY

NMR Methods For Product Analysis

The NMR experiments were performed on a Bruker AMX-400 spectrometer interfaced to Bruker X32 computer. One of the major advantages of NMR is that many different nuclei can be detected separately. A probe dedicated to ^1H observation provided optimum signal-to-noise. Normally the ^1H NMR spectra were obtained at 400.14 MHz

using the following acquisition parameters: an observation pulse of 4 μ s and 4 s relaxation delay for an acquisition time for small molecules, and 9 μ s observation pulse, 2.5 s relaxation delay for proteins. Most of the spectra were obtained with suppression of the solvent peak by applying a low-power saturation pulse during the relaxation delay.

Aside from NMR observation of the ^1H nucleus, there are other nuclei with non-zero nuclear spin that can be observed. The ones that were used in this study are ^{19}F and ^{31}P . Both have spin 1/2, a large magnetogyric ratio, and 100% natural abundance. A 5 mm switchable probe was used to obtain these spectra. The ^{19}F spectra were acquired at a frequency of 376 MHz with an observation pulse of 20 μ s and 4 s relaxation delay. The ^{31}P spectra were acquired at a frequency of 162 MHz with 4 μ s observation pulse and 3 s relaxation delay. Decoupling of protons was done by the standard WALTZ-16 procedure (Shaka et al., 1983), which results in dramatic reductions in line widths and enhancement of signal to noise ratio. The sequence for the power-gated proton decoupling starts with a relaxation delay during which low-power decoupling is applied to maintain the NOE and then increased decoupler power is used during signal acquisition to guarantee all collapse of coupling constants. For ^{19}F , inverse gated proton decoupling was used, which followed a similar pulse sequence as above but without the decoupling to eliminate the NOEs.

Two-Dimensional NMR methods

All two-dimensional FT-NMR correlation experiments operate on the same principle. Acquisition of data requires a preparation period, then an evolution period (t_1) during which the spins are labeled according to their chemical shifts, a mixing period during which the spins are correlated with one another, and, finally, a detection period (t_2). The experiment is repeated several times with successive increments of the evolution period t_1 to yield a data matrix $S(t_1, t_2)$, which upon Fourier transformation in both

dimensions gives the 2D spectrum $S(\omega_1, \omega_2)$ (Wüthrich, 1986). This is demonstrated schematically in Figure 5. In all 2D plots presented in this document, the diagonal corresponds to the 1D spectrum and the off-diagonal elements correspond to nuclei that are either connected through bond or through space, depending on the experiment.

Structure Determination Of Free Antigen

Complete and detailed NMR determination of the conformation of an intact antibody with its bound antigen is not yet feasible because of the large size of such a complex. Resonances in the antibody have large linewidths (>20 Hz), while those in the small antigens used in this study are narrow (<5 Hz). Studies on the conformation of the bound antigen give, in principle, information on the specific involvement of groups of the antibody combining site and about the different strengths of these interactions. There are two main classes of 2D experiments, each providing different information.

Information From Spin-Spin Coupling: COSY. In two-dimensional correlated spectroscopy (COSY) (Bax and Freeman, 1981), strong cross peaks occur only between neighboring protons that are separated by not more than three covalent bonds. This is called J-coupling, and reflects the dihedral angle through the C or N nuclei to which the protons are attached. The basic pulse sequence is $(90^\circ-t_1-90^\circ-t_2)$, where t_1 is the incremented delay for the first dimension and t_2 is the time during which the data is acquired. Another technique which is of the same class is total correlation spectroscopy (TOCSY). TOCSY spectra display both direct and relayed crosspeaks (Braunschweiler and Ernst, 1983). In this type of experiment, a short spin-lock field is applied followed by a composite pulse sequence known as MLEV17 to transfer the magnetization among scalar coupled homonuclear spins (Bax and Davis, 1985b). The advantages of TOCSY are that coherence transfer is efficient and data are largely pure phase. The pulse sequence used with solvent suppression was $(t_0-90^\circ-t_1-SL-MLEV17-SL-Acquisition)$ where t_0 is

the relaxation delay during which the solvent is suppressed, SL is the low power level used for spin lock, MLEV17 is a composite pulse sequence composed of 16 composite pulses of 90° - 180° - 90° followed by 60° 17th pulse. This pulse sequence will be discussed in more detail in Chapter VIII. Figure 6 shows expected magnetization transfer through bonds.

Coupling Through Space: NOESY. The other type of information is from 2D nuclear Overhauser effect spectroscopy (NOESY). These spectra provide information about protons that are close in space (less than about $4.5 \text{ \AA}$). Each proton spin possesses a property known as magnetization. Magnetization is exchanged between the spins by a process termed cross relaxation. The rate constant for this process is directly proportional to r^{-6} , where r is the distance between the two nuclear spins. The nuclear Overhauser effect (NOE) is a consequence of modulation of the dipole-dipole coupling between different nuclear spins by molecular tumbling. Figure 6 shows an example of magnetization transfer through space. The pulse sequence used with solvent suppression by presaturation during relaxation delay and mixing time was (t_0 - 90° - t_1 - 90° - τ - 90° -Acquisition), where t_0 and τ are the relaxation delay and mixing times. Cross relaxation can also be measured using a 1D experiment by perturbing the magnetization of a particular spin and watching its effect on the neighboring spins by difference spectroscopy.

For small molecules, the ^1H - ^1H NOE has a positive sign with a maximum intensity of 0.5. This intensity for small molecules decreases as a function of molecular weight and increases in negative intensity for large molecules. For intermediate size molecules, the NOE is small or zero. For large molecules, the NOE has a maximum intensity of -1.

NOESY of Intermediate Size Molecules: ROESY. Peptides are generally flexible and undergo rapid conformational averaging in water (Bothner-By et al., 1984; Otting and Wüthrich, 1987). For molecules that have an effective motion correlational time equal to reciprocal of the angular Larmor frequency, the NOE is near zero and no NOESY cross

peaks are observed even with a long mixing time. As an alternative, a rotating frame NOESY (ROESY) can be used to obtain distance information. The ROESY experiment is done under spin-locked conditions and has the advantage of producing positive NOEs for molecules no matter what their size is, so the passing through a zero stage for intermediate size molecules is eliminated (Bax and Davis, 1985a). Spin locking is applied during the evolution period, during which spin exchange among spin-locked magnetizations of different nuclei can occur. ROESY cross peaks have opposite phase to the diagonal, as do small molecule NOESY cross-peaks. The pulse sequence used with solvent suppression was (t_0 -90°- t_1 -SL-Acquisition) where SL is the low power pulse for the ROESY spin lock.

ANTIBODY ANTIGEN COMPLEXES WITH LARGE OFF-RATES

Transferred Nuclear Overhauser Difference Spectroscopy: TRNOE

For weakly binding ligands with large k_{off} (fast exchange between bound and free hapten), the approach developed by Clore and Gronenborn (Clore and Gronenborn, 1982; 1983) was used to study the conformation of the ligand in its bound form. This approach is referred to as transferred nuclear Overhauser effect (TRNOE) spectroscopy. The theory will be explained briefly below. The advantages of this technique are that it is not limited by the large size or the small concentration of the antibody. Information can be obtained with large excess of the small molecule antigen, thus reducing the amount of antibody required.

The standard NOESY spectrum of the intact antibody, Fab or Fv in the presence of excess ligand contains TRNOE cross peaks. These cross peaks reflect magnetization transfer that occurs between protons in the bound state. Information regarding the conformation of the bound state is transferred to the easily observed free state. If the exchange of ligand molecules on and off is fast compared to the spin lattice relaxation of

the free ligand, magnetization that is transferred between protons in the antigen-antibody complex is efficiently further transferred to the free ligand by the exchange between bound and free. In other words, NOE experiments are performed on the ligand but the results are dominated by the conformation of the bound protein-ligand complex. For this to work, the off-rate should be fast relative to spin lattice relaxation of both the antibody and ligand proton.

Kinetics. The kinetics of the TRNOE are illustrated in the following scheme.

where $k_{\text{on}}/k_{\text{off}} = K_a$ which is the binding constant

I and S are two nuclei of the ligand related by cross relaxation. σ is the cross relaxation rate relating the magnetization between the free and bound ligand. ρ is the spin lattice relaxation rate. E is the free macromolecule to which the ligand binds.

Factors Influencing the TRNOEs. The observed TRNOEs depend on the following factors. First, the rate constant for exchange between free and bound states of the ligand affects transfer of magnetization between bound and free. Second, TRNOEs depend on the σ s, which are the cross relaxation rates in free and bound ligand. Third, TRNOEs depend on the relative concentration of the free ligand to protein. The increase in free ligand concentration can increase the magnitude of the negative TRNOE and hence

results in gain in sensitivity. Fourth, the spin lattice relaxation rates (ρ) of free and bound ligand have to be slow relative to the exchange off-rate. This transfer between bound and free should occur before the nuclear spin system decays to equilibrium. Fifth, the molecular weight of the protein affects the cross relaxation rate. As the molecular weight increases, the correlation time increases and this causes an increase in cross relaxation rate.

In the most common form of the TRNOE experiment, the free and bound ligand resonances are averaged due to fast exchange. The information is in the form of negative NOEs between the free ligand resonances. Another type of cross-peaks observed in highly concentrated antibody samples is between bound antibody protons and the free ligand.

Spin Diffusion. If the off-rate is too slow, spin diffusion occurs for bound ligands. In multispin systems the cross-peak volumes in 2D data sets can be affected by their neighboring protons if dipolar coupling is strong enough to cause transfer of relaxation to many protons in the molecule (Macura and Ernst, 1980; Keepers and James, 1984). The indirect paths such as $A \rightarrow B \rightarrow C$ give inaccurate distance indication of $A \rightarrow C$, and can result even when A and C are far apart. Olejniczak et al. (1986) gave experimental verification of the importance of this indirect path to the contribution of inaccurate distance information. Thus under this condition, accurate distance information cannot be calculated from the volume of the cross peaks. The contribution from the indirect NOE increases with the size of the protein, the mixing time used, and the reciprocal of the off-rate. It is possible to distinguish between direct magnetization transfer, from one proton to another (primary NOE), and indirect magnetization transfer through an additional spin (spin diffusion), by checking if the NOE magnitude extrapolates to zero at zero mixing time (Wüthrich, 1986). However, as shown by Olejniczak et al., even if this is true, only a portion of the curve may be due to direct magnetization transfer. For the purpose of this work, where it is not necessary to obtain very accurate distance constraints, the accuracy

of the data was checked by extrapolation of NOE percent to zero with zero time as done by Koide et al. (1989). Also, keeping mixing times significantly less than spin lattice relaxation time (T_1) helps to get direct NOEs (Sanders and Hunter, 1987).

Examples of Previous Work Using TRNOEs. TRNOEs between a ligand and a protein was first observed by Bothner-By and Gassend (1972). Interestingly, the protein in that work was NP, which is central to this document. Resonances within the ligand giving rise to TRNOEs were first observed by Albrand et al. (1979). Clore and Gronenborn (1982, 1983) further developed the technique as discussed above.

Anglister and his group contributed immensely to the understanding of antigen-antibody interactions by using 1D and 2D TRNOE experiments (Anglister, 1990; Anglister and Zilber, 1990 ; Anglister et al., 1989). They extended this technique to complexes which had slow ligand off-rates by using modified peptide ligands to increase the off-rate. In studying these interactions, they increased the resolution in the TRNOE difference spectra by deuterium labeling of the aromatic amino acids in the Fab in order to assign the cross-peaks of a type of amino acid. To assign specific residues, they used calculated models to determine what amino acids in the combining site are exposed to the solvent, and therefore can interact with the antigen.

Glaudemans and coworkers used TRNOEs to study the interaction of anti-carbohydrate antibodies with sugars (Glaudemans et al., 1990). They compared the conformation of the free ligand with the bound ligand to see that the antibody alters the conformation of its ligand upon binding. The conformation of the free ligand was determined by J-coupling and 2D NOESY methods. The conformation of the bound ligand was determined by TRNOE experiments.

Figure 4. Column chromatography of NPs. (A) Gel filtration using Sephadex G-75. The absorbance was set to 2.0, chart speed 0.6 cm/hr, 8 mL / fraction. The crude NPs were pooled from the middle peak. (b) Ion-exchange separation on a DEAE-Sephadex A-50 column. Elution was performed with a linear 0-0.4 M NaCl gradient at a rate of 12 mL/hr. The absorption was set to 2.0, chart speed 0.3 cm/hr, 8 mL/fraction at a wavelength of 254 nm.

Figure 5. Generalized representation of a 2D NMR experiment. The first pulse prepares the system. The second pulse is a mixing step which transmits the effects of the evolution to the magnetization that is detected. t_1 is the incremented delay. Fourier transformation is done in both dimensions. ω_1 and ω_2 are the frequency axes in both dimensions (Clare and Gronenborn, 1989).

$$N_{ij}(t) \sim \sigma_{ij} t$$

Figure 6. Transfer of magnetization through bonds and through space. (A) Through bond magnetization transfer within the same spin system of an amino acid residue between α and β protons gives rise to COSY cross-peak. Transfer of magnetization from the α proton to the CH_3 protons gives rise to a TOCSY cross-peak. The identified spin system of nonlabile protons is then connected to the neighboring residues by NOE cross-peaks (Wüthrich, 1986). (B) The basis of the NOE. r_{ij} is the distance between the protons i and j ; σ is the cross-relaxation rate; N is the NOE (Clare and Gronenborn, 1989).

CHAPTER III

OVERVIEW OF ANTIBODY RESEARCH

THE ANTI-PC RESPONSE

Background On Immune Maturation

Immune maturation as shown in Figure 3 is accompanied by substantial changes during the interval between the primary and the memory responses, including changes in affinity (Berek and Milstein, 1987), and specificity (Azuma et al., 1987; Chang et al., 1982) which allow the immune system to respond to a broader array of potentially harmful antigenic structures. The precise molecular interactions underlying changes in affinity and specificity are not entirely understood, and most of what is known originates from a limited set of X-ray crystallographic analyses (Davies et al., 1990; 1988).

It is important to understand how epitope recognition allows the immune memory response to evolve into a highly effective and specific mechanism. The immune response to phosphocholine-keyhole limpet hemocyanin (PC-KLH) provides a unique model to study the interrelationships between epitope recognition and the evolution of the specificity found in the primary and memory responses because both primary antibodies and those in the memory response bind to PC-protein. The immune response to PC-KLH has been studied in the laboratory of our collaborator, Dr. Rittenberg, and the brief review that follows is largely based on his published work.

The memory response to PC-KLH consists of two antibody populations, Group I and Group II. Group I predominates in the primary response where Ig molecules are mainly IgM, while Group II codominates with Group I in the memory response. The distinction between Groups I and II antibodies was originally based on the observation

that binding to PC-protein by all of the antibodies in the primary response could be inhibited by PC, while binding to PC-protein by IgG antibodies from the memory response cannot be inhibited by PC but was inhibited by p-nitrophenylphosphocholine (NPPC) (Chang et al., 1982; Chen et al., 1992). In the case of Group II antibodies, the phenyl moiety, a negatively charged phosphate ester, and the trimethyl structure of the choline are required for binding. The hapten aromatic ring is essential to binding, presumably because it forms the basis for the diazophenyl linkage of PC to the carrier protein. It was shown that PC is an integral part of the epitope since Group II antibodies did not bind to p-diazophenylarsonate conjugated to bovine serum albumin, BSA, that contained the same diazophenyl ring and tyrosyl and/or histidyl carrier determinant, but not the PC moiety (Wicker et al., 1982). In Group I antibodies, the phenyl group is not essential for binding, but the positive charge in the trimethylammonium group is required. Group II antibodies bind at least 100 fold better to PC-protein than do Group I antibodies (Stenzel-Poore et al., 1988).

Germline Diversity

The germline encodes a large but selected repertoire of antibody binding sites. Each variable region is encoded by two or three different genetic elements, variable chain (VL) and joining chain between multimers (JL) for the light chain and VH, diversity segments (DH) and JH for the heavy chain. Germline diversity is derived from multiple gene segments arising from different combinations of the VDJ gene fragments that encode the heavy and light chains, random pairing of these segments, and junctional diversity caused by imprecise DNA rearrangement (Tonegawa, 1983). Group I antibodies expressed by primary B cells were shown to have restricted gene usage: a single VH gene and three VL segments (V κ 8, V κ 22 and V κ 24). The VH1 gene combined with V κ 22 is present in a prototype PC-binding myeloma protein TEPC15 which expresses a dominant

idiotype called T15 idiotype. Antibodies that share the same determinant are said to belong to the same idiotype. Similar T15+ molecules comprise approximately 90% of the primary antibody response to phosphocholine. Group II antibodies lack the T15 idiotype (unique determinant of Ig hypervariable regions) and exhibit greater VH diversity, as well as light chain diversity in V κ and V λ light chains. Two basic mechanisms, somatic mutation and recruitment of new clonotypes, play dominant roles in producing antibody diversity in the immune response (Berek and Milstein, 1987).

Somatic Mutation

Somatic mutations are structural alterations in the heavy and light genes that occur after stimulation by the antigen, leading to more diversity in the B cell repertoire (Weigert et al., 1970; Bernard et al., 1978). Clones that express V regions with higher antigen affinity are preferentially selected over those with lower affinity. Few mutations in the CDR occur in antibodies of Group I, while Group II shows extensive mutations, resulting in multiple replacements in three CDR regions of the light chain λ (Stenzel-Poore et al., 1988). Changes in CDR2 are a dominant path to a high affinity for PC-protein (Brown et al., 1992).

Clonal Recruitment

Since Group II antibodies use V genes different from those used by Group I, they are not derived from Group I but are actively recruited from a different precursor pool (Stenzel-Poore et al., 1988). Another observation that led to this conclusion is the behavior of immunodeficient mice; they do not produce normal anti-PC T15+ responses (Group I) (Quintáns, 1977), but can produce normal Group II memory antibodies (Wicker et al., 1982). These results suggest that the Group II response originates from a different B cell subpopulation. Group I antibodies also lack the potential to diversify, which would

explain the selection of Group II antibodies from a minor population in the primary response to being co-dominant in the memory response.

Group II Antibodies

The Group II population has been divided into two subgroups based on their ability to bind NPPC analogs. Group IIA antibodies require the positively charged choline nitrogen for binding, while Group IIB antibodies can also bind to p-nitrophenyl-3,3-dimethylbutylphosphate (NPDBP), which lacks the positive charge since the nitrogen is replaced by a carbon atom (Bruderer et al., 1989). All Group IIA antibodies used in this work use κ light chains and all Group IIB antibodies that were studied use λ light chains, while the same VH gene is associated to both groups. This shows that the generation of these subgroups is linked to light chain, and not to heavy chain usage. Therefore various regions of the PC-protein epitope contribute to binding to various Group II antibodies.

Groups other than Rittenberg's recognized the presence of Group II antibodies (Wicker et al., 1982; Heusser et al., 1984; Feeney and Mosier, 1984; Lotscher et al., 1992). Lotscher and coworkers studied the specific VDJ gene combinations that contribute to the shift from PC to phenyl-PC specificity. They divided Group II into two main specificities; one that recognizes the entire azophenyl-PC hapten and the other is directed against the phenyl group of the PC hapten.

The anti-PC protein response illustrates the process of diversification because the primary response produces an antibody population of which Group I is dominant, and diversification results in the development of a second population of Group II antibodies, which can recognize the PC moiety only when connected to different structures (Stenzel-Poore et al. 1988). Having two subpopulations of antibodies helps to evaluate the importance of the variations in epitope recognition and affinity that determine the memory B cell pool composition.

Other groups are studying the evolution of the antibody variable region during the immune response using different haptens. Manser and coworkers used antibodies specific to p-azophenylarsonate (Manser et al., 1987) and 2-phenyl-5-oxazolone (Berek and Milstein, 1987). They found that the maturation of the immune response results from hypermutation and antigenic selection of variants of higher affinity. The hypermutation affects both framework residues and the CDRs.

Environmental Aspects of the Anti-PC response

Another reason that makes anti-PC antibodies interesting to study is that PC is a component of the cell walls of many organisms including species of fungi, nematodes, and bacteria (Potter, 1970). The possibility arises that most common germ line VH and VL genes have evolved to provide antibodies to common pathogen(s). This is inferred from the relationship between the idiotype of an anti-PC antibody and its ability to protect mice from fatal pneumococcal infection (Briles et al., 1982). As indicated above, almost 95% of the primary antibodies produced in Balb /c mice in response to PC arise from B cells that express one VH gene (that of T15) (Claflin and Rudikoff, 1977), the same gene usage as in Group I. The presence of these clones in a germ-free environment is lower than in conventionally raised mice (Gearhart and Cebra, 1979). This shows that it is likely that these mice have been exposed to antigenic forms of PC, and hence produced Group I antibodies, because it is very unlikely to have mice already exposed to the diazophenyl PC (Wicker et al., 1982).

Aging of mammals is accompanied by changes in antibody response. Aging of mice in particular is accompanied by changes in antibody responses to PC (Nicoletti et al., 1991). All young mice express T15 idiotype, which is characteristic of Group I antibodies, while aged mice produce antibody with T15 genes that are somatically altered, or there may be individual T15 genes in new combinations of VH1 and different VL. This

suggests that aged mice respond to antigens by producing antibodies that are different from those produced by young mice, and show preference for an antigenic response to PC in the context of the carrier.

HISTORICAL DEVELOPMENT

Since the 1950s, research on the molecular basis of immune responses and their diversity attracted the attention of molecular biologists, immunologists and protein chemists. Among the investigated systems by X-ray crystallography is the Group I myeloma protein McPC603, which has been studied both in the absence and presence of free PC (Segal et al., 1974; Padlan et al., 1976; Satow et al., 1986). These studies showed that PC binds in a pocket on the antibody McPC603 Fab combining site, with the choline buried and the phosphate on the surface. Based on this crystal structure, specific antibody residues have been postulated to interact with PC. The PC-McPC603 crystal structure shows PC in an extended conformation, with the negatively-charged phosphate hydrogen-bonded to Tyr 33(H), and electrostatic interaction with Arg 52(H) and Lys 54(H). The positively-charged ammonium appears to interact with Glu 35(H). Interactions involving Tyr 33(H), Arg 52(H), Tyr 100(L), Glu 35(H) and the negative charge of either Asp 97(L) or Asp 101(H) was verified by site-directed mutagenesis of McPC603. The unimportance of the tyrosine hydroxyl group, however, was shown by site directed mutagenesis of Tyr 33(H) to Phe (Jackson et al., 1991). There are minimal structural changes in McPC603 relative to the complex PC-McPC603, although this result may have been compromised by a sulfate ion that was present in the PC-phosphate site of the McPC603 sample when the hapten was absent (Satow et al., 1986).

Other techniques have been employed to study the structural and dynamic details of interaction between antigens and antibodies. These include circular dichroism (Rockey

et al., 1972) and chemical modification (Grossberg et al., 1974) to identify the relative importance of residues of the antibody to binding .

Using NMR (Dwek et al. 1975; 1976), Dwek and his coworkers showed that the antibody MOPC315 (a myeloma protein with specificity for di- and trinitrophenyl haptens) undergoes minor conformational changes upon binding dinitrophenyl haptens. Also, the combining site contains many aromatic residues and very few non-aromatic protons in dipolar contact with the haptens. They used NMR techniques to refine the model of the combining site of the Fv fragment of the dinitrophenyl-binding mouse myeloma protein MOPC315 constructed by Padlan and coworkers (Padlan et al., 1976). For this study, Dwek et al., used one-dimensional difference spectra between the Fab complexed with the hapten, and the Fab itself, and ring-current shift calculations. Since the model constructed by Padlan et al. has two histidine residues near the combining site, monitoring the pKa values of the three histidine residues in the Fv fragment, in the presence and absence of the hapten, allowed the assignment of these histidines (Wain-Hobson et al., 1977). A specifically deuterated tryptophan residue was incorporated into the Fv fragment in an effort to assign the ^1H NMR spectrum of MOPC315 (Gettins and Dwek, 1981). Comparison with the non-deuterated case gave the assignments of the Trp protons. The effect of binding was then monitored by observing the changes in the chemical shift difference and the broadening of these signals.

The interaction of PC with antibodies was also studied in solution using the signals from ^{31}P in the phosphoryl group and ^{13}C in the trimethylammonium group (Goetz and Richards, 1977) by monitoring line broadening and chemical shift differences in NMR signals. ^{31}P can be used to monitor the ionization state of the phosphate ester sensitively, and hence learn about the electrostatic nature of the groups in the phosphorous environment. Kooistra and Richards (1978) also used ^{19}F NMR to study the interaction of CF_3 -nitrophenyl- γ -aminophenylbutyric acid, lysine, or glycine haptens with MOPC315,

and hence probe the environment of the bound hapten. Their conclusion was that the trifluoromethyl group on the phenyl ring experiences a downfield shift upon binding due to proximity to an aromatic system.

Bennett and Glaudemans (1979) studied a variety of PC analog ligands in their interaction with an Fab from the myeloma IgA H8 which is highly homologous to McPC603. They studied the nature of the binding site in its dielectric compatibility with the various ligands, and the possible role of water displacement from the combining site on hapten binding. The choline $-N(CH_3)_3^+$ proton resonance was shifted downfield and broadened on binding to the Fab. These effects were interpreted as a decrease in mobility relative to unbound ligand. They stressed the importance of the steric, as well as electronic, nature of the charged groups by showing the importance of the hydrophobic bulky groups around the charged nitrogen of the choline. Also, the binding of the antibody to different analogs was taken as evidence that the binding site has substantial flexibility.

Others have now studied in detail the specific interactions between an antibody and its peptide antigenic ligand by using NOE measurements, and especially 2D TRNOE difference spectroscopy (Anglister et al. 1989, Glaudemans et al. 1990). The magnitudes of the TRNOE cross-peaks depend on the structure of the peptide in the bound state and the restrictions of the complex's mobility as discussed in Chapter II.

Other NMR techniques such as total correlation spectroscopy (TOCSY) and photo-CIDNP can be applied to selectively deuterated antibodies (Kato et al., 1989). Photo-CIDNP measurements of the antibody and its complexes were used to obtain information about the solvent exposure of aromatic residues in the combining site region. TOCSY experiments in combination with selective deuterium labeling were done to establish connectivity between the C_2H and the C_4H of the His residue of the flexible

portion of an antibody. The change in flexibility induced by the binding of the antigen can hence be detected.

2D COSY is another NMR technique used to probe the binding site of tightly bound ligands (Cheetham et al., 1991). Resonances that correspond to the bound peptide may be monitored directly. Since the magnitudes of the COSY cross peaks decrease with increasing linewidths because of anti-phase cancellation, the residues on the small antigen interacting strongly with the antibody can be identified by monitoring the decay of the intensity of cross peaks. Thus differences in linewidths distinguish between antigen residues that do not interact strongly with the antibody combining site from those that become immobilized upon binding. The intensity of the cross peaks also depend on the spin-spin coupling constant (J) which is a function of the dihedral angle between the proton pair, and hence provides molecular conformation information. The cross peak intensity is very weak when the linewidth exceeds J . Therefore, a difference in either the linewidth or dihedral angle will cause a change in the intensity.

Isotope edited studies are used for tightly bound ligands. Isotope editing experiments have been used to make use of the less crowded ^{13}C , ^{15}N or ^{31}P NMR antigen spectra to study the conformation of the bound Fab-antigen complex and the nature of the combining site (Tsang et al., 1988). Most of these experiments were done by ^{15}N labeling of a peptide antigen and then the parts of the peptide that interact strongly with the antibody were determined by linewidth and chemical shift difference. Another type of isotope-edited experiment involves producing specifically isotopically labeled Fv to get assignments of the aromatic proton resonances.

CHAPTER IV

ANTIBODY-RELATED METHODS

ANTIBODY PRODUCTION AND PURIFICATION

This portion of the work is done using the facilities in Dr. Rittenberg's lab at the Oregon Health Science University.

Hybridoma Production

This is a technique for large scale preparation of antibodies with predetermined specificity. The advent of hybridoma technology (Kohler and Milstein; 1975) allowed thousands of monoclonal cell lines to be obtained from a single immunized mouse. Figure 7 illustrates this technique. A myeloma line which is a monoclonal tumor derived from a B lymphocyte and does not make antibody is the fusion partner for an immune B cell and should lead to the production of stable clones secreting large amounts of antibody. To eliminate the non-fused cells from the fusion mixture, a parent myeloma that is sensitive to hypoxanthine, aminopterin, and thymidine (HAT) medium is selected. Aminopterin inhibits the enzyme dihydrofolate reductase and therefore blocks the main biosynthetic pathways for purine and pyrimidine synthesis. The salvage pathways involve the enzymes thymidine kinase for pyrimidine synthesis and hypoxanthine phosphoribosyl transferase for purine synthesis. Cells can be made deficient in these enzymes and will therefore die in HAT medium, and those secreting the specific antibody can be selected by limiting dilution techniques. If normal cells are fused to mutants, they will provide the necessary enzymes and the hybrids synthesize DNA and grow in HAT medium. Desired clones can then be selected and propagated.

Expansion of Hybridomas

To grow large amounts of antibody, hybridomas were first expanded in vitro as tissue culture, and then in vivo as ascitic fluids.

Media used. Hybridoma production requires a particularly high standard of sterile technique. Materials needed are sterile hood, carbon dioxide incubator, inverted microscope to monitor the cells and the appearance of clones, a centrifuge, sterilized solutions and glassware, and a haemocytometer for counting cells. Two main types of media were used: RPMI 1640 (Gibco-BRL, Grand island, NY) and Iscove's (Gibco-BRL) media. Iscove's medium was preferred because it contains an additional buffer that helps stabilize the pH. The medium was supplied without glutamine because of its instability in aqueous solution, and so it was added to a level of 2 mM. Pyruvate, nonessential amino acids, 2-mercaptoethanol, and antibiotics such as gentamycin that inhibits the growth of bacteria were added. Both media were bicarbonate buffered with phenol red indicator. The pH was adjusted with sterilized NaOH to pH 7.2, producing a bright orange color. The serum free medium was stored at 4°C. Foetal calf serum (FCS) that contains growth factors required by the cells was added before use to make up 30% of the complete medium.

Growing Cells. Five different hybridoma cell lines were used: D16 (IgM), D18, and D9 (IgG1), N8 and N9 (IgG3). Cells were removed from liquid nitrogen and thawed in a 37° water bath. The contents were then transferred to a small plastic flask containing 5 mL of complete medium. Next, the suspension was centrifuged at 1000 rpm for 7.5 min, and the supernatant that contained 10% DMSO was discarded. 5 mL of medium were added to the cells, and this mixture was placed in a tissue culture flask and incubated at 37°C and 10% CO₂. The cells were fed daily by adding 5 mL of medium. Growth of cells in the flask was monitored with a microscope. To remove floating dead cells, the

medium was replaced by fresh. To transfer to a larger flask, the smaller first flask was incubated with a 0.25% trypsin for 7 min at 37°C to cleave the surface proteins. Then the flask was tapped lightly to suspend the cells, then washed with 5 mL of medium, and centrifuged at 1000 rpm for 7 min. The supernatant was transferred to the larger flask and suspended in the medium.

The cells were harvested after a week and counted using a haemocytometer. The cells were checked with 0.4% Trypan Blue dye, which differentiates dead from live cells by staining the dead cells. The line cells were resuspended in medium to a cell concentration of 6×10^6 cells/mL. The cells were centrifuged and resuspended at 6×10^6 cells/mL in 60% Iscove's medium, 30% FCS and 10% DMSO. 1 mL aliquots of this suspension were stored in plastic vials, then frozen slowly at -70°C for 12 hr, and then transferred to liquid nitrogen.

Expansion of Hybridomas in Mice

12 Balb/c mice were primed intraperitoneally with 0.5 mL pristane. Pristane depresses the normal immune function so that injected myeloma cells are able to grow without rejection (Frund and Blair, 1982). 14 days later, each mouse was injected intraperitoneally with 0.5 mL (3×10^6) hybridoma cells. Mice were inspected daily to monitor the growth of the ascites tumor. Animal handling techniques such as immunization, injection and sacrifice are described in sufficient detail elsewhere (Campbell, 1991).

After 7-14 days, enough time for the tumor to grow and swell, the mice were harvested. They were quickly killed by cervical dislocation. Dissection took place immediately. The whole peritoneal wall was exposed by removing the skin and flooded with 70% ethanol. The ascitic fluid was slowly drawn into a sterile pasteur pipette. Care was taken to avoid clogging the pipette with fat. The suspension was centrifuged for 10

min. at 3000 rpm to remove blood cells, and the supernatant was stored in a preserving medium of 1 mM EDTA and 0.1% sodium azide. The ascites were then removed from suspended lipids by extraction with Freon. An equal volume of 0.1 M phosphate buffer at pH 8 was added and the solution was passed through a 0.8 μ filter to filter out suspended material.

Affinity Purification

Gel Preparation. Antibodies from the mice were then affinity purified on phenyl phosphocholine-Sepharose gels. For 20 mL of gel, 5.72 gm CNBr-activated Sepharose 4B (Pharmacia) were suspended in 100-150 mL of 1 mM HCl for 15 min. HCl preserves the activity of reactive groups that could be hydrolyzed at high pH. The gel was washed with 1 mM HCl (10 $\times$ 100 mL) on Whatman 50 hardened filter paper using suction. The gel was then washed with 5 mL NaHCO₃ of pH 8.3 (coupling buffer) for each gram of gel. The gel was transferred immediately to a tube containing Gly-Tyr dipeptide dissolved in coupling buffer, then mixed overnight at 4°C using end over end gentle stirring. The suspension was centrifuged at 2000 rpm for 5 min and the supernatant was discarded. To block excess active groups, 30 mL of 1 M ethanolamine buffer was added, and the mixture was incubated at room temperature for 2 hours with end over end mixing. Next, the gel was successively washed with coupling buffer and solutions of 0.1 M, pH 4.0 acetate buffer and 0.05 M and pH 8.3 borate buffer. To wash away the unreacted blocking agent, the gel was washed and suspended in borate buffered saline (BBS) made up of 7.04 g Na₂B₄O₇, 4.67 g NaCl in 1 L at pH 9.0. The hapten p-diazophenyl phosphoryl choline (DPPC) was added and incubated for 2 hours. The resulting gel was then stored in excess borate saline at 4°C.

Purification. Five 1 mL Isolab "Quick-Sep" polypropylene columns were packed with the gel, and equilibrated with 0.1 M phosphate buffer at pH 8.0. The ascitic fluid was

added after the eluant absorbance (OD) at 280 nm was less than 0.05. The columns were washed with phosphate buffer to remove proteins that were loosely bound to the column until the absorbance was less than 0.05. 1 mL of 0.05 M CBG (n-carbobenzoxy glycine) (Sigma) in 0.05 M borate buffer, pH 8.3 was applied to the column, then washing with 0.1 M phosphate buffer at pH 8.0 was performed until the OD at 280 nm was less than 0.05. Next, 4 mL/column of 0.1 M NPPC, pH 8 was added. NPPC competed with the immobilized DPPC for the antibody combining site, thus stripping antibody from the column. The antibody-NPPC mixture was dialyzed extensively in PBS buffer to remove NPPC. The dialysis tubings were treated with sodium bicarbonate and EDTA to eliminate bacterial growth.

Yield. The approximate concentration of each cell line was calculated spectrophotometrically taking the molar absorptivity to be $1.4 \text{ mL cm}^{-1} \text{ mg}^{-1}$ (Harlow and Lane, 1988). The N series gave very poor yield: 0.32 mg of N9 and 0.8 mg of N8. Among the D series, D9 gave the best yield of 40 mg, D18, 14.8 mg; and D16, 5.6 mg.

RELATIVE AVIDITY DETERMINATION

Affinity Assay

The solid phase assay ELISA (Engvall and Perlman, 1971) was used to test the ability of the hybridomas to produce active antibodies in vitro before expanding these hybridomas in mice. The basis of this type of assay is shown in Figure 8. The procedure is as follows: The solid-support in the form of a 96 well polystyrene plate was coated with 50 μL of 1 $\mu\text{g/mL}$ of the antigen PC-histone at pH 9.6. The plate was incubated overnight at room temperature in a humid chamber. The liquid was removed from the wells by shaking. Since the solid support adsorbs protein non-specifically, the blocking reagent, 1% BSA in PBS buffer, was added and incubated for one hour to prevent the nonspecific adsorption of the antibody to the solid support. The plate was then washed

four times with the non-ionic detergent Tween 20, and then four times with phosphate buffer saline (PBS) made up of 8 g NaCl, 0.2 g KH_2PO_4 , 2.16 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g KCl, 0.2 g NaN_3 in 1 L at pH 7.4. The background binding to BSA-coated plates was determined for each antibody. The antibody to be screened was used both undiluted and diluted 1:2 in PBS-1% BSA, pH 7.4, added in 50 μL aliquots, incubated at room temperature for one hour in the humid chamber and washed as before. A polyclonal antibody directed against the constant regions of the tested antibody and covalently bound to an enzyme specific for a chromogenic substrate was diluted 1:1000 to 1 $\mu\text{g/mL}$ into PBS containing 1% BSA, added in 50 μL aliquots and incubated for one hour and then washed as before. The enzyme used was alkaline phosphatase, and the substrate was pNPP (p-nitrophenyl phosphate) which was dissolved in diethanolamine buffer, pH 9.8 just before use. The reaction was stopped after 30 min by adding 60 mM EDTA. The concentration of the antibody present was proportional to the OD at 405 nm, the wavelength at which p-nitrophenol absorbs. ELISA assays were read using a spectrophotometer adapted for microtitre plates.

Affinity Test of Purified Ascites. ELISA was done on purified ascites for two reasons. First, it was necessary to check the activity of antibodies in both the light and heavy chains. Duplicate measurements were done with a different secondary antibody; one specific for the heavy chain (anti $\gamma 2b$) diluted 1:500, and one to check the activity of the light chain (anti λ), diluted 1:300. The antibody M3C65 tissue culture supernatant was used as a control. The second reason was to assay over a wide range of antibody concentration to determine the concentration that will give an absorbance of 0.5 relative to the background. This value is important for determining the relative affinity constants. The five ascites were used in duplicates of the following concentrations: 10, 1, 0.1, 0.01 $\mu\text{g/mL}$. From the plot of OD versus concentration, the concentration of the antibody at 0.5

absorbance was computed. These concentrations in $\mu\text{g/mL}$ gave OD = 0.05 for D9, 0.02; for D16, 0.01; for D18, 0.72; for N9, and for N8 0.33.

Relative Avidity Determination by ELISA

A competitive inhibition immunoassay based on ELISA was used to assess the relative affinity among the series of antibodies for a given ligand. The two ligands used as inhibitors were PC-BSA and NPPC. Following a standard ELISA procedure and varying the antibody concentration, a standard curve was constructed to check that the absorbance change was linear through the concentration range. Taking the concentration of the antibody that gave an absorbance of 0.5 relative to the background as 50% as one concentration, three other concentrations were used: a double of that concentration as the 100%, 1/5 as the 10%, and 1/50 as the 1%. The standard procedure for ELISA was followed using PC-histone as the coating antigen. The only difference was that the inhibitor was added at each concentration, while the concentration of the antibody was kept constant and equal to 50% (see above). A range of the inhibitor NPPC concentrations from 10^{-2} to 10^{-6} M was added with the antibody in one triplicate measurement. In another triplicate measurement, the other inhibitor PC-BSA was added also in a range from 10^{-6} to 10^{-10} M. Following this incubation, the second antibody was added, and color was developed and analyzed by the usual system.

A plot of the binding percentages, which was directly proportional to absorbance, versus concentration of inhibitor, gave relative binding affinities (I50 values). I50 values represent the molar concentration of free hapten (NPPC) or hapten bound to the carrier (PC-BSA) required to inhibit the binding to PC-histone by 50%. An example of these measurements is shown in Figure 9. Table I shows the I50 values for the five antibodies purified with their respective V-gene usage.

TABLE I
V-GENE USAGE AND I50 VALUES FOR PC-PROTEIN BINDING HYBRIDOMAS

D9	γ 1	κ 1	3.9 E-7	1.3 E-5
D16	μ	κ 1	1.8 E-7	5.2 E-4
D18	γ 1	κ 1	1.8 E-7	4.9 E-6
N8	γ 3	κ 1	4.0 E-10	4.1 E-4
N9	γ 3	κ 1	6.0 E-10	7.1 E-4

It is interesting to note the difference in the I50 values between D16 and D18 in their binding to NPPC but not the PC-BSA. D16 is a germ line clone that appears in the early response, while D18 which is a sister clone that was produced in the same fusion but has several mutations in the V region that alter its ability to bind NPPC appears in the secondary response.

Affinity Determination by Fluorescence Quenching

This technique is of great sensitivity (in the order of 1.0^{-8} M of antibody is needed). Fluorescence quenching is a liquid phase assay that gives a direct measurement of binding affinity. Quenching is the reduction of fluorescence by a competing deactivating process, that results from interaction between a fluorophore and another substance. The antibody's tryptophan residues are quenched upon binding to the hapten as a result of energy loss from excited tryptophan residues to aromatic systems by charge transfer. The quenching is increased upon successive addition of the hapten. The excitation and emission wavelengths were 295 and 345 nm, respectively. 25 μ L aliquots

from stock solution of 10^{-4} M NPPC were added with stirring in 10 steps to 2.9 mL antibody samples. Maximum quenching occurs when the binding sites are saturated. This is obtained by linear regression analysis of the reciprocal of quenching vs the reciprocal of ligand concentration ($1/Q$ vs $1/[L]$). The binding constant K is equal to the negative slope of a Scatchard plot of r/L vs r where r reflects the extent of the reaction and L is the concentration of the free ligand. The affinity constants for four of the antibodies were determined for NPPC. These results are given in Table II.

TABLE II
AFFINITY CONSTANTS DETERMINED BY FLUORESCENCE QUENCHING

HYBRIDOMA	AFFINITY CONSTANT K $\times 10^4 (M^{-1})$
N8	1.3
N9	1.7
D16	1.6
D18	4.9

FAB PREPARATION AND PURIFICATION

The reason for generating the Fab fragment is that it has a smaller molecular weight, and thus easier to study by NMR. Another reason is the Fab has a reduced degree of aggregation and precipitation due to deletion of Fc. This fragment, however lacks the ability to kill cells because it cannot activate complement. Activated complement components mediate cytolysis and opsonization. The Fab fragment also has lower avidity due to the loss of bivalency. In IgG molecules, the most susceptible region for proteolytic cleavage is the hinge located between the constant regions C_H1 and C_H2 of the heavy

chain. Enzymatic digestion with papain gives two identical antigen-binding fragments (Fab) and the Fc shown in Figure 1. Papain is a nonspecific protease with a sulfhydryl group in the active site. IgG molecules are incubated with papain in the presence of a reducing agent to keep the enzyme active in the reduced form.

Materials Needed

The materials were supplied by Pierce ImmunoPure Fab Kit, and details of their chemical identities are not available. These are binding buffer, elution buffer, AffinityPak prepacked columns of immobilized protein A coupled to agarose, immobilized papain, cysteine, and phosphate buffer to prepare digestion buffer the day of use.

Fragment Generation

The procedure below is taken from the Pierce standard protocol of generation and purification of Fab fragments from IgG. D18 was the antibody used. 4 mL of 3 mg/mL of D18 were dialyzed (3×2 L) against a 20 mM phosphate / 10 mM EDTA buffer at pH 7.0. The solution was then concentrated to 20 mg/mL by ultrafiltration. The immobilized papain was equilibrated and washed with digestion buffer, then resuspended in 0.5 mL of buffer. The solution of 0.5 mL of antibody and an equal volume of digestion buffer were added to the immobilized papain. The mixture was incubated overnight in a shaker water bath at 37°C. The crude digest was separated from immobilized papain gel using a supplied separator.

Fab Purification

The protein A column was equilibrated with 13 mL of binding buffer. The crude digest (3 mL) was applied to the column. Protein A, which has the ability to bind to the Fc region and undigested IgG, allowed the Fab fragment to pass through the column. The

column was washed with 6 mL of binding buffer. The eluate (9 mL total) containing Fab was collected.

Analysis

The resulting Fab was analyzed for its purity and activity as discussed below.

Activity. The binding of the Fab fragment was compared to the intact antibody by ELISA. For the Fab, the Fc region is missing, hence a secondary antibody specific to the light chain κ was used. The procedure for direct ELISA was followed as above using a series of dilutions. For the Fab, 50, 20, 10, 1, and 0.1 $\mu\text{g/mL}$ were used. The same color reaction as before was observed as expected for active antibodies. A higher concentration of Fab was needed to give the same color reaction as the intact antibody. This was expected because the Fab has only one binding site.

Purity. Liquid chromatography was done using Sephacryl 200 on both the intact antibody and the Fab for comparison with standards for molecular weight determination. The results obtained for both were unreliable because it seemed that the protein was interacting with the gel, and hence no clear separation was observed.

The cleaved Fab fragments were analyzed under reducing conditions by SDS-PAGE and compared to intact IgG. The papain generated Fab fragment showed a major band on the gel at 50,000 D (data not shown). The Fab was further purified over a Sephadex G-25 column and eluted with PBS buffer. NMR spectroscopy was used to confirm that the small organic molecules were removed from each sample.

Yield. Spectrophotometric determination of concentration with molar absorptivity of 1.48 gave a mass of 3 mg Fab. A more reliable method for protein concentration determination is the Bradford's assay. Following the procedure outlined in Bradford, (1976), the mass of Fab determined was determined to be 2.2 mg.

PREPARATION OF FV FRAGMENT

Cloning and expression of active antibody fragments in bacteria is an alternative technique to hybridoma production because of the relative ease of bacterial fermentation (Cheadle et al, 1992). This also allows the use of recombinant DNA technology, which is better suited for studies of fragments than intact antibodies. For physicochemical studies such as NMR, the Fv is a very useful fragment. *E. coli* has been extensively used for the expression of Fv fragments. This portion of the work is done exclusively by people in the lab of our collaborator, Dr. Rittenberg, where more work is still in progress for optimizing the yield. The discussion that follows is a brief overview of the procedure that was used (Cheadle et al., 1992). The approach that worked so far was the coexpression of the heavy and light chain fragments as a single chain Fv (sFv), in which recombinant VH and VL were linked by a polypeptide spacer region.

The steps involved in cloning are the following. SFv gene was assembled from the individual synthetic VH (M141) and VL (λ 1) genes using polymerase chain reaction (PCR) techniques. Coding cDNA molecules were made from the poly(A) + cytoplasmic mRNA population isolated from mouse hybridoma cells. The specifically primed cDNA was used as a template for amplification using PCR. Plasmid DNA from these clones was transformed into an *E. coli* strain BL21(DE3) competent cells. These constructs included pET sFv where both VH and VL were derived from synthetic assembly origin. The second step is protein synthesis in inclusion bodies followed by recovery in 8 M urea and refolding by chaotrope removal. The final step was affinity purification of the Fv fragments. For this preparation, the sFv of M3C65, a Group II B antibody was used.

INTRODUCTION TO PC-ANALOGS

Small molecule models for PC-KLH differing in size and charge were used to evaluate the influence of antibody binding on PC-based hapten and to determine the epitope(s) recognized by Group II antibodies. The small molecule models were phosphocholine (PC), p-nitrophenylphosphocholine (NPPC), p-nitrophenyl-3,3-dimethyl butylphosphate (NPDBP), p-fluorophenylphosphocholine (FPFC), and p-trifluoromethylphenylphosphocholine (TFPPC). Amino acids that were coupled to PC were tyrosine, histidine, lysine, and valine. Diazophenylphosphocholine (DPPC) was the coupling agent used to couple phenyl-PC to amino acids, peptides, and proteins. DPPC can react through its diazonium functional group with the phenol of tyrosine, the imidazole group of histidine at either the C₂, or both C₂ and C₄, the sulfhydryl group of cysteine, and primary α and ϵ amino groups. Secondary reactions involving guanidino groups of arginine and indole groups of tryptophan are possible as well. The different amino acids were chosen to be coupled to PC in order to understand the effects of the heterogeneity in peptide-PC and protein-PC molecules. The peptides used were Gly Tyr Ala, and Gly His Gly, and an octapeptide with a single Tyr residue in the middle of its sequence. The proteins of choice were neurophysins (NP-I and NP-II), and ubiquitin (Ub).

SYNTHESIS OF HAPTENS

Materials

The ligands PC and NPPC were obtained from Sigma. NPDBP was supplied by Dr. Rittenberg's lab and was synthesized as described by Bruderer et al. (1989), shown in this scheme:

NPDBP

Synthesis of FPPC

The purpose of the fluoro group in serving as a mimic for the nitro group is in the use of ^{19}F NMR to probe the binding interactions between hapten and PC-KLH antibodies. This can be done by observing the chemical shift of the ^{19}F nucleus of the fluoro group of the hapten. ^{19}F NMR is particularly useful for studying ligand-macromolecule interactions because the ^{19}F nucleus is very sensitive to environmental changes (Gerig, 1989). Also, ^{19}F - ^1H NOEs transferred from ligand to protein is useful in locating the binding site of ligands (Cairi and Gerig, 1985). FPPC was prepared by modifications and combinations of procedures described by others (Turner and Khorana, 1959; Chesebro and Metzger, 1972). As a summary of the synthetic steps, the following reaction scheme is given

p-fluorophenylphosphorodichloridate. 10 gm of p-fluorophenol (Aldrich) was changed to sodium p-fluorophenoxide in the presence of excess NaOH (23 mL of 10 N). The phenoxide was filtered and dried over phosphorous pentoxide for at least a week.

The resulting finely powdered solid (5 g) was further dried at low pressure, then slowly added at 0°C to stirred phosphorous oxychloride (Aldrich) in a flask equipped with a stirrer, reflux condenser, and a N₂ bubbler which provided dry atmosphere. A vigorous reaction occurred and was moderated by cooling in an ice bath. When addition was complete half an hour later, sodium chloride was removed by vacuum filtration. The unreacted phosphorous oxychloride was removed by rotary evaporation, and the residue was finally maintained at 10-15 µm for one hour. The resulting oil was distilled in a short-path apparatus at 30 µm. Three fractions were collected. The third fraction was the desired product that had a boiling point range of 91-105°; yield: 0.85 g.

p-fluorophenylphosphocholine. 1 g of choline iodide (Sigma), p-fluorophenylphosphodichloridate (0.85 g; 3.7 mmoles), and dry quinoline (516 µL; 4 mmoles) were dissolved in 3 mL of dry acetonitrile, mixed, and stirred at 0°C in the dark for 8 hr. Then 2.0 mL of pyridine and 400 µL of water were added, and the solution was incubated at room temperature for 2 hr. The solvents were removed by rotary evaporation. The viscous residue was then dissolved in 10 mL of water, and the pH was adjusted to 7. The solution was passed through a 70 mL TMD-8 (Sigma) column equilibrated with water. TMD-8 is a mixed-bed resin that has attraction for charged substances, and changes color when exchange is reached to capacity. At neutral pH, p-fluorophenylphosphorylcholine is the only neutral substance that will elute with the water. The column was washed with 100 mL of water. The pooled and lyophilized white crystals weighed 110 mg. The pure product was stored at 4°C in the dark.

Analysis. The product was analyzed by thin-layer chromatography on cellulose (Analtech) using the mobile phases isopropyl alcohol-NH₄OH-H₂O (7:2:1, v/v), which gave a single component as detected by fluorescence quenching.

With respect to NMR analysis, ¹⁹F, ³¹P NMR spectra (²H₂O) were obtained; these spectra are shown in Figure 10. The ¹H spectrum shown in Figure 25 of Chapter V

showed a singlet (9H), and two multiplets (2H) characteristic of the choline portion and extensive splitting in the aromatic region partly due to through bond coupling from the F and P nuclei. The ^{19}F spectrum showed a multiplet (triplet of a triplet of a doublet, with a coupling constant of 7 Hz, 4 Hz, and 1.8 Hz) due to coupling from the 2,6 H, 3,5 H and ^{31}P . ^{31}P spectra were obtained both as proton-coupled and proton decoupled. The decoupled spectrum showed a doublet of 1.8 Hz due to long range coupling from the F nucleus. Proton decoupled ^{19}F spectrum was also acquired (not shown) that showed a doublet due to splitting from ^{31}P nucleus. The ^{19}F and ^{31}P nuclei are coupled to each other because they are connected by an intervening conjugated network.

Synthesis of TFPPC

The trifluoromethyl group, chosen as a mimic for the nitro group, is also useful because ^{19}F NMR can be used to probe the binding interactions between hapten and PC-KLH antibodies. The CF_3 group has an additional advantage over the F group in having more intensity from three F nuclei instead of one. Also, the overlap in the aromatic region of the ^1H spectrum of FPPC is eliminated. TFPPC was prepared the same way as FPPC, with 5 g of trifluoromethylphenol (Aldrich) as the starting material. 1.84 g of anhydrous phenoxide was recovered and treated with 9.8 mL of POCl_3 . One fraction was collected after distillation with boiling point of 120°C at less than 100 μm . The product was viscous, waxy liquid, with a tinge of purple color probably due to slight decomposition. Trifluoromethylphenylphosphodichloridate weighing 380 mg (1.5 mmoles) was added to 0.4 g of choline iodide, and 194 μL (1.5 mmoles) dry quinoline, in 374 μL dry acetonitrile as above. The product obtained after mixed bed chromatography was further purified from free choline by thin-layer chromatography on cellulose plates.

NMR Analysis

^1H , ^{31}P NMR spectra ($2\text{H}_2\text{O}$) were obtained as shown in Figure 11. The ^1H spectrum showed a singlet (9H), and two multiplets (2H) characteristic of the choline portion and a double of doublets in the aromatic region. The ^{19}F spectrum showed a single peak (Figure 32E of Chapter V). ^{31}P spectra were obtained both with proton coupled and proton decoupling. The decoupled spectrum showed a singlet as an evidence that little or no long range coupling to the ^{19}F is occurring.

SYNTHESIS OF AMINO ACIDS-PC

To evaluate the feasibility of assigning phenylphosphocholine resonances in hapten-protein conjugates, and the contribution from the carrier molecule, coupled amino acids were prepared. The amino acids that were coupled to PC were Tyr, His, Lys, and Val. The amino acids were first N-acetylated (except for Val) because diazonium salts can react with free amino groups (Vaughan and Ahern, 1973). In the Val case, the reaction of the N-terminal was desired. For all these reactions, the same coupling reagent, p-diazophenyl phosphocholine (DPPC) was used. DPPC was synthesized from p-aminophenylphosphocholine (APPC) purchased from Sigma as described by Rodwell et al., (1983) and illustrated in this equation

Tyrosine-PC

This molecule served as a model for the immunogen PC-Protein. It was important that the PC should only label the position ortho to the hydroxyl group on the phenyl ring. Two other possible sites are the free amino and carboxyl groups. To eliminate these possibilities, both N-acetyl tyrosine (N-AcTyr) and N-acetyl tyrosine ethyl ester were used.

Preparation of Mono-(p-Azophenylphosphorylcholine)-N-Acetyl-L-Tyrosine. This reaction was done at alkaline pH with the amino acid in large molar excess (Chesebro and Metzger, 1972). 0.1 mmole of N-Ac Tyr (Sigma) (23 mg) was treated with 0.01 mmole of p-diazonium phenylphosphorylcholine (DPPC) (2.82 mg) in 3.0 ml 24 mM borate buffer (pH 9) with continuous stirring at room temperature for 12 hours. The pH was then adjusted to 7.0 and the reaction mixture lyophilized. The resulting solid was dissolved in methanol and developed on thin-layer cellulose Analtech 250 μ plates using an isopropyl alcohol-ammonia-water (7:2:1) mobile phase. Two bands were detected by UV quenching, one with R_f value of 0.6 (unreacted N-Ac Tyr) and 0.3 for the colored N-Ac Tyr-PC. The yellow product was obtained by scraping and eluting with water. The eluent was lyophilized, then passed over Sephadex G-10 equilibrated with water. The following reaction took place:

where Ph-PC is

The same synthesis procedure was followed for the N-Ac Tyr ethyl ester (Sigma). The ester portion underwent transesterification when mixed with isopropyl alcohol in the purification stage. This was not important however because the free carboxyl group does not react with the haptenation reagent under my reaction conditions, as shown by the pure monolabeled product formed above, and thus N-Ac Tyr became the target compound for subsequent larger scale preparation and all binding studies.

NMR Analysis. A very clean ^{31}P proton decoupled spectrum showing a single resonance was an evidence of a pure monolabeled product as shown in Figure 12A. More conclusively, the ^1H NMR spectrum showed the choline moiety and the characteristic aromatic portion very clearly in Figure 12B. Peaks A and C correspond to the protons of the phenyl ring of Ph-PC. 2D nuclear Overhauser effect (NOE) spectrum was obtained to

assign peaks without ambiguity, even with the overlap of the nine proton singlet of the choline with one of the β protons of the Tyr. The assignments are shown in Figure 13.

Histidine-PC

His-PC is another hapten that models the PC-histidyl linkage to KLH. There are three possible sites for the reaction, at the N terminal, at the C2, and at the C4 of the imidazole ring. Since our interest was in modeling the imidazole linkage, the N terminal site was blocked.

Preparation of Mono-(p-Azophenylphosphorylcholine)-N-Acetyl-L-Histidine. 400 mg N-Ac His (Sigma) was treated with continuous stirring with 16 mg DPPC added in 8 aliquots of 100 μ L of 20 mg/ml stock solution in 8 mL of BBS at pH 8.5, to make a 35 fold excess of N-Ac His. The reason for this large excess was to minimize the reaction at the C₄ of the imidazole ring. The reaction was allowed to go for 3 hours in the dark. The pH was then adjusted to 7.0 and the reaction mixture lyophilized. The resulting solid was dissolved in methanol and developed on 3 thin-layer cellulose Analtech 1000 μ plates using isopropyl alcohol-ammonia-water (7:2:1) mobile phase. The same plates were developed three times for better separation. Two bands were separated, eluted from the cellulose with water. Final purification was done by elution through a column of Sephadex G-10 with water. The following is the reaction scheme:

NMR Analysis. NMR analysis showed that the faster moving band on the TLC plates was the desired product, while the other bands showed spectra of a hydrolyzed product and a product labeled at both C₂ and C₄. The aromatic region of the second product showed evidence of a mixture of double and single labeling (not shown). This impure product was rerun over TLC plates twice. A weak brownish band eluted first, followed by a yellowish broader band, with a streaking background of brown color. After isolation and subsequent purification through Sephadex G-10 as above, NMR analysis of ³¹P and ¹H spectra showed that the second band was the desired pure product, as shown in Figure 14.

The presence of broad peaks mainly in the aromatic region, which broaden as the concentration was increased, was an evidence of self-association of His-PC. A very dilute sample gave sharper peaks, and so coupling of the 1,4 disubstituted phenyl group in the aromatic region was observed. This sample was too dilute to allow reliable NOE analysis. Use of acetonitrile as a cosolvent proved to be efficient in breaking up the self-association. 42% acetonitrile gave what seems to be a monomer with sharp peaks and resolved

coupling in the aromatic region, showing homogeneous monolabeling at C₂ of the imidazole ring, as shown in Figure 15.

Lysine-PC, Valine-PC

Coupling the diazo linkage to an α or ϵ amino groups are two other ways that PC might couple to KLH and cause an immune response in immunized mice. N-Ac Lys was used (Sigma) to restrict the reaction to the ϵ amino group. Val was chosen because it does not have functional groups other than the N terminus, to restrict the coupling to the α amino group. The above procedure for preparation and purification was followed identically. However, neither expected triazene was formed in high enough quantity and purity for detailed NMR analysis. An evidence of formation of product from NMR was the presence of the characteristic singlet around 3 ppm, and the appearance of broad and non-resolved peaks in the aromatic region. Another evidence is the yellow color of the obtained product. The possible reaction to the free amino group is illustrated in the following reaction:

SYNTHESIS OF PEPTIDES-PC

The tripeptides Gly Tyr Ala and Gly His Gly, purchased from Sigma were coupled to PC using the same coupling agent as before. The octapeptide N-Ac Ala Gly Ala Gly Tyr (PC) Ala Gly Ala, was custom synthesized.

Gly Tyr(PC) Ala

Preparation of Mono-(p-Azophenylphosphorylcholine)-N-Trifluoroacetyl-GY(PC)A. N-terminal protection was done by reacting 50 mg of the tripeptide with a 50 fold excess of ethyltrifluorothioacetate (ETFA) as the blocking agent in 7 mL of BBS buffer at pH 9.5 overnight with continuous stirring. Excess reagent was removed on a rotovap, and then the residue was passed over Sephadex G-10. The protected peptide was separated from the unreacted tripeptide by chromatography on 1000 μ Analtech cellulose TLC plates. The product was eluted with water, then dissolved in methanol-water mixture and developed in isopropyl alcohol, ammonia, water mixture. Two bands were detected. Both were checked by ^{19}F NMR in $^2\text{H}_2\text{O}$ solvent to observe the fluorine signal. The faster band belonged to the protected peptide. The unreacted material was treated again by the same procedure. To maximize the yield, this step was repeated 4 times. Some hydrolyzed product was observed as yellowish trails that stuck to the cellulose support. Apparently the trifluoroacetyl group is not ideal for protecting the terminal amine. For this reason, another group might be used for future synthesis of N-protected peptides.

The reaction with DPPC was done in the dark at pH 9.0 in 4 mL BBS and 140 μL DPPC. The reaction mixture was left stirring for 8 hours at room temperature. Then the pH was adjusted to 7.0 and the mixture was lyophilized. The crude product was dissolved in methanol and loaded over 1000 μ and 500 μ TLC plates. Three bands were detected with the following R_f values: 0.9 for the protected amino group that was not coupled to

PC; 0.73 for the native peptide, this fraction was protected and mixed with the first band for PC coupling; and 0.6 for the broad band of the pure protected and PC monolabeled peptide. The total yield was 5 mg. The product was then stored at 4°C in the dark as a 14 mM solution.

NMR Analysis On The Pure PC-Peptide. ^1H and ^{31}P showed a pure monolabeled product (not shown). From a COSY spectrum, the Ala and Tyr spin systems were assigned unambiguously. The 2 α -protons of Gly were bleached under the residual water resonance as shown in Figure 16.

Gly His (PC) Gly

The same procedure as above was followed to form Gly His (PC) Gly starting, by blocking the N-terminus. Coupling with DPPC introduced an additional complication because of the presence of two sites for labeling, and hence caused lower yield for a monolabeled product and difficulty in purification. The same chromatography method was used for purification. No satisfactory NMR analysis was done on the product because of the low yield and impurity. However binding studies that may not require absolutely pure products, were performed and analyzed in Chapter VI.

SYNTHESIS OF PROTEINS-PC

The reason for generating homogeneous proteins-PC is eventually to use them as antigens that are small enough to be studied by NMR, and yet act as models for more complex proteins typically used to immunize mice to generate antibodies. Every kind of biological molecule can serve as an antigen, however, only macromolecules can initiate lymphocyte activation necessary for an antibody response (Stenzel-Poore et al., 1988). In order to generate antibodies specific for small molecules, these small molecules are covalently linked to macromolecules such as KLH before immunization. The following

model antigens were used to provide an experimental probe of antibody diversity and specificity of the immune response. Ub was available from Sigma. NP-I and NP-II were isolated from lyophilized bovine pituitaries (discussed in detail in Chapter II). The same coupling reagent is used as above. Diazonium salts readily couple with proteins to form colored derivatives at alkaline pH 8 or 9, in borate buffer. Several attempts were made to optimize the conditions of the reaction to get homogeneous product. Several methods were tried for purification. Below is a brief overview of the method that gave the best result for preparation of protein-PC. This procedure is outlined for only one protein; the others followed a similar path.

Protein-PC

NP-II is a 10 KDa protein that has one tyrosine and no histidines. Free amine groups on the protein (two lysines and the terminal amine) offer other sites for reaction. Another possible interference is from the seven guanidino groups from the arginines.

Blocking of Amino Groups. 40 mg NP-II were dissolved in 4 mL of 0.14 M phosphate buffer (prepared from 2 N stock solution of sodium phosphate, 6.9 NaH₂PO₄·H₂O, 13.4 g Na₂HPO₄·7H₂O in 500 mL) at pH 8.5. 80 µL (50 fold excess) of the N-blocking reagent, ethyltrifluorothioacetate (ETFA) (Ludblad, 1984, Fanger and Harbury, 1965) was added in 4 aliquots over one hour period, constantly adjusting the pH with 5% NaOH. Once the pH stabilized (after about 2 hours), the reaction mixture was passed over Sephadex G-25 to remove mercaptan and excess ETFA. This is illustrated in the following scheme:

The desired fractions were then pooled and concentrated to 2 mL. It is recommended not to lyophilize ETFA derivatives due to polymerization problems (Ludblad, 1984). The separation of partially modified NP from the completely modified was done by chromatography on the cation exchanger carboxymethylcellulose (CM) in phosphate buffer. ETFA modification made NP more anionic so it would not stick to the cation exchange column, and it eluted first, followed by monomodified and native NP. The concentration gradient used was 0.01-0.08 M phosphate at pH 7.6. The first fraction was pooled and concentrated. Since ^{19}F NMR was used to monitor the modification, three peaks were expected for complete modification of the two lysines and the terminal amine. Figure 17 B and C shows ^{19}F NMR spectra of both NP-I and NP-II. NP-I showed a purer product due to a more resolved separation on ion exchange (not shown).

Blocking of Guanidino Groups. The resulting product was mixed with 8 mL of borate buffer at pH 7.5. 3.6 mL of 10% 2,3 butanedione was added (150 molar excess). The pH was adjusted to 7.5 and the reaction was left overnight in the dark (Riordan, 1973). The resulting mixture was passed over Sephadex G-25 in PBS buffer. The protein fraction was pooled and concentrated. Figure 17A shows the ^1H NMR spectrum of NP-II with Arg blocked. Notice the extra sharp methyl peaks. This is the scheme followed:

DPPC Coupling. The purified blocked material was then reacted with 0.6 mg of the haptening reagent in 2 mL borate buffer, pH 8.5 with the protein in 2 fold excess. The reaction was allowed to go for 2 hours at 4°C. The resulting mixture was neutralized to pH 7.5 and passed over Sephadex G-25 for desalting. The final purification was done by affinity chromatography using immobilized anti-PC antibodies. Figure 18 shows evidence of PC-linking at the tyrosine as shown by extra peaks of the phenyl PC ring in the aromatic region. This can be seen by comparing to the aromatic region of Tyr-PC in Figure 13. It should be noticed that after all these modification procedures, the protein retained its tertiary structure, as shown by the overall form of the 1D spectrum.

MATERIALS AND METHODS FOR NMR ANALYSIS

Sample Preparation

The antibodies were prepared as described in Chapter III. The antibodies were supplied by the Rittenberg lab as 3 mL aliquots of 1.65 mg/mL concentration for PCG1-2 and PCG2b-3 and at varying concentrations of the Fv. This volume was concentrated to

40 μM by ultrafiltration on a membrane with M_r 30 KD cutoff (Amicon YM-30) for the antibody and 5K(Amicon YM-5) for the Fv. Successive exchanges with $^2\text{H}_2\text{O}$ were done in 400 μL of $^2\text{H}_2\text{O}$ in deuterated PBS buffer (137 mM NaCl, 1.5 mM KH_2PO_4 , 8 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 3 mM KCl, 3 mM NaN_3 , pH 7.4). Antibodies and their fragments denatured upon freezing and lyophilization so this method of solvent drying was avoided. A typical NMR sample contained 450 μL of 40 μM intact antibody and 0.1 to 0.6 mM Fv. The protein concentration of the Fv was determined from the protein's absorption at 280 nm (molar absorptivity of $54 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$). Spectra were acquired at 40°C for the antibody and at 25°C for the Fv. The molar ratios of ligand to antibody were on the average of 50 : 1.

NMR Measurements

COSY and NOESY spectra were performed on a Bruker AMX-400 spectrometer in the phase-sensitive mode using a proton-selective probe. Most of the NOESY spectra were acquired as duplicates and sometimes triplicates of 80 ms, 100 ms and 300 ms mixing times for the intact antibody and 350 ms for the Fv. The HDO line is presaturated using high power for 2 sec during the relaxation delay before the 90° pulse is applied. The data was acquired with a spectral window of 4808 Hz. For most of these samples, a total of 242 t_1 values were obtained and free induction decays for t_2 were recorded in 1024-point blocks, summing 84 acquisitions each. The data sets were zero-filled to 512×1024 data matrix.

One-dimensional NOE difference experiments were performed using a preirradiation time of 2 or 4 s in the sequence $[-\tau_{\text{wt}} - \tau_{\text{irrad}} - (\pi/2)_{\text{obs}} - \text{FID}]_n$ or the inversion recovery sequence $[-\tau_{\text{wt}} - (\pi)_{\text{select}} - \tau - (\pi/2)_{\text{obs}} - \text{FID}]_n$. The $\pi/2$ pulse reads the state of the system to measure the size of the NOEs. At least 128 scans were accumulated

for each irradiation frequency, by cycling through the decoupling list after each 16 or 32 scans, and 5-Hz line-broadening factor was applied before Fourier transformation.

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Figure 7. The general procedure for monoclonal antibody production in mice (Campbell, 1991).

Figure 8. The basis of the ELISA assay. (Top) The Mab that binds to the antigen attached to the solid surface is detected by a second antibody directed to the Fc region. (Bottom) A comparison between direct and competition ELISA. The free antigen competes with the plate bound antigen for the antibody and prevents it from being detected by the second antibody (Campbell, 1991).

Figure 9. Binding curve of D18 with PC-BSA as an inhibitor. The vertical axis represents binding to the antigen, PC-histone. The I50 value is the concentration of the inhibitor in M that gives 50% inhibition.

Figure 10. ^{31}P decoupled (A), coupled (B) and ^{19}F spectra of FPPC (C). The ^{19}F spectrum shows a triplet of a triplet of a doublet with coupling constants of 7 Hz, 4 Hz and 1.8 Hz.

Figure 11. ^{31}P decoupled (A), coupled (B) and ^1H spectra of TFPPC (C).

Figure 12. Spectra showing monolabeling of Tyr-PC. (A) ^{31}P spectrum, (B) unreacted N-AC-Tyr, (C) Tyr-PC.

Figure 13. NOESY of Tyr-PC. Only the cross peaks are plotted because they are of opposite sign to the diagonal (characteristic of a small molecule). The mixing time used was 500 ms and a temperature of 40°C. All primes indicate protons of the Tyr residue.

Figure 14. Spectra showing monolabeling of His-PC. (A) ^{31}P spectrum, (B) ^1H spectrum of 0.4 mM concentration at 40°C.

Figure 15. Acetonitrile titration of His-PC. (A) 21%, (B) 30%, (C) 40%;
Note the increase in resolution between 7 and 8.5 ppm.

Figure 16. Spectra of Gly Tyr (PC) Ala. (A) ^1H , (B) Magnitude calculation COSY spectra showing the assignments.

Figure 17. Spectra showing blocking of amino and guanidino groups. (A) ^1H NMR spectrum of NP-II after amino and guanidino blocking. The arrow points to the methyl peak of the blocking reagent. (B) and (C) are ^{19}F NMR spectra of NP-I and NP-II respectively. Notice the better resolution in NP-I.

Figure 18. ^1H NMR spectra of NP-II before (A) and after (B) linking it to PC. Note the changes in NP-II-PC:

- * Sharp peaks between 7.3-8.2 ppm labeled by *.
- * Disappearance of NP tyrosine aromatic protons around 7 ppm.
- * Presence of the nine proton singlet of the choline at 3.2 ppm.

CHAPTER V

ANTIBODY-HAPTEN INTERACTIONS

Nuclear Overhauser effect (NOE) studies were performed on some of the molecules synthesized to establish whether the haptation produces a “bent” structure and to understand the overall conformation of the ligands especially when the hapten is covalently attached to a carrier protein, peptide, or amino acid. This conformation in the free ligand was compared to the conformation of the bound antibody. The difference observed gives information about the restrictions imposed by the antibody upon binding, and hence about the structure of the antibody combining site. A variety of model haptens PC, NPDBP, NPPC, FPPC, TFPPC were used throughout this work to probe the antibody diversity and specificity.

CONFORMATION OF PC

The conformation of phosphoryl choline (PC) was studied in detail by others. These studies showed that O-C-C-N system prefers a gauche conformation. In the choline moiety, the dihedral angle around the C-C bond is distorted from the usual gauche angle, 60°, to a larger one (Dufourcq and Lussan, 1972). The ethanolamine group in comparison is freely rotating and therefore does not give rise to any restriction in conformation. Earlier studies suggested that there is an electrostatic interaction leading to restriction of molecular mobility in PC (Akutsu and Kyogoku, 1977, Richard et al., 1974).

Binding to the Group IIB Antibody M3C65 (γ 2b, λ 1)

M3C65 is Group IIB antibody that does not bind to PC, which lacks the phenyl moiety, according to solid phase assays. Since NMR is more sensitive than ELISA to

lower binding constants, NMR was used to test for binding. This is done by monitoring the broadening of the hapten peaks as it is titrated with the antibody. Line broadening is generally observed in NMR spectra of excess ligand bound to macromolecules if the exchange rate is fast enough between free and bound ligand. This broadening is caused because of the restricted mobility upon binding that leads to short values of the transverse relaxation time. PC (0.15 mM) was titrated with an increasing amount of the antibody M3C65, from 100 fold excess to 5 fold excess of the hapten. There was no evidence of line broadening nor chemical shift difference, indicating PC does not interact with the antibody M3C65 even at this high concentration (0.03 mM) as shown in Figure 19.

CONFORMATION OF NPPC AND NPDBP

Free Haptens

These haptens were synthesized as described in Chapter IV. They differ in the choline moiety where NPDBP has a carbon atom in place of the charged ammonium ion. These two haptens were used to probe the effect of charge on the structure of the hapten and its subsequent effect on antibody binding.

Molecular Conformation Calculations. A calculation according to the MM2 protocol was performed to obtain the energetics for the bent and extended structures for NPPC and NPDBP shown in Figure 20. Calculations were performed in both geometries for both molecules in the absence of solvent in order to obtain first approximations of the structure. The distance between the aromatic ring and the trimethylammonium end of the NPDBP molecule in the extended conformation was found to be about twice that of the bent conformation. The bent structure for NPPC was calculated to be ~ 0.5 kcal/mol lower in energy than the fully extended structure, while the extended and bent conformations for NPDBP were nearly equal in energy. The calculations of Pullman et al.

(1975) indicated that the PC molecule has a similar conformation to the PC portion of NPPC in that the O-C-C-N system is gauche, and the bond angle was not affected by the presence of a solvent.

NMR Analysis. The averaged solution conformation of the free hapten can be obtained by analysis of the intensities of the 1D NOE spectrum. Figure 21 A, C, E demonstrates the appearance of the ^1H NMR spectra of PC, NPPC, and NPDBP at 37°C and pH 7.0. NPDBP showed a first-order spectrum in the upfield region, including coupling arising from the ^{31}P to the αCH_2 . The ^{31}P appeared as a triplet with $J \sim 7$ Hz as shown in Figure 22A. From this observation it was concluded that the ^{31}P -O-C α H $_2$ -C β H $_2$ spin system corresponds to an open chain system, in which there was sufficient internal mobility to render the methylene protons equivalent within each pair. The fast averaging process due to motion in solution resulted in sharp lines in the case of NPDBP. In contrast, the upfield region of both NPPC and PC ^1H NMR spectra showed a complex set of overlapping multiplets for each methylene pair. This was an indication that averaging is slow and at least on a time scale on the order of the NPPC coupling constants; hence it was concluded that both NPPC and PC have a restricted conformational mobility. The ^{31}P NMR spectrum of NPPC in Figure 22B supported this interpretation because it had the appearance of a distorted triplet. The ^1H NMR spectrum of PC is similar to that of NPPC. Thus, NPDBP, compared to NPPC or PC, was shown to be relatively unrestricted in the internal conformational mobility between its conformers.

To assign which methylene group is next to the N and which is next to the P, a $^1\text{H}/^{13}\text{C}$ inverse detection 2D experiment was done, where the ^{13}C spectrum was displayed on the F1 axis, and the ^1H spectrum was displayed on the F2 axis and the cross peaks arise from coupling between the two. Correlation of the 2D spectrum with the 1D splittings from a $\{^1\text{H}\}^{13}\text{C}$ spectrum showed a difference in splitting between the two

methylenes. The C of the methylene that showed a doublet is the one close to the P, split by the $(2I+1)$ where $I=1/2$ for a spin-1/2 nuclei. The C of the methylene group close to N showed a triplet because N is a spin-1 nucleus.

Figure 21B, D, F shows different spectra resulting from irradiating the nine-proton singlet peak δ of each analog. Positive NOEs were observed from each δ -(CH₃)₃ group to both methylene groups (α CH₂, β CH₂). This showed that these proton sets have averaged positions not more than about 0.4-0.5 nm from the δ -(CH₃)₃ group. A positive NOE ($\sim$ 0.3%) was observed between the ortho-aromatic and the δ -protons of NPPC. The presence of this NOE, combined with the fact that NPPC is conformationally restricted indicated that the major fraction of NPPC existed in a conformation that placed the two ends of the molecules in close proximity.

NPDBP has an NOE ($\sim$ 0.2%) which was $\sim$ 30% less than the NOE of NPPC from the δ -protons to the ortho-aromatic ring protons. Since NPDBP was shown to have a large degree of internal mobility, this NOE presumably arose from a minor fraction of the molecule which had its termini near each other. To eliminate the effect of self association at high concentration, spectra were obtained over a wide concentration range (1-100 mM). Neither the appearance of the spectra of NPPC and NPDBP nor their NOEs changed, indicating that these results did not arise from self-association. The electrostatic interaction between the positively charged nitrogen and the phosphate group was judged to be a major contributor to the bent structure in NPPC. Without this electrostatic effect, there seems to be less incentive for the two ends of NPDBP, on the time average, to approach each other and to maintain a conformation similar to that of NPPC.

Bound Hapten

ELISA Binding Studies. Table III shows a comparison of the I50 values in mM concentration of antibodies of Group I, Group IIA, and Group IIB to PC, NPPC, and NPDBP.

TABLE III
I50 VALUES OF ANTI-PC-KLH ANTIBODIES

GROUP TYPE	PC	NPPC	NPDBP
GROUP I	0.002-0.13	0.01-0.27	4.6->10
GROUP IIA	>10	0.026-6.6	>10
GROUP IIB	>10	0.06-0.8	0.28-6.5

Group IIA and IIB do not bind PC, as shown by the large number of concentration (>10) needed to show any inhibition. Only Group IIB antibodies bind to NPDBP, but with less affinity compared to NPPC.

NMR Analysis. In order to assess ligand conformations and internuclear distances when bound to the antibody, the transferred nuclear Overhauser effect (TRNOE) spectra were analyzed. Figure 23A shows the NMR spectrum of 100-fold excess of NPPC in the presence of the Group IIA antibody PCG1-2 (γ 1, κ 24). The linewidth of the nine-proton singlet was broadened relative to that of the free hapten; this broadening was much larger at lower ratios of hapten to antibody (e.g., 1:30, not shown). Broadening was also obvious in the ortho and meta protons, in causing less resolution in the splittings. Since no two sets of NPPC resonances were observed even at lower temperature, the hapten was determined to be exchanging between free and bound forms in the fast exchange

regime. Figure 23B is a representative NOESY spectrum for this NPPC/PCG1-2 system, with a mixing time of 300 ms. The bound $\leftrightarrow$ free exchange was sufficient to produce the observed negative TRNOEs. Negative TRNOEs were observed between protons of the hapten indicating that the hapten was bound to the antibody and was acting as a big molecule, giving rise to negative TRNOEs as was explained in Chapter II. The antibody concentration was very little compared to that of the hapten, so no NOEs were observed between the bound hapten and the antibody.

TRNOE Buildup Curves. Direct cross-relaxation effects may be distinguished from spin diffusion by plotting NOEs as functions of irradiation time. This is illustrated in Figure 24A, which presents a set of NOE-buildup curves for TRNOE cross peaks between the meta-, α -, β -, δ -proton sets and the ortho-phenyl protons. The NOE percent is defined as the ratio of the cross peak volume when one of the peaks is irradiated to the equilibrium single proton volume (Olejniczak et al., 1986). Since these TRNOE-buildup curves can be extrapolated to zero intensity at zero time, it can be concluded that the observed TRNOE between each of the proton sets is at least in part from a primary NOE (Koide et al., 1989), although the observed TRNOEs may still also have a spin-diffusion component.

Binding Studies to Group IIB Antibody PCG2b-3 (γ 2b, λ 1). PCG2b-3 can interact strongly with both NPPC and NPDBP, as shown in Table III. These interactions can be examined by TRNOEs. Results similar to those obtained for NPPC interacting with PCG1-2 were obtained with PCG2b-3 interacting with either NPPC or NPDBP, except that both negative and positive intramolecular hapten TRNOEs were observed. The positive TRNOEs were of lower magnitude than in the free hapten, indicating that there is binding of the hapten to the antibody that decreased the intensity of the TRNOEs but the exchange was too slow to decrease the intensity to negative values. Since the TRNOEs between the δ -protons and the aromatic ortho-protons were negative, the bound

hapten should then be in a bent conformation with the ends of the molecule within 0.5 nm of each other. However, the bound $\rightleftharpoons$ free exchange seemed to be significantly lower than in the NPPC/PCG1-2 case described above. This was inferred from the magnitude of broadening of hapten peaks when bound. This slow off-rate suggested that spin diffusion may contribute significantly to the results. The tight binding (slow off-rate) manifested by this Group IIB to NPPC is consistent with the observation that antibodies with a λ light chain have high affinity for hapten.

Binding Studies to Group IIB Antibody Fab PCG1-9 (γ 1, λ 3). In order to address the possible contribution of spin diffusion, the interaction of NPDBP with the Fab fragment of PCG1-9 was analyzed. Because of the Fab's smaller molecular weight, its contribution to the negative TRNOE of the hapten was diminished as compared with NPDBP bound to the intact antibody. Therefore, only a set of 1D NOE data was reported. Figure 24B shows the TRNOE buildup curve resulting from inverting the singlet from $-C(CH_3)_3$ and observing the intensity of perturbation to the ortho-phenyl NMR signal. As in each of the previous cases, there was a primary TRNOE between the δ - and ortho-protons. The presence of the primary NOEs verified the observation that the bound NPDBP geometry is bent with its δ -proton tail within dipolar contact distance (<0.5 nm) of the phenyl ortho-protons. Even if the exchange were quite slow, and spin diffusion a strong contributor to the observed TRNOEs at longer mixing times, the presence of a negative NOE at the shortest mixing time (20 ms) for this only 50 KDa system demonstrated that a major part of the TRNOE was primary and not a result of spin diffusion, as discussed in Chapter II. Thus the conformation of NPDBP, when bound to the PCG1-9 Fab fragment, resembled that of NPPC or NPDBP when bound to PCG2b-3 or to intact PCG1-9.

Binding Studies to Group IIA Antibody Fab D18 (γ 1, κ 1). Fab D18 is expected not to show any binding to NPDBP since it is a Group IIA antibody fragment. The

interaction with NPPC was monitored by increasing the temperature to 47°C to increase the exchange rate in order to maximize the TRNOEs. Also, the linewidth of the nine-proton singlet was monitored at increasing temperatures for any evidence of broadening. No obvious broadening or any TRNOEs were observed. The work with D18 was discontinued because binding evidently took place with a very slow exchange off-rate.

Binding of NPDBP to M3C65 Group IIB ($\gamma 2b, \lambda 1$). M3C65 was titrated with NPDBP to study the effect of this antibody on the conformation of the ligand. The 1D TRNOE difference spectrum showed weak negative peaks for the aromatic protons upon irradiation of the $-C(CH_3)_3$. This shows proximity in the bound state of the $-C(CH_3)_3$ group to the aromatic ring. Here again the antibody is acting as a template to stabilize the bent conformation out of many conformations of unbound hapten.

INTERACTION OF FPPC AND TFPPC WITH SFV

Free Hapten

The purpose of the fluoro and trifluoro methyl groups in serving as a mimic for the nitro group is in the use of ^{19}F NMR to probe the binding interactions between hapten and PC-KLH antibodies. FPPC and TFPPC are the fluorinated haptens synthesized in Chapter IV. Both haptens show the same restricted conformational mobility as NPPC. This can be seen by the set of complex multiplets of the methylene protons in the upfield region of the 1D spectra and the proximity of the $[-N(CH_3)_3^+]$ to the aromatic region shown by 1D and 2D NOE hits, as illustrated in Figure 25.

Binding Studies to Group IIB M3C65 Fv ($\gamma 2b, \lambda 1$)

In these studies, the sFv of M3C65 was titrated with FPPC and TFPPC. The words Fv, antibody or protein were used interchangeably to mean the sFv fragment.

Group IIB antibodies were difficult to study using TRNOE method because of their tight binding to PC based haptens and hence the resulting slow exchange. Working however with the relatively small Fv fragment (25 KDa), TRNOE peaks can be observed even for antibodies that have quite tight binding.

Proton NMR Titration. 0.1 mM of the Fv of M3C65 was titrated with an increasing concentration of FPPC to 30-fold excess, monitoring by ^1H NMR. Broadening of the methylene peaks was observed at lower concentrations. No obvious change in chemical shift of the hapten resonances was observed.

Fluorine NMR Titration. The effect of binding was studied by observing the change in the ^{19}F signal as the Fv was added. In the FPPC case, the multiplet caused by coupling from the aromatic protons was reduced to a doublet by broadband Waltz-16 decoupling of the protons. The doublet with $J \sim 1.8$ Hz was due to long range coupling with the phosphorous. The decoupling simplified the spectrum and increased the intensity of the peak. Observing the ^{19}F signal in TFPPC was much easier because of the larger intensity attributed from three fluorine nuclei and the absence of short and long range couplings. 0.08 mM hapten was titrated with increasing amount of the Fv to get to 20 fold excess of the hapten. The ^{19}F signal was shifted upfield by 0.1 ppm during this titration. The upfield chemical shift upon addition of Fv cannot be attributed to pH difference because all spectra were acquired under the same conditions. The effects of hydrogen bonding and local electronic effects from close aromatic side chains probably gave rise to the chemical shift difference. The shift is then an indication of the change in environment experienced by the fluorine nucleus as it leaves the aqueous phase and enters the antibody binding site. ^{19}F NMR was also used to determine the dissociation rate constant, as will be shown later.

NOESY of Bound FPPC. NOESY spectra were obtained for FPPC bound to 0.08 mM Fv at 25°C with 350 ms mixing time and 30 fold excess of hapten. Figure 26 shows

that there are TRNOE cross peaks within the bound hapten and TRNOEs between the hapten and the protein. The observation of TRNOE cross peaks between the bound hapten and the protein, for the first time in this study, was made possible by the smaller size of the Fv and its higher concentration. The TRNOEs within the bound hapten were indicative of the restricted conformation. The αCH_2 of the choline showed TRNOE to the ortho aromatic protons. The 9-proton choline singlet showed proximity to the α and β of choline at 4.38 and 3.15 ppm, respectively.

There were visible TRNOEs between the bound hapten and the Fv. The 9-proton choline singlet resonance gave strong NOE cross-peak to protons at 1.4 ppm. It also showed NOEs to the ortho protons of two different aromatic spin systems; one corresponds to two protons at 6.75 and 6.48 and the other at 6.9 and 6.5 as shown in Figure 27. These could belong to either a phenylalanine or a tyrosine ring in the antibody combining site. There are cross-peaks that increased in intensity at 6.8 and 7.1 ppm. The ortho protons of the hapten ring at 7.1 showed an NOE cross-peak to the αCH_2 of the choline.

βCH_2 of the choline showed NOEs to the bound Fv at 3.90, 2.75, and 2.18 ppm. αCH_2 of the choline showed a TRNOE to 3.35 ppm.

The extra cross peaks observed between protons of the bound protein in the aromatic region (Figure 27A compared to 27B) indicated a decrease in flexibility of the antibody fragment upon binding. Cross peaks were observed between resonances at 7.1 and 7.7 ppm that likely belong to the protons on the imidazole ring of a histidine. The peak at 7.7 ppm showed NOE cross-peak to the 9-proton singlet. This observation is consistent with the fact that a decrease in flexibility causes an increase in the effective correlation time, which gives rise to the increased NOEs (Harris, 1986).

NOESY of Free SFv. The antibody SFv fragment has a molecular weight of 26,000 D. For this reason, the resonances observed in the 1D ^1H NMR spectrum in

Figure 28A were much better resolved than those of the intact antibody. NMR studies on the Fv fragment are very limited due to the difficulty in obtaining these fragments, either proteolitically or by genetic engineering techniques. The increased resolution in the spectra, relative to intact antibody, might allow direct observation of NOE cross peaks between the hapten and the Fv. A NOESY spectrum for 0.6 mM of the free Fv was obtained with a 70 ms mixing time and shown in Figure 28B. This short mixing time was used to reduce the possibility of NOEs due to spin diffusion. The majority of the cross-peaks observed were due to intramolecular interactions in the Fv. The upfield shifted resonances, between 1 and 1.5 ppm, indicated interaction of aliphatic residues with an aromatic ring system. The cross peaks between 5 and 6 ppm indicated β sheet structures. The α protons of random coil residues normally show resonances at around 4.5 ppm. A downfield shift indicates the presence of β sheet and an upfield shift indicates the presence of helices (Wishart, 1991).

Interaction of Fv with TFPPC. A NOESY spectrum of 0.5 mM Fv was obtained in the presence of four fold excess of TFPPC at pH 8.0 and 25°C as shown in Figure 29. With 70 ms mixing time and with this low excess of TFPPC, it was not easy to identify the hapten aromatic protons since they can be nearly as broad as the protein resonances. Figure 30 shows 1D spectra of 0.15 mM of Fv titrated with an increasing concentration of TFPPC. Spectra E and F are difference spectra between C and B, and D and C respectively. Spectrum F shows the bound complex with very little excess hapten. Spectrum E shows the excess free hapten. The presence of differential interaction was concluded. The meta protons interact the least since it was the first peak to appear upon titration. The ortho protons and the 9-proton singlet protons are very tightly bound and hence broadened and disappeared as shown in spectrum E. Spectrum E also showed some peaks from the Fv. These peaks were due to change in chemical shift in the protein peaks upon binding. A change in chemical shifts reflects a change in the chemical and magnetic

environment of those resonances. This change could be partially due to hydrogen bonds formation and due to some conformational change in the protein.

In the NOESY spectrum of the TFPPC-Fv complex of Figure 29, the resonances belonging to the choline portion were resolved and showed NOE cross peaks from the δ protons to the α and β protons within the bound hapten. The 2,6 and 3,5 aromatic protons of the hapten were assigned to 7.7 and 7.3 ppm. The 3,5 protons peak at 7.3 ppm was very broadened as discussed above and hence hard to observe. The 2,6 protons showed NOE to the δ protons at 3.2 ppm. This closeness in space between the ring and the quaternary ammonium ion is an indication of the bent conformation of the bound hapten as observed in other antibodies with other haptens. The connections above the diagonal in Figure 29 represent the NOEs within the bound hapten.

There are NOEs observed between the bound hapten and the antibody fragment in Figure 29 as shown by connections below the diagonal. The δ protons singlet at 3.18 ppm showed a strong NOE to a proton at 1.4 ppm, and a weaker NOE to 0.35 ppm. The resonance at 0.35 ppm showed an NOE to the meta protons of the hapten at 7.7 ppm. The resonance at 1.4 ppm showed an NOE to an aromatic proton at 7.0 ppm. This NOE was not observed in the free Fv. The δ and β protons of the choline portion showed NOEs to aromatic protons of the Fv at 6.85 ppm. The Fv protons at 6.85 and 6.65 ppm showed NOE cross-peaks to each other and could belong to the same spin system.

Figure 31 shows a comparison of the aromatic region between the free and bound Fv. Assignment of some aromatic residues was possible even without specific deuteration. The following assignments were based on chemical shifts and are very preliminary. In the free Fv, a sample of each aromatic ring was assigned. The protons at 7.1 and 8.25 ppm very likely belong to the 4H and 2H of the imidazole ring of the histidine residue. The protons at 7.0, 7.35 and 7.7 ppm may be from the 5H, 6H and 4H of the indole ring of a tryptophan. The protons at 7.3 and 7.5 ppm were assigned to the 2,6 and 3,5 protons of a

phenylalanine ring. The protons at 6.4 and 6.8 ppm were assigned to 3,5 and 2,6 protons of a tyrosine ring. On hapten binding several changes in cross-peaks intensity were observed, indicating that there is a change in mobility at the binding site and probably a significant tertiary structural change.

Measurement of the Dissociation Rate Constant. The presence of the fluorine nuclei in the TFPPC hapten provided a convenient way for measuring the hapten-antibody fragment dissociation rate, k_{off} . Both free and bound-hapten signals were observed. The calculation of the dissociation rate depends on the relative mole ratios of free and bound, the linewidth of the bound-ligand resonance and the degree of overlap from other resonances (Sandstrom, 1983). Bound-ligand signals are often detected in ^{19}F spectra because of no overlap from other resonances (Craik and Higgins, 1989; Glaudemans et al., 1990). CF_3 group is a good substituent because the three equivalent F nuclei give rise to a sharp signal that is not complicated by large coupling as in the F group of FPPC. Furthermore, rotation about C_3 axis is rapid even when attached to a macromolecule causing the signal to stay reasonably sharp (Gerig, 1989).

Figure 32 shows ^{19}F spectra of mixtures of 0.5 mM Fv and TFPPC at mole ratios indicated in the figure caption. The broadened peak for the bound complex ($\delta \sim 57.6$ ppm or 21692 Hz) is in fast exchange with the free hapten ($\delta \sim 57.8$ ppm or 21742 Hz). Spin-spin relaxation time T_2 is affected by ligand binding. T_2 can be calculated from the linewidth of the bound and free ligand to give 0.013 s for the bound and 0.096 s for the free. The relative population of the bound hapten to the free was taken as 0.62 to 0.38 in spectrum C of Figure 32. Measurement of peak broadening as a function of Fv-TFPPC mole ratio allowed k_{off} to be determined. The complete band shape method was used (Binsch 1968) to calculate an off-rate that was in the range between 130 and 150 s^{-1} . This indicated that the exchange was fast enough to cause the contribution from spin diffusion in Figures 29 and 31 to be minimal.

ELISA Results of Fluorinated Haptens. I50 values were obtained for FPPC and TFPPC and compared to NPPC in their binding to M3C65 and a Group IIA antibody PCG1-1. These values are listed in Table VII of Chapter VI. Similar I50 values were obtained indicating that substitution of the nitro group by a fluoro or trifluoromethyl did not perturb the antibody binding pocket. The slight reduction in binding affinity of TFPPC compared to NPPC (7.60 to 2.65) is probably due to weaker hydrogen bonding interactions of the trifluoromethyl groups (Kooistra and Richards, 1978). Another reason for this decrease might be the difference in geometry between the two groups, spherical for the trifluoromethyl and planar for the nitro group. With PCG1-1, no such difference was observed (5.69 to 5.18) indicating that hydrogen bonding interactions are probably not as important. However, the differences in both cases are not large enough to conclude any valid differences in the nature of the interaction. The conclusion is that we are justified in using either F or CF₃ moieties in place of the NO₂ group.

DISCUSSION

Preliminary Assignments

In the antibody M3C65, Thr 55 of the CDR2 of the light chain is one of the important residues for antigen binding (Brown et al., 1992). This residue in λ 1 light chain was shown to be important for high affinity binding by site specific mutagenesis. In the Fv-TFPPC complex, the resonance at 0.35 ppm could be the γ CH₃ resonance of Thr 55 that is shifted upfield to ring current effects from the ligand ring. An NOE cross-peak between 0.35 ppm and the meta protons of the aromatic ring of the hapten supports this observation.

In antibodies in general, tyrosine is the most commonly occurring residue in the heavy chain CDRs and second in overall CDR occurrence, suggesting that many tyrosine residues may be important to antibody antigen interaction (Kabat et al., 1987).

Examination of the antibody M3C65 CDR sequence showed that there are four tyrosine residues in the heavy chain (Brown, private communication). The other aromatic residues are one tryptophan in the heavy chain and two tryptophan, two histidine, and one phenyl alanine residues in the light chain. Aromatic peaks that showed NOE cross-peaks to the hapten peaks, were assigned to antibody aromatic protons in the binding site. Since the chemical shifts of these aromatic peaks differed from those of the hapten phenyl group, the assignment of these peaks to antibody protons was unambiguous.

The significant shift of the 0.35 peak observed upon binding could be due to ring current effects due to direct interaction with the hapten ring or due to structural changes in the protein associated with hapten binding. The assignment to a specific type of amino acid can be obtained in the future by specific deuteration of the aromatic amino acids or by point mutation.

In the Fv-FPPC complex, there were NOEs from the δ protons to the ortho protons of two different aromatic spin systems, one corresponded to two protons at 6.48 and 6.75, and was assigned to a tyrosine ring or a phenylalanine ring. Similar cross-peaks were observed for the more concentrated Fv-TFPPC system. The Fv protons at 6.92 and 6.52 ppm that showed NOE cross peaks to each other and to the δ protons of the choline could belong to a tyrosine spin system. The observations in the Fv-FPPC complex were consistent with that of the Fv-TFPPC complex.

Flexibility

Comparing the aromatic region of the bound Fv to the free in Figures 27 and 31 showed that some cross-peaks gained in intensity. This is an evidence that the residues giving rise to these peaks are strongly influenced by the hapten. This loss in flexibility is due to an increased effective correlation time, that increased the NOE. There is definite gain in intensity in the spin systems of 7.0 and 7.7 ppm and 6.8 and 7.1 ppm of Figure 27.

The presence of extra peaks and loss of others suggests the possibility of induced conformational changes in the combining site upon binding.

Both of these systems showed less intense negative NOE cross-peaks as compares to the intact antibody-NPPC complex. There are several reasons for these less intense bound hapten peaks. One reason is the lower temperature used for the Fv-hapten interaction that lowered the exchange rate and the other reason is the smaller size of the Fv compared to the intact antibody. At lower temperature the Fv resonances become broader from increased correlation time, whereas those of the bound hapten become narrower due to slower exchange. Another reason for this difference in hapten linewidths could be the tighter binding characteristic of Group IIB antibodies with the λ light chain.

Figure 19. Titration of PC with the antibody M3C65. (A) 50 fold excess of the hapten to 20 fold excess (E). These spectra were acquired at 40°C and pH of 7.5.

Figure 20. Conformations of NPPC and NPDBP as calculated using the MM2 parameters; bent for NPPC; extended for NPDBP.

Figure 21. ^1H NMR spectra of PC, NPPC, NPDBP. (A) PC. (B) NOE difference spectrum of PC resulting from irradiating at peak δ . (C) NPPC. Similar to PC in having complex unresolved multiplets for the α and β CH_2 's. (D) NOE difference spectrum. (E) NPDBP. Note the resolved multiplets for the α and β CH_2 's. (F) NOE difference spectrum. The structure for NPPC ($\text{X} = \text{N}^+$); for NPDBP ($\text{X} = \text{C}$); for PC ($\text{X} = \text{N}^+$) and lacks the nitrophenyl moiety

Figure 22. ^{31}P NMR spectra of NPDBP (A) and NPPC (B). Note the distorted triplet in NPPC due to restricted mobility. The reference in both axes is arbitrary.

Figure 23. Two-dimensional TRNOE ^1H NMR spectrum for NPPC in the presence of PCG1-2 at 37°C and pH 7.0. (A) Reference spectrum. (B) NOESY spectrum with a 300 ms mixing time. Note the cross peaks between peak δ and each of the other NPPC resonances.

Figure 24. TRNOE buildup curves. (A) TRNOE buildup for NPPC in the presence of PCG1-2. The meta to ortho TRNOE data were multiplied by 0.1 in order to fit the data on the same plot. The meta to ortho TRNOE data were fit to a logarithmic function. (B) TRNOE buildup for NPDBP in the presence of PCG1-9 Fab fragments. The data in arbitrary intensity units were fit to a logarithmic function. Note that in both cases, each of the NOEs had an intercept at time ~ 0 ms and so had at least a primary NOE component.

Figure 25. ^1H NMR spectra of FPPC and TFPPC. (A) FPPC. (B) NOE difference spectrum of FPPC resulting from irradiating at peak δ . (C) TFPPC. Similar to FPPC in having complex unresolved multiplets for the α and β CH_2 's. (D) NOE difference spectrum. Notice the small NOEs to the region between 7 and 8 ppm indicating proximity of the δ protons to the aromatic ring.

Figure 26. NOESY of 0.08 mM Fv bound to FPPC. The connections above the diagonal are between the hapten's protons and the bound Fv. The connections below the diagonal are connections within the bound hapten. The hapten protons at 7.1 ppm showed cross peaks to the αCH_2 indicating a bent conformation. The 9-proton singlet showed cross-peaks to aromatic residues of the Fv at 6.75, 6.9 and 7.7 ppm

Figure 27. NOESY spectra showing the aromatic region of the bound (A) and free (B) Fv of 0.08 mM concentration. Notice the appearance of cross peaks at 7.0 and 7.7 and the increase in intensity of cross peaks at 6.8 and 7.1 ppm. The aromatic hapten protons were not resolved and gave rise to a broad diagonal peak around 7.1 ppm.

Figure 28. NOESY of 0.6 mM free Fv. (A) The 1D spectrum shown directly above the NOESY spectrum (B). Spectra were obtained at 25°C and 70 ms mixing time. Notice the peaks around 5 ppm indicating the presence of β -sheet structures.

Figure 29. NOESY of M3C65 Fv with a four fold excess of TFPPC. 0.5 mM Fv with TFPPC at pH 8.0, 25°C and 70 ms mixing time. The connections above the diagonal are between resonances within the bound hapten, while those below the diagonal connect resonances of the antibody to the hapten.

Figure 30. ^1H NMR spectra showing titration of 0.15 mM Fv with TFPPC. Spectra were obtained at pH 7.0 and 25°C. (A) Free, (B) 0.5:1, (C) 2:1, (D) 5:1 TFPPC-Fv complex. (E) and (F) are difference spectra of C-B and D-C respectively.

Figure 31. Comparison of a portion of the NOESY spectra of the free Fv (A) and the Fv-TFPPC complex (B). Preliminary assignments of aromatic protons are shown on the spectrum of the free Fv. The positions of the intra-hapten peaks are pointed out in the complex.

Figure 32. ^{19}F NMR spectra of M3C65 Fv-TFPPC system at various ratios. The Fv concentration was 0.5 mM at pH 8.0 in $^2\text{H}_2\text{O}$ at 25°C . The ratio of the hapten to the Fv is as follows (A) 0.4:1, (B) 1.3:1, (C) 2.2:1, (D) 3.2:1, and E is for the free hapten. Spectra were acquired with broadband decoupling and processed with line broadening of 5 Hz.

CHAPTER VI

CONTRIBUTION FROM CARRIER MODELS

The aim of this work is to identify residues on the carrier protein that contribute to epitope recognition. Even though the importance of the carrier protein for inducing antibody formation is well accepted, there has been little done to evaluate specific structural interactions between the carrier protein and the antibody. Furthermore, the procedures often used to produce hapten-carrier protein conjugates must yield heterogeneous products, with many haptens bound for each carrier protein. While this is advantageous for antibody production, it makes studying the binding site impossible with a high-resolution technique like NMR. This chapter will focus on the interactions between Group II antibodies and mono-labeled hapten-carrier amino acids, peptides, and proteins.

CONFORMATION OF TYR-PC

Free Ligand

NMR Analysis. A NOESY spectrum at 500 ms mixing time was obtained to determine the structure of the PC portion when linked to the amino acid tyrosine. Table IV presents the proximity between the various groups. In this table the letter Y was used to differentiate the tyrosine protons from the PC protons. A and C are the ortho and meta protons to the diazo linkage of the phenyl (Φ) ring. In the tyrosine, E' represents the ortho protons to the hydroxyl group. B' and D' are the meta protons with D' being on the same side of the ring as E'. Also the abbreviations, st, md, and wk mean NOE cross-peaks of strong, moderate and weak intensities. From the spectrum, it was shown that the acetyl

group (CH₃) is not in close proximity to any other tyrosine protons and the bent restricted conformation discussed before is also a characteristic of the PC part of this molecule.

TABLE IV

NOE DATA OF FREE TYR-PC

	Y-CH ₃	Y-α H	Y-β H,H'	Y-Φ (B')	Y-Φ (D')	Y-Φ (E')	Φ (A)	Φ(C)	C _α	C _β	C _δ
Y-CH ₃											
Y-αH			st.	st.	st.						
Y-βH,H'		st.		st.	st.						
Y-Φ(B')		st.	st.								
Y-Φ(D')						st.					
Y-Φ(E')					st.						
Φ(A)								st.	wk.	wk.	md.
Φ(C)							st.		st.	st.	st.
C _α										st.	st.
C _β									st.		st.
C _δ									st.	st.	

Bound Hapten

Binding to PCG2b-3 and M3C65 (Group IIB). Titrations of these two antibodies at 25 μM, 500 μL at pH 7.5 and 35°C with an increasing concentration of Tyr-PC were carried out from 1:1 ratio to 100:1 (5, 10, 30, 50, and 100 fold- excess). The nine-proton singlet was not broadened demonstrating that the hapten exchanged very slowly between the free and bound forms reflecting very tight binding. These results are consistent with the previous data of λ light chain antibodies, that exhibited very slow k_{off} in every case

when bound to NPPC and TFPPC. Under these conditions, a meaningful TRNOE analysis was not possible. A 1D NOE hit was nevertheless performed on the nine proton singlet. The difference spectra showed small negative NOEs, indicating that binding took place although with a slow k_{off} .

Binding to PCG 1-2 (Group IIA). A 40 μM sample of this antibody at pH 7.0 and 40°C was titrated with an increasing concentration of Tyr-PC ligand, from 1:1 to 50:1 fold excess. The linewidth of the nine-proton singlet was strongly broadened relative to the free Tyr-PC. Differential broadening was observed in the aromatic region. The broadened peaks and the absence of individual free and bound ligand peaks suggested that the exchange between free and bound forms was in the “fast exchange” regime, making TRNOE analysis possible. Figure 33 illustrates the difference in 1D spectra between fast and slow exchange cases.

Figure 34 is a 2D NOESY spectrum for this Tyr-PC/PCG1-2 system with a mixing time of 300 ms. The size of the antibody (150 KDa) made seeing reliable NOEs from the bound ligand to the antibody impossible, especially at the large excess of ligand used. Only NOEs within the bound ligand were observed, and these gave information about the change in conformation of the ligand as it binds to the antibody. Table V shows the observed cross-peaks between the protons of the NOESY spectrum of Figure 34.

The main difference between the bound and free Tyr-PC ligand is the proximity of the acetyl portion to the rest of the molecule in the bound form. The CH_3 from this group showed cross-peaks to A, B', C, D', E', α' , and β' . These cross-peaks were primary, and not due to spin diffusion, because they were also observed at 100 ms mixing time (not shown). The off-rate is sufficiently fast in this system based on comparison with the TFPPC-Fv system. This spectrum was obtained at higher temperature and showed broader hapten peaks indicating an off-rate greater than 150 s^{-1} . It was also observed that the PC portion assumes the same “bent” conformation already observed for PC-based

haptens in the absence and presence of the antibody. From the differential broadening of the peaks in the bound form, such as peaks A and B', one may conclude that different forces to different nuclei of the Tyr-PC molecule are contributing to the binding. Another observation is the chemical shift difference in the bound ligand that led to the resolution between α and α' resonances. These resonances were not resolved in the spectrum of the free ligand as shown in Figure 12 of Chapter IV.

TABLE V

NOE DATA OF BOUND TYR-PC

	Y-CH ₃	Y- α H	Y- β H,H'	Y- Φ (B')	Y- Φ (D')	Y- Φ (E')	Φ (A)	Φ (C)	C $_{\alpha}$	C $_{\beta}$	C $_{\delta}$
Y-CH ₃		md									
Y- α H	md		st								
Y- β H,H'	md	st.									
Y- Φ (B')	md	st	st.								
Y- Φ (D')	wk	md	st								
Y- Φ (E')	wk	wk	md		st						
Φ (A)	md							st.	wk		md
Φ (C)	md								st.	st.	st.
C $_{\alpha}$										st.	st.
C $_{\beta}$											
C $_{\delta}$											

The bold letters represent the additional changes in the bound ligand with respect to the free.

Molecular Mechanics Calculations

Molecular conformation calculations (as in Chapter V for NPPC and NPDBP) were performed to obtain the energetics of a fully extended structure of Tyr-PC, a “bent” structure with the choline portion in close proximity to the phenyl ring and a “bent U” with the acetyl group bent closer to the ring. The “bent U” structure was calculated to be ~ 2.5 kcal/mol lower in energy than the fully extended and ~ 3 kcal/mol lower than the bent. In the presence of the solvent however, NMR analysis showed a preference of the free ligand to the bent structure with the methyl group at a distance greater than 0.5 nm. In the presence of the antibody, NMR analysis showed a preference of the bound ligand to the “bent U” structure.

Bent Structure

Bent U Structure

CONFORMATION OF HIS-PC

Free His-PC

Since His-PC dimerizes at high concentrations as discussed earlier, a NOESY was obtained at a high concentration in 42% acetonitrile to study the conformation of the monomer. The mixing time used was 500 ms at 40°C. Table VI shows the proximity between various groups. The symbol H before the group means that the group is part of the histidine residue structure. Similar to Tyr-PC, Ho and Hm are the protons on the phenyl PC ring ortho and meta to the phosphate group.

TABLE VI

NOE DATA OF FREE HIS-PC

	H-CH ₃	H-αH	H-β H,H'	H-C ₄	ΦHm	ΦHo	C _α	C _β	C _δ
H-CH ₃									
H-αH	wk.								
H-βH,H'		st.							
H-C ₄	wk.	st.	st.						
ΦHm					st.				
ΦHo							st.	st.	st.
C _α								st.	st.
C _β									
C _δ									

Bound His-PC to PCG 1-2

PCG1-2 was titrated with an increasing concentration of the hapten His-PC, starting with 1:1 concentration, and ending with 65:1 excess. At 5:1 excess, a sharp

methyl peak at 2 ppm started to appear indicating little interaction of the antibody with the acetyl group. The aromatic peaks started to appear clearly at 10:1 excess. At 15:1 excess, the broad 9-proton singlet peak at 3.2 ppm appeared and became more pronounced at 22:1 fold excess, then merged with an antibody proton at 65:1 fold excess, as shown in Figure 35. A 2D TRNOE spectrum with 300 ms mixing time was obtained of the 65:1 complex. To check if there was self-association at this large excess, a spectrum of the free hapten was obtained (without the acetonitrile cosolvent) at this concentration, and still showed the narrow peaks of a monomer. The TRNOE peaks are shown in Figure 36. Within the bound hapten, the meta proton of the phenyl group showed strong NOEs to the ortho, and weaker NOEs to the δ , β , and α of the choline, characteristic of a bent conformation. In the free His-PC, the C4 proton of the imidazole ring showed weak NOEs to the β protons of the same residue as shown in Table VI. In the bound ligand, the C4 proton of this imidazole ring did not show an NOE to the histidine β protons. This could be due either to change in conformation upon binding that made the ring C4 proton and the β protons > 0.5 nm apart, or due to broadening of the C4 proton because of direct interaction with the antibody. The interaction of the hapten with the antibody was observed in the form of cross peaks between the β and δ protons and an antibody proton at 7 ppm. A strong NOE was observed between what looked like the β H of His at 3.05 and an antibody proton at 1.75 ppm. There were other cross-peaks that arose from antibody protons that were not assignable at that stage.

Discussion and ELISA Binding Data of Amino Acids Coupled to PC

Figure 37 shows the binding curves of PC-coupled amino acids in comparison to NPPC. Both ligands were used as inhibitors with two antibodies: PCG1-1 which is a Group IIA antibody representative, and M3C65 which represents Group IIB. With PCG1-1, Tyr-PC and NPPC showed very similar binding curves, while His-PC showed

less inhibition and higher I_{50} values. This was consistent with NMR experiments that showed a faster off-rate for His-PC. With M3C65, His-PC and NPPC showed identical binding curves and less inhibition than Tyr-PC. The N-acetylated tyrosine residue appeared to be interacting favorably with this antibody to produce a better inhibition than even NPPC.

CONFORMATION OF GLY TYR (PC) ALA

Free Peptide

The assignments of the free peptide GlyTyr(PC)Ala were determined in Chapter IV and illustrated in Figure 16 by the 1D and COSY NMR spectra. Both the α H of the tyrosine and the α H's of Gly were masked by the water signal. The α H of Ala was assigned due to its coupling to the β CH₃ of the same spin system. The NOESY spectrum showed the characteristic bent conformation of PC-based haptens, demonstrated by the presence of NOE cross-peaks between the δ protons of the choline portion and the aromatic peaks of the phenyl ring. NOE cross peaks were also observed between the α and β protons within the same spin system of Ala and Tyr. The proximity of the tripeptide portion to the phenyl PC hapten was illustrated by a weak NOE cross peak between the Ala β CH₃ and the singlet ortho proton of Tyr ring (not shown).

Tripeptide Bound to PCG1-2

PCG1-2 was titrated with an increasing concentration of the hapten N-CF₃ Gly Tyr(PC) Ala, starting with 1:1 concentration and ending with 55:1 peptide excess. At 12:1 excess, a sharp methyl doublet started to appear, indicating no strong specific interaction with the Ala β CH₃. The hapten aromatic peaks started to appear clearly at 35:1 excess. At this excess, the choline δ -proton resonance started to become obvious. An upfield shift was observed for the δ protons of the choline relative to the free, from 3.2

to 3.1 ppm indicating probable proximity to an aromatic ring. Figure 38 shows a 2D TRNOE spectrum acquired with 300 ms mixing time on the 55:1 sample. Another spectrum was acquired at 80 ms mixing time to verify the presence of primary NOEs as opposed to those arising from spin diffusion. The bent structure of the phenyl PC portion was still obvious. No NOE cross-peak was observed between the βCH_3 of Ala and the Tyr ring proton that was observed in the free peptide suggesting a possible conformational change upon binding. A strong NOE was observed between what looked like the βH of Tyr at 3.05 and an antibody proton at 1.75 ppm.

ELISA Results of Tripeptides

Figure 39 shows the effect of PC coupling on binding as illustrated by the binding curves of the coupled tripeptides to the antibodies (M3C65 and PCG1-1) discussed before. Neither the Tyr nor the His tripeptide showed inhibition of binding even at high concentration, which showed that the phenyl-PC portion was what introduced the recognition. The Gly Tyr(PC) Ala showed the same binding curve shape as NPPC but with lower affinity; the I_{50} value was obtained to be 1.9×10^{-5} as compared to 8.3×10^{-6} M of NPPC. This same trend was observed with PCG1-1, with an I_{50} value of 1.6×10^{-5} compared to 3.1×10^{-6} of NPPC. The results from the histidine tripeptide were probably less reliable because of the impurity in the sample (discussed in Chapter IV). However, the impure coupled tripeptide showed some inhibition relative to the uncoupled, but apparently much lower than NPPC, with an I_{50} value of 1.2×10^{-4} compared to 8.3×10^{-6} of NPPC with the antibody M3C65. Table VII shows a comparison of the I_{50} values of the ligands discussed above in their binding to these Group IIA and Group IIB antibodies.

TABLE VII

150 VALUES IN M OF VARIOUS PC BASED LIGANDS

	NPPC	FPPC	TFPPC	Y-PC	H-PC	G-Y(PC)- A	G-H(PC)- G
M3C65	8.3 E-6	6.3 E-4	7.6 E-4	4.6 E-6	9.0 E-6	1.9 E-5	1.2 E-4
PCG1-1	3.1 E-6	8.8 E-5	5.7 E-5	3.1 E-6	9.6 E-6	1.6 E-5	5.9 E-5

CONFORMATION OF OCTAPEPTIDE-PC

Free Peptide-PC

The octapeptide studied was N-terminal acetylated and PC coupled to the one tyrosine. The conformation of the free peptide-PC was studied by NMR. Using TOCSY in $^1\text{H}_2\text{O}$, the assignments of the 4 Gly, 1 Tyr and 3 Ala were done as shown in Figure 40A. The amide protons between 7 and 9 ppm showed relayed connectivities to α protons between 4 and 5 ppm and to βCH_3 's at 1.4 ppm for the Ala residues and β,β' H's at around 3 ppm for the Tyr residues. However, the positions of these residues in the sequence was not determined. Three spin systems that could only belong to a Tyr residue in this peptide-PC were assigned as shown in Figure 40A. The α protons showed no significant upfield or downfield shifts, indicating the absence of secondary structure.

To determine the conformation of the phenyl-PC in the peptide, a rotating frame NOESY was obtained (ROESY) in $^2\text{H}_2\text{O}$. ROESY was acquired because the size of this peptide is in the intermediate range, and hence no strongly positive or negative standard NOEs can be expected. The ROESY spectrum gave indication of the characteristic bent conformation of the phenyl-PC portion as shown in Figure 40B, in the form of NOE cross-peaks between the ortho proton of the phenyl ring and the αCH_2 of the choline. No strong NOE cross-peaks were observed to the β and δ protons of the choline as was

observed in the smaller ligands studied before. However, the obvious proximity of the ortho proton to the αCH_2 still indicated the bent conformation of the phenyl PC portion.

It was noticed that at least two major different conformations exist for the tyrosine residue. This was shown in the COSY spectrum of the free peptide-PC in Figure 41. In the aliphatic region, one conformation gave rise to an α proton at 4.5 coupled to the β protons at around 3.1 ppm, and the other at 4.2 ppm coupled to the β protons at around 2.6 ppm. In the aromatic region the two conformations were also obvious in the form of coupling between meta and ortho protons at 7.0 and 7.3 ppm, and 7.3 and 7.6 ppm as shown in Figure 41A. In the TOCSY spectrum of Figure 40A, the amide protons and hence the entire spin system of three Tyr conformers of the octapeptide-PC were assigned. Since there is only one Tyr residue in this peptide-PC, the existence of two major and one minor conformations was predicted. The upfield shift in one of the β protons showed that the tyrosine residue in this conformation is in sufficiently close proximity to the PC phenyl ring to experience a ring current shift.

Binding Studies to PCG1-2

PCG1-2 (30 μM) was titrated with the peptide-PC at pH 7.0 and 40°C. Tight binding causing very slow exchange was observed since the peaks did not broaden significantly. TRNOE cross-peaks were not observed and hence could not be used to give any indication of the conformation of the bound structure. The limitations of the TRNOE in this system may be overcome by working with a smaller antibody fragment. At this stage in the research, no Fvs were available in sufficient quantity.

However, some information about the conformations of the peptide-PC was given from comparison of the COSY spectra of the free and the antibody-bound peptide. Contribution of the antibody protons to the COSY spectrum is negligible because of the broad linewidth of the antibody protons and therefore only the contribution of the much

smaller peptide were observed. The comparison of COSY spectra of free peptide and bound is shown in Figure 41. Other than decrease in intensity of the peaks due to binding, only one set of Tyr cross-peaks was seen, reflecting only one conformation. Two mechanisms could account for this observation. In both of these mechanisms, the exchange between the free peptide conformers H and H' is slow relative to the exchange between antibody-bound and free peptides. In one case, the antibody preferentially binds one conformer H' and stabilizes it, hence drives the equilibrium between H and H' towards H' causing observation of H' peaks in the COSY spectrum. The other mechanism is the antibody binding to the conformer H and causing its peaks to broaden and hence only peaks from the unbound conformer H' are observed. Both are consistent with the antibody having a restrictive binding site

BINDING RESULTS OF PROTEINS

The small quantity of NP-PC available permitted only ELISA binding studies of this coupled product. From the NPI-PC binding curves in Figure 42, several observations were made. First, native NP-I and NP-II did not bind to the antibody, and therefore any binding that occurred was due only to the PC portion. Second, there was a significant decrease in binding caused by lysine-blocking. This is expected because the blocked NP may have up to three moles of PC less than the unblocked NP. This technique therefore was useful in checking both the affinity and the level of purity of the protein. The affinity purified NP-II PC showed significant binding to M3C65 as shown by ELISA tests.

DISCUSSION

Some of the forces observed in this chapter that are contributing to binding are electrostatic interactions. This is inferred from the broadening of the nine-proton resonance of the choline when interacting with Group IIB antibodies.

From the study of the various ligands, there is evidence that the combining site is restrictive since one conformation is tolerated. This is shown in both NPDBP and octapeptide-PC. A single conformation was observed upon binding even when there is a mixture of conformations in free solution. This observation is similar to other works on antibodies (Clare et al., 1986). The presence of all these models illustrates the continuity of a specific behavior, where the restrictive effect of the antibody combining site is manifested. A single conformation for a bent structure of phenyl PC is observed in all the bound ligands.

The effect of the carrier is demonstrated by comparison of Tyr-PC to octapeptide-PC. In free Tyr-PC, there is only one conformation of Tyr which shows that the ring rotations are not restricted. In the peptide where there is a longer chain carrier, averaging between conformations is restricted giving rise to three different observed conformations. When the octapeptide-PC binds to the antibody, the antibody stabilizes one conformation.

The ability to bind analogs of different sizes is an evidence that the binding site has substantial flexibility since the binding pocket can accommodate a number of ligands of different size, shape and electronic nature. The latitude that the antibody combining site has for a ligand suggests the presence of a mechanism by which antibodies can still recognize different structures as long as they can access a particular structure which they prefer to bind. The interaction of an antibody to its antigen with no conformational change is consistent with the "lock and key" hypothesis where specificity arises from geometrically complementary shapes. TRNOE studies, however, have shown slight conformational changes in a number of ligands. Changes were observed in bound Tyr-PC, His-PC, Gly Tyr(PC) Ala. TRNOE studies have shown slight conformational changes in a number of ligands. These conformational changes could be either due to the antibody binding inducing a conformational change or the antibody selecting one conformation that already exists but is undergoing averaging with other conformations.

Antigenicity is the ability of antigens to bind to antibodies. Immunogenicity is the ability of antigens to generate immune responses. Immunochemical binding studies were correlated with the NMR results. Both showed that within the series of ligands studied, the relative binding affinity (I_{50} values) decreased as the size of the carrier molecule increased. This was shown by comparing Tyr-PC to GlyTyr(PC)Ala and then to NP-II-PC. Only macromolecules can generate immune responses. This shows that strong binding of the antigen is not correlated with immunogenicity.

Figure 33. ^1H NMR spectra illustrating slow and fast exchange. (A) Tyr-PC in the presence of PCG2b-3 obtained at 35°C and (B) PCG1-2 obtained at 40°C . Both antibodies have 30 fold excess hapten. Notice the difference mainly in the nine proton singlet at 3.2 ppm.

Figure 34. TRNOEs of Tyr-PC in the presence of PCG1-2. The connections above the diagonal represent cross peaks within the tyrosine residue. The connections below the diagonal show cross peaks within the bound haptin portion. Notice the strong TRNOEs between protons (C) and α , β and δ protons of the haptin.

Figure 35. Titration of PCG1-2 with His-PC. 40 μ M of the antibody was titrated with 10:1 (A), 15:1 (B), 22:1 (C), 65:1 (D) fold excess of the ligand. Notice the appearance of the methyl peak at 2 ppm and the 9-proton singlet at 3.2 ppm.

Figure 36. TRNOEs of His-PC in the presence of PCG1-2. Note the connection between the ortho-proton and α , β and δ protons of the hapten. The connections in the aliphatic region above the diagonal are between α and β protons of the histidine residue. The connections below the diagonal are between α , β and δ protons of the hapten.

Figure 37. Binding curves of amino acids coupled to PC. (A) Binding curves of NPPC, His-PC and Tyr-PC to M3C65. (B) Binding curves to PCG1-1. As the hapten's concentration (x-axis) is increased, the % binding decreases. The I50 values are given in molar concentration of the hapten. The greater than 100% binding is due to artifacts caused by light scattering.

Figure 38. TRNOEs of Gly Tyr (PC) Ala in the presence of PCG1-2. 30 μ M of the antibody were present with 55 fold excess of ligand at pH 7.0 and 40°C. The connections below the diagonal are due to NOEs within the phenyl PC hapten. The connections above the diagonal are for NOEs between the peptide portion of the ligand.

Figure 39. Binding curves of tripeptides coupled to PC. (A) Binding curves of Gly Tyr (PC) Ala and (B) Gly His (PC) Gly to M3C65 compared to NPPC and the uncoupled peptide. (C) Binding curves of Gly Tyr (PC) Ala to PCG1-1.

Figure 40. TOCSY and ROESY spectra of free peptide PC. (A) A portion of the TOCSY spectrum in $^1\text{H}_2\text{O}$ showing assignments of the amino acid residues. (B) ROESY spectrum phased with the cross peaks as positive. Note the connection between the ortho protons and the choline moiety indicating a bent conformation. The assignments of Gly (G), Ala (A) and Tyr (Y) are shown on the spectra.

Figure 41. Comparison of the COSY spectra of free (A) and bound (B) octapeptide-PC. Note the presence of two cross-peaks between αH and $\beta,\beta'\text{H}$ of the Tyr in the free peptide-PC while there is only one cross-peak in the bound peptide-PC.

Figure 42. Binding curves of NPI-PC. (A) and (B) show the reduced inhibition of the lysine blocked NPI-PC-ETFA compared to the nonblocked NPI-PC. The x-axis represent the log of the concentration of the ligand.

CHAPTER VII

INTRODUCTION AND SIGNIFICANCE OF NEUROPHYSINS

SIGNIFICANCE

The posterior pituitary produces two important regulatory hormones, oxytocin and vasopressin. Both have structural similarities including the presence of a cyclic hexapeptide ring connected by a disulfide bond, and the substitution of the free carboxyl groups by the amide group. Oxytocin mediates milk ejection by causing contraction of myoepithelial cells on receptors of the mammary gland. It also plays a role in uterine contraction and sexual behavior and response. Vasopressin affects kidney function, blood pressure and body fluids (Acher et al., 1956). These hormones are nonapeptides found in 0.1 M concentration in neurosecretory granules of the posterior pituitary (Dreifuss, 1975). They are complexed in 1:1 ratio to a class of disulfide-rich proteins called neurophysins (NPs). NPs have a very low content of aromatic amino acids and a high proportion of cysteine and proline. NPs and hormones are synthesized in the hypothalamus as precursor proteins which are packaged into neurosecretory granules. These hormones and NPs interact to form noncovalent complexes which are dissociated upon secretion into the blood when the hormone is demanded. NPs as shown earlier in this document are suitable carrier protein models for KLH. In order to identify the residues on the carrier protein that contribute to epitope recognition, the conformation of the unbound protein should be determined. For this reason, detailed structure analyses were attempted.

In addition to their role as carrier proteins, there are other reasons that make NP molecules interesting to study. The physiological role of NPs includes hormone storage and stabilization of oxytocin and vasopressin against proteolytic degradation within the

posterior pituitary neurosecretory granules (Breslow and Burman, 1990). Since these complexes dimerize at high concentration, and even tend to form aggregates, packaging becomes possible because the limited solubility reduces hormone osmotic activity. This remarkable packaging of the hormone and NP forms a model for other biological molecules which are stored in very high concentration against a strong concentration gradient (Palade, 1975).

In addition to biological significance, the availability of NP purification methods in high purity and yields encouraged detailed studies of structure and function. These studies have used NPs as probes of hormone release into the blood (Sinding and Robinson, 1977), and as immunological probes of neuronal pathways relating to hypothalamus and pituitary using light microscopic studies (Zimmerman et al., 1975). Other reasons that make NPs interesting molecules to study are the usefulness of the NP-hormone system as a model to analyze the noncovalent interaction between peptides and proteins. Since NPs have a relatively low molecular weight (monomer $M_r = 10,000$), the self-association of NPs provides a model to study protein-protein interactions. Moreover, the cooperativity exhibited between NP-hormone interaction and NP self association provides a tractable model for the study of protein-protein interactions, coupled allosterically to ligand binding.

REVIEW OF THE LITERATURE |

There are two major classes of bovine NPs; NP-I and NP-II (Hollenberg and Hope, 1968) which have very similar physical and chemical properties. Circular dichroism and binding properties of the two are almost identical (Breslow and Burman, 1990). NPs' structure, function and interactions have been studied in many areas. The purpose of my work relates to understanding two important questions; NP interaction with hormones, and protein-protein interaction leading to self association, and its allosteric effect on binding.

Protein-Hormone Interaction

The principal interactions between hormones and proteins depend on several factors. Figure 43 shows the binding pocket of NP with the first three residues of vasopressin and the types of interactions involved (Breslow and Burman, 1990).

First, the presence of a salt bridging of a carboxyl group in NP with the α -amino group of the hormone is deduced from pH titration (Blumenstein et al., 1977). The important role of α -amino group shows that the salt bridge is formed in a non-polar environment (Breslow et al., 1973).

Second, there is an apolar π - π interaction involving Tyr-2 of the hormone. ^1H NMR studies showed a chemical shift difference in the ring protons of Tyr-49 of the protein induced by the aromatic ring of bound peptide (Breslow and Burman, 1990). In aromatic systems, the delocalized π electrons give rise to a ring current when the field is perpendicular to the molecular plane. The presence of an aromatic residue in the peptide was necessary since substitution of Tyr to several other nonaromatic residues reduced binding to undetectable levels (Breslow and Abrash, 1966). ^{13}C NMR and hydrogen bonding observations showed the effect of hydroxyl group for a tighter binding (Sur et al., 1979; Blumenstein et al., 1980).

Third, the peptide backbone between hormone residues 2 and 3 is hydrogen bonded to the protein. This was shown in large downfield chemical shift changes of ^{15}N NMR of the peptide upon binding (Live et al., 1987). Substantial deshielding leading to chemical shift changes is normally observed for intermolecular hydrogen bonding.

Fourth, the side chains of residues 1 and 3 bind in an apolar pocket. This was shown in large chemical shift changes in the ^{13}C for the α -carbon of residue 3 which is Phe in vasopressin and Ile in oxytocin. The weak specificity of the binding pocket to residue 3 suggests that the principal interactions of the side chain are at the βCH_2 which is

common to both Ile and Phe, as observed from NMR data (Balaram et al., 1973). The contribution of the side chain of Cys 1 is shown by the reduced binding affinity upon deletion, by more than a factor of 100 (Breslow et al., 1973). Substitution by Phe showed similar binding, indicating that the side chain of Phe mimics the disulfide bridge of Cys in providing an apolar environment.

For most of these studies, small dipeptides and tripeptides that resemble the amino terminus of the hormones were used as hormone analogs. They were shown to bind with identical qualitative interactions as the hormones (Bothner-By et al., 1980; Breslow, 1970). The di- and tri-peptides showed the same ligand-induced change in the environment of Tyr 49 as measured by pH titration, as well as by absorbance, circular dichroism, and NMR spectroscopy. Also, the above peptides showed the same ligand-induced change in dimerization (Nicolas et al., 1978; Breslow, 1973), and the presence of the same bonding interactions that were outlined above.

Protein-Protein Interaction

Another area of importance is the investigation of the positive allosteric coupling that exists between NP-hormone binding and NP dimerization. The NP hormone binding shifts the monomer-dimer equilibrium toward the dimer (Breslow and Garguilo, 1977). The affinity of each NP-I dimer site for the hormone is at least 10 times that of the monomer (Breslow et al., 1991). The dimerization increases with the binding of hormones and related peptides by an unknown mechanism (Nicolas et al., 1980; Breslow and Burman, 1990). Different mechanisms for the peptide-induced increase in NP dimerization have been proposed (Breslow and Burman, 1990). NMR studies suggested that the peptide stabilizes the dimer by binding across the two monomer subunits since it was observed that the Tyr-49 residue contributed both to hormone binding and to dimerization (Peyton et al., 1986). However, the crystal structure of bovine NP-II

complexed with Phe-Tyr amide hormone analog that has recently been solved at 2.8 Å resolution (Chen et al., 1991) showed that the dimerization contact surface and hormone binding site are spatially remote.

The monomer-monomer interface is mainly comprised of β -sheet interactions that stabilize the dimer by hydrogen bonding. Models to explain the cooperative ligand binding with dimerization were suggested. The most recent one to explain the stronger ligand binding by the dimer is that binding induces conformational changes in the monomer that lead to unfavorable interactions with the solvent; this is relieved by intersubunit contact (Breslow et al., 1992). Circular dichroism and fluorescence polarization studies however provided no evidence of a large change in secondary or tertiary structure associated with dimerization (Rholam and Nicholas, 1981). Recent fluorescence polarization studies of pressure-induced dimer dissociation have shown differences in the regions involved in dimerization in the liganded and unliganded states (Breslow et al., 1991). For this reason NMR studies were done in this study on the unliganded state and then compared to the liganded to determine changes in conformation upon binding.

Since an increase in ionic strength and concentration seems to favor the dimer form, studies of the concentration dependence of bovine NP NMR spectra suggested some residues to contribute to the intersubunit contacts, which are expected to be largely apolar. These residues are Phe 22 or 35, Lys 18 or 59, an Ala, Thr, Val, Ile, and Leu residues (Breslow and Burman, 1990). Both the crystal structure and NMR studies gave evidence of the participation of His-80 and Phe-35 in dimerization in the unliganded state (Breslow et al., 1992). The observed broadening of Tyr-49 ring protons during dimerization could be due to dimerization-induced heterogeneity in the environment of the ring protons because of a change in conformation, and not due to monomer-monomer interaction. These results however are not strictly established (Breslow et al., 1992).

In reviewing NMR studies of NP, it should be noted that the majority of the work done was with one-dimensional NMR. Most of the NMR studies have been done on NP-I because its complexes are more soluble (Peyton et al, 1987; Lord and Breslow, 1978). The few recent attempts to make use of two-dimensional spectroscopy for assigning proton resonances were very limited (Breslow et al., 1992). The difficulty arose because of the largely dimeric state of the protein at high concentrations in normal solvents (dimer $M_r=20,000$) and broad linewidths (30-50 Hz) for at least the resolved α -proton resonances. However, the available crystal structure and the recent advances in multi-dimensional NMR now provide the opportunity for studies with a new direction: a detailed structural investigation of NP and its functioning at atomic resolution.

Figure 43. Principle interactions between the hormone, vasopressin, and neurophysin. The shaded area defines the protein. Dotted lines represent hydrogen bonds. The difference between oxytocin and vasopressin is the Ile residue instead of Phe in oxytocin. This Figure is adapted from Whittaker et al., 1985.

CHAPTER VIII

NMR METHODS SPECIFIC FOR NP ANALYSIS

Sequence-specific resonance assignments are essential for the study of the solution structure of proteins by NMR. The largest proteins structures determined by 2D ^1H NMR experiments are *E. coli* (Dyson et al., 1990) and human thioredoxin (Forman-Kay et al., 1991) which have 108 and 105 residues respectively. The most direct approach to solving the assignment problem for proteins of 100 amino acids and greater is isotopic labeling of the protein. Heteronuclear 2D NMR pulse sequences make use of ^{13}C and / or ^{15}N labeling to extend the practical limit to M_r of 20,000 and perhaps higher. The crowding is overcome in the 2D ^1H - ^1H spectra by dispersing the spectra in a third or fourth dimension (Bax et al., 1990). This technique however could not be applied for isotope labeling of NP because it has not been cloned and overexpressed in bacteria systems. For this reason, NMR experiments have been restricted to ^1H - ^1H 2D experiments.

The NMR experiments were performed at 400 MHz on a Bruker AMX-400 spectrometer interfaced to Bruker X32 computer. Clean-TOCSY (MLEV-17) (Bax and Davis, 1985; Griesinger et al., 1988), DQF-COSY (Rance et al., 1983), TQF-COSY, and NOESY (Jeener et al., 1979) experiments were conducted. Basic NMR methods and techniques were discussed in Chapter II. For NP analysis however, and due to the complexity of the work, more sophisticated techniques were investigated. Some of the techniques that were used to obtain more resolved spectra are discussed below.

SPIN SPIN COUPLING

Multiple Quantum Filters

Double quantum filter DQF-COSY corrects for the different lineshapes of the diagonal and the cross-peaks obtained by conventional COSY where the diagonal peaks are in pure dispersion mode and the cross-peaks in pure absorption. The elimination of dispersive character of the diagonal peaks helps to identify cross-peaks that are very close to the diagonal. Both DQF and triple quantum filter TQF-COSY are the most popular filtered experiments that use multiple-quantum transitions. These are transitions in which several nuclei participate simultaneously in a coherent manner. These experiments use special properties of multiple-quantum transitions to select certain types of amino acids. As an example, the amino acid glycine (Gly) in $^2\text{H}_2\text{O}$ has only two equivalent protons, which prevent the generation of double or triple-quantum transitions. For this reason a spectrum free of Gly cannot be obtained using a double or triple-quantum filter. TQF-COSY is useful especially in simplifying NP COSY spectra because it eliminates the resonances of 14 Gly. Figure 44 compares a TQF COSY to a DQF COSY of NP-I to show the simplification in the spectrum. Notice the disappearance of cross-peaks in the TQF-COSY around the diagonal between 4 and 5 ppm where the α protons of the Gly residues are expected to show. These are visible in the DQF-COSY because, in the asymmetric protein environment, they are not equivalent. The pulse sequence used in a conventional COSY experiment is $(90^\circ-t_1-90^\circ-t_2)$ where t_1 is the evolution period and t_2 is the detection period. The second 90° pulse is the mixing time. Multiple quantum filters insert a third 90° immediately after the second 90° pulse to give a pulse sequence of $(t_0-90^\circ-t_1-90^\circ-d-90^\circ-t_2)$ where t_0 is the relaxation delay of 2.3 s while applying solvent saturation using low power for samples run in $^2\text{H}_2\text{O}$ and high power for samples run in

$^1\text{H}_2\text{O}$. d is a short delay to allow time for switching rf phases between second and third pulse.

Clean TOCSY

TOCSY spectra involving net magnetization transfer among scalar coupled homonuclear spins can be obtained by applying a phase-alternating spin-lock field with a composite pulse cycle termed MLEV-17 (Bax and Davis, 1985). An improvement of this technique is Clean TOCSY (Griesinger et al., 1988). Low RF pulses were used in this experiment (90° pulse $\sim 21 \mu\text{s}$) with short delays equal to 2 times the 90° pulse inserted into the 180° -composite pulse in the MLEV-17 sequence to reduce rotating-frame cross relaxation effects as shown in Figure 45. The improvement of clean TOCSY is in eliminating rotating-frame cross relaxation effects that give rise to negative cross-peaks. The intensity of the standard TOCSY cross-peaks would be reduced if they were to overlap with these cross relaxation cross-peaks. Spectra were obtained at 298 K, 308 K, and 338 K with mixing times ranging from 10 ms to 100 ms, including two trim pulses of 2.5 ms duration before and after the MLEV-17 mixing. The majority of the spectra however were recorded at 50 ms and 308 K. The pulse sequence using the MLEV mixing with two power levels, for excitation and spin lock was

$$[t_0-90^\circ-t_1\text{-SL-}(90^\circ\text{x-}\Delta\text{-}180^\circ\text{y-}\Delta\text{-}90^\circ\text{x})\text{-}60^\circ\text{-SL-acquisition}].$$

SL is the low power used for spin locking, Δ is the delay introduced by the Clean TOCSY. The composite pulse cycle consists of 16 composite pulses of different phases followed by the 17th 60° pulse to remove the effect of pulse imperfections. The two trim pulses, 2.5 ms each defocus magnetization that is not parallel to the x-axis. t_0 is the preparation time of 2.3 s during which saturation of the solvent occurs with high power and low power for samples in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ respectively. Some spectra were obtained using a range of

mixing times from 5 ms to 100 ms, to identify primary coherence transfer from coherence transfer relay.

SOLVENT SUPPRESSION

Semi-Selective Jump-Return

Some of the problems of the resonance assignment process are overlap of peaks and missing data such as in bleached areas due to water suppression. To overcome these problems, acquisition at different temperatures is often carried out. Since, as stated earlier, one requirement for determining sequence specific assignments is running in $^1\text{H}_2\text{O}$ as solvent, solvent suppression techniques were investigated for optimal results. Some of these techniques were utilized in the course of this study because they lead to better resolved spectra. In the NOESY ($^1\text{H}_2\text{O}$) experiments, the solvent resonance was suppressed by either continuous irradiation or by applying a semi-selective jump-return pulse sequence ($90^\circ_x - \tau - 90^\circ_{-x}$ -Acquisition) (Hore, 1983; Plateau and Gueron, 1982). Figure 46 shows a comparison of two NOESY spectra with the different saturation techniques. Figure 46A shows the result of saturating the solvent with strong power that causes bleaching of αH 's that resonate at similar frequency to the water, and loss of cross-peaks due to rapid amide hydrogen exchange with solvent. The pulse sequence used is (t_0 - 90° - t_1 - 90° - τ - 90° -acquisition). Solvent saturation takes place during both t_0 and τ , the preparation period and the mixing time respectively. Typically, pulses of 9 μs were used with 3 s relaxation delay and 175 ms mixing time. For Figure 46B the result of using selective excitation pulse sequence is seen to be far superior. In this sequence, the first and second pulses have opposite phases separated by a delay that is short compared to relaxation time of the water. Typically, pulses of 6 μs were used with a delay of 200 μs and mixing time of 150 ms.

Pre-TOCSY

Pre-TOCSY COSY spectra (Otting and Wüthrich, 1987) were also collected for samples run in $^1\text{H}_2\text{O}$. In these experiments a short MLEV-17 mixing sequence was applied after the 90° pulse, during which magnetization is exchanged between scalar coupled spins. This restores some of the αH resonances that are bleached out by the strong irradiation of the water peak upon suppression. An example of the usefulness of this technique is shown in Figure 47. Two disadvantages, however, are the loss of signal to noise ratio, and that cross-peaks are no longer purely in phase since the insertion of the pre-TOCSY period gives rise to dispersive antiphase coherences. The pulse sequence used is (t_0 - 90° -MLEV-17- t_1 - 90° -acquisition). The MLEV-17 pulse sequence is similar to the above discussion, but with 25 ms duration.

SEQUENTIAL ASSIGNMENTS

The most successful procedure for sequential assignments was proposed by Wüthrich and coworkers (Wüthrich, 1986), based on the observation of sequential NOESY cross-peaks between the spin systems of the individual residues ($d_{\alpha\text{N}}$, d_{NN} , and $d_{\beta\text{N}}$) from analysis of the regions containing the NH- αH , NH-NH, NH- βH cross-peaks. For example, the $d_{\alpha\text{N}}$ cross peak connects α carbon proton, αH , of the one residue with the amide proton, NH, of the following residue. Since amide protons are labile, and hence exchange with $^2\text{H}_2\text{O}$, the solvent normally used for proteins, acquisition in $^1\text{H}_2\text{O}$ is essential. This is because sequential assignments are based completely on the NOEs to the labile backbone NH protons. Experiments are then done under conditions of pH and temperature where the exchange with the solvent is minimized. For this analysis, both NOESY and COSY (or TOCSY) giving a suitable combination of information on through space and through bond connectivities are needed.

EXPERIMENTAL METHODS

Data Acquisition

At the same temperature, a set of NOESY spectra, as discussed in Chapter II, was obtained with mixing times of 50, 100, 150 and 200 ms. The majority of the spectra were obtained with a mixing time of 150 ms. The cross-peaks consist of responses corresponding to the size of the NOE which has built up during the mixing time. Typically data sets consisting of 450 t_1 increments for TOCSY and 400 t_1 for NOESY and COSY, of 64 transients each with 1024 data points in the t_2 domain were accumulated. The transmitter offset was placed on the water resonance, and the spectral width of 5.4 KHz was used in both dimensions. All spectra were collected in the pure phase absorption mode, using the time-proportional phase incrementation (TPPI) technique (Marion and Wüthrich, 1983) for signal accumulation in the t_1 dimension. All spectra were resolution enhanced. After zero-filling, the digital resolution was 2.4 Hz/pt in F_2 and 4.7 Hz/pt in F_1 dimension. Base-line corrections were performed using polynomial functions of standard Bruker software installed on the Bruker X32 computer.

Sample Preparation

For NMR samples, the protein was isolated and purified as discussed in Chapter II. NP was further purified over Sephadex G-25, eluted with water. The concentration of NP-I in the NMR samples was 5 mM. Using aliquots of dilute ^2HCl , the pH was adjusted to 5.6-5.8. Extra care was taken for preparation of NMR samples in $^2\text{H}_2\text{O}$. The lyophilized protein was dissolved in 2 mL $^2\text{H}_2\text{O}$ at neutral pH and lyophilized. The pH was reduced to 5.7 in $^2\text{H}_2\text{O}$ and the resulting solution was lyophilized, redissolved in $^2\text{H}_2\text{O}$, the pH was readjusted and the solution was lyophilized. The sample was finally

dissolved in 99.996% $^2\text{H}_2\text{O}$ and placed in Wilmad 535PP NMR tubes which had been prerinsed twice with $^2\text{H}_2\text{O}$. $^1\text{H}_2\text{O}$ samples had 10% $^2\text{H}_2\text{O}$ to provide the lock signal.

Chemical shifts were referenced to the residual water peak to 4.76 ppm at 25°C, 4.66 ppm at 35°C, and 4.43 ppm at 55°C. No correction was done to the reported assignments for the offset introduced by the upfield shift of residual water peak upon addition of acetonitrile. An offset of 0.03 was introduced upon addition of 5% acetonitrile and 0.28 upon addition of 30% acetonitrile at 35°C. At 55°C, an offset of 0.37 was introduced upon addition of 30% acetonitrile. These offset determinations were done relative to water soluble TMS (DSS) which is 2,2-dimethyl-2-silapentane-5-sulfonate.

Chemically modified and proteolytically clipped NPs were prepared and purified by our collaborator, Professor Esther Breslow, of the Cornell University Medical College. NMR sample preparations of these proteins followed the same procedures as above.

Figure 44. Multiple-quantum-filtered ^1H COSY spectra. Spectra were obtained at 400 MHz for 5 mM NP-I in 99.9% $^2\text{H}_2\text{O}$, 30% $\text{C}_2\text{H}_3\text{CN}$. (A) Double-quantum-filtered spectrum. (B) Triple-quantum-filtered spectrum.

Figure 45. Phase-sensitive 400 MHz ^1H TOCSY spectra. 5 mM NP-I in 90% H_2O /10% $^2\text{H}_2\text{O}$, 30% $\text{C}^2\text{H}_3\text{CN}$ with a mixing time of 50 ms. (A) Mixing process with the MLEV-17 pulse sequence; (B) Same as (A) but only negative levels are plotted. (C) Mixing process with the clean TOCSY. (D) Same as (C) but only negative levels are plotted

Figure 46. Phase-sensitive ^1H NOESY spectra showing different solvent suppression techniques. Spectra were obtained for a 5 mM NP-I in 90% $^1\text{H}_2\text{O}$ / 10% $^2\text{H}_2\text{O}$, 30% $\text{C}^2\text{H}_3\text{CN}$, pH 5.8, $T = 35^\circ\text{C}$, 400 MHz., mixing time 150 ms. (A) NOESY with solvent irradiation suppression technique. (B) NOESY with a semi-selective jump-return pulse sequence. Notice the appearance of cross-peaks in (B) between 4.5 and 5.0 ppm that were bleached in (A).

Figure 47. Double-quantum-filter ^1H COSY spectra with two different solvent suppression techniques. Spectra were obtained for 5 mM solution of NP-I in 90% $^1\text{H}_2\text{O}$ / 10% $^2\text{H}_2\text{O}$, 30% $\text{C}^2\text{H}_3\text{CN}$, pH 5.8, $T = 35^\circ\text{C}$, 400 MHz. (A) COSY. (B) COSY with pre-TOCSY using MLEV-17 during 25 ms. Notice the appearance of cross-peaks in (B) between 4.5 and 5.0 ppm that were bleached in (A).

CHAPTER IX

SEQUENCE-SPECIFIC ^1H NMR ASSIGNMENTS FOR NEUROPHYSIN

INTRODUCTION

For large molecules, there would be extensive spectral overlap even in two-dimensional contour plots. Assignments under this condition would be ambiguous. The 2D spectrum of NP-I dimer in Figure 48 provides a good illustration of the challenge in making assignments. Another problem inherent to large molecules is the sharp decrease in cross-peak intensity for the three-bond J-couplings as the linewidths become larger than the coupling constants because of increasing rotational correlation time. This effect would make identification of spin systems impossible. Because of these two disadvantages, no attempt was made to assign the entire ^1H spectrum for the dimer of NP, a 93 residue protein that dimerizes at concentrations needed for 2D NMR studies.

Because of the interesting properties of NP discussed in Chapter VII and what sequence specific assignments might contribute to the understanding of hormone interaction and dimerization, we decided to attempt partial assignments. The total assignments, and hence the determination of the tertiary structure, is perhaps an impossible task at present; nevertheless, partial assignments of the protein can still give important information about the interactions between NP and peptide hormones, and the NP monomer-monomer interface.

These partial assignments were made possible by using acetonitrile as a cosolvent. Acetonitrile was found to be essential to obtain more resolved spectra, in part because it lowered the dimerization constant, and in part decreased the solvent viscosity. Another way to increase the spectral resolution was by doing the assignments on the proteolytically

cleaved des (1-8, 92, 93) NP-I, hereafter referred to as des NP-I. These ten residues can be deleted without losing dimerization or hormone binding capability (Breslow and Burman, 1990). Since the overall fold of the protein remains the same, it was expected to be possible to correlate the assignments to the native. Moreover, the crystal structure shown in Figure 49 was a very helpful guide to focus on the residues that might contribute to protein and hormone interactions, and to dimerization.

MATERIALS

Mononitrated bovine NP-I and des NP-I derivative were generously provided by our collaborator E. Breslow and were prepared by published methods (Sardana et al., 1987; Lord and Breslow, 1979; Peyton et al., 1986). The native NP-I was isolated and purified in our lab as discussed before. C^2H_3CN and 2H_2O were available from Cambridge Isotope Laboratory.

RESULTS

Effect of Cosolvent

Acetonitrile as a cosolvent was used to drive the NP equilibrium toward the monomer because the interface contact is hydrophobic. To determine the amount of acetonitrile needed for the best spectral resolution without causing a major conformational change, a set of 1D spectra of NP-I were acquired with an increasing percent volume of acetonitrile. Since circular dichroism studies had indicated that NP still binds to the hormones in the presence of acetonitrile (E. Breslow, private communication, and from our results shown in Chapter X), it was assumed that the presence of this cosolvent would not disrupt the native protein structure significantly. Moreover, evaluation of the 1D NMR spectra in pure water and in cosolvent showed that this assumption was valid. Figure 50 shows a comparison between the 1D spectra of NP-I without acetonitrile and

with 30% C^2H_3CN (90% 1H_2O /10% 2H_2O), at pH 5.8, 35°C, and 2 mM protein. The increase in spectral resolution was manifested by narrower peaks and increased resolution of downfield-shifted α protons in the region of 4.8 to 6.2 ppm. More resolved α protons are crucial for determining sequence specific assignments.

Effect of Temperature

Increasing the temperature decreases the rotational correlation time. This gives sharper peaks, and therefore more connectivities can be found through the spin systems. A set of 1D spectra was acquired on 2 mM of NP-I at pH 5.8 and 30% C^2H_3CN / 1H_2O from 25°C to 65°C, as shown in Figure 51. The final spectrum was acquired after two hours of incubation at 65°C to check whether the sample was stable for a time at this high temperature. Note that the peaks sharpened and the coupling constants were more resolved with the increase in temperature. On the other hand, there were no major changes in the 1D spectra; this indicated that the overall fold of the protein was preserved. This stability for NP was expected because of its seven disulfide bonds (Breslow and Burman, 1990).

A 200 ms 2D NOESY and 50 ms Clean TOCSY pair of spectra were obtained for des NP-I at 55°C. 1D spectra obtained after both runs showed that the protein was still intact. These high temperature spectra gave more resolved peaks, and hence were very helpful in confirming the assignments that were done at 35°C, the standard temperature used for assignments. Figure 52 compares the 2D TOCSY of des NP-I obtained at 25°C, 35°C and 55°C. These spectra were obtained at 5 mM concentration, pH 5.8, 30% C^2H_3CN , (90% 1H_2O /10% 2H_2O) using the Clean TOCSY pulse sequence discussed earlier. At low temperature, the peaks were broader than the coupling constants which led to disappearance of cross-peaks at 25°C. Comparing these three spectra showed that the easiest way to get assignments was by analyzing the 2D spectra at elevated temperatures.

Comparison of Native NP-I to Des NP-I

A set of 1D and 2D spectra were obtained for both native NP-I and des NP-I. The presence of acetonitrile and the elevated temperature gave a similar increase in the resolution in both proteins. Figure 53 shows a comparison of the native and des NP-I at the optimized conditions described above. Because both proteins follow the same pattern, starting to assign des NP-I and then correlating to NP-I seemed to be a reasonable approach because des NP-I gave much better resolved spectra, as shown in the 2D spectra in Figure 54.

Partial Assignment of the Proton NMR Spectrum of Des NP-I

As an aid to this section, the primary sequence of NP-I (Breslow, 1979) is included. *Italic* residues are deleted upon proteolytic cleavage to form des NP-I. According to the crystal structure of NP-II hormone complex (Chen et al., 1991), which as stated earlier is taken to be very similar to NP-I, residues 39-49 comprise a 3-turn 3_{10} -helix (bold face). The amino-terminal domain consists of a four-stranded antiparallel β -sheet of residues 11-13, 19-23, 25-29, 32-37. The carboxyl-terminal domain consists of a four-stranded antiparallel β -sheet of residues 59-61, 65-69, 71-75, and 78-82. The β -structures in the following sequence are in small caps.

*Ala Val Leu Asp Leu Asp Val Arg*8 Thr Cys LEU PRO CYS GLY Pro Gly Gly Lys
Gly₁₉ ARG CYS PHE GLY₂₃ Pro SER ILE CYS CYS GLY Asp Glu₃₁ LEU GLY CYS PHE
VAL GLY Thr₃₈ **Ala Glu Ala Leu Arg**₄₃ Cys **Gln Glu Glu Asn Tyr**₄₉ Leu Pro
Ser Pro Cys Gln Ser Gly Gln LYS₅₉ PRO CYS Gly Ser Gly₆₄ GLY ARG CYS ALA
ALA GLY ILE CYS CYS SER₇₅ Pro Asp GLY₇₈ CYS HIS GLU ASP Pro Ala Cys Asp
Pro Glu Ala Ala₉₁ *Phe Ser*

Identification of Spin Systems. The first stage of the assignment process involved identification of the spin systems. This was established primarily by using Clean TOCSY

spectra at 55°C and 35°C, at pH 5.8 and 30% C²H₃CN, 99% ²H₂O, and 30% C²H₃CN, 90% ¹H₂O/10% ²H₂O. COSY spectra were used to obtain unambiguous identification of direct scalar connectivities.

In ¹H NMR, the resonance frequencies of amide and aromatic protons are between 6 and 10 ppm. The CαH protons resonate between 4 and 6 ppm, and the methyl groups between 0 and 2 ppm. Subtle deviations from expected chemical shifts have been recognized as a sensitive measure of molecular conformation and environment. α protons and amide protons experience an upfield shift when placed in a helical conformation, and a downfield shift when placed in a β structure (Wishart et al., 1991). Also, helix residues are distinguished from β sheet and random coil residues in having NOE cross-peaks between sequential amide protons, the so-called d_{NN} connectivities. In the following section, residues were assigned to be part of the helix depending on the upfield shift of the α protons and the presence of d_{NN} cross-peaks (described in Chapter VIII).

Gln and Glu have the same spin system of AM(PT)XY. There are three Glu and one Gln residues in the 39-49 helix of the protein. These residues contain NH, α, β, and γ protons. The β protons from these four residues in the helix give TOCSY cross-peaks to the γ protons, NH protons, and to the α proton of the same residue. The α-proton is not always observed because of the presence of the water signal in that range. Figure 55 shows the assignment of a Glu residue in the TOCSY spectrum of des NP-I in ¹H₂O at 55°C. The NH at 8.77 ppm gives cross-peaks to the α proton at 4.11, the γ protons at 2.43, and the β protons at 2.18 ppm. The broadness of the peaks in F1 for the β and γ protons are due either to the presence of sharp intense peaks or due to overlap of the two βH's and two γCH₂'s.

The two overlapped δCH₃ resonances of a Leu are at 0.90 ppm, and gave cross-peaks to its γ proton that also overlaps with its β protons at 1.76 ppm, and to the amide protons as shown in Figure 56. γ and β protons are expected to overlap because they have

very close chemical shifts. In a random coil, these protons resonate at 1.64 and 1.65 ppm respectively (Wüthrich, 1986). This residue was easily identified because of the unique upfield chemical shift of the δ protons. The cross-peak at 3.7 ppm does not align exactly with the rest of the spin system, so it could belong to a different spin system in such a crowded region. This cross-peak shifts considerably from the rest of the Leu cross-peaks at lower temperature, indicating that it belongs to a different spin system. Since these spectra were acquired in 90% $^1\text{H}_2\text{O}$, disappearance of α protons under the huge water signal is expected between 4.6 and 4.8 ppm. From the spectrum in $^2\text{H}_2\text{O}$ at 35°C, a TOCSY cross-peak was observed between the β and α protons. This α -proton was then assigned to 4.62 ppm. This large deviation in chemical shift of the α proton compared to α protons in a helix could be due to the presence of additional local fields from aromatic rings or charged neighbors.

A combination of NOESY and COSY of des NP-I at 35°C were used to assign the ring protons of Tyr 49, which has AA'XX' spin system, as shown in Figures 57 and 58. His 80 is the only His residue in NP-I, and was also assigned as shown in the same two figures. The His 80 assignments will be discussed in a later section. The 3,5 and 2,6 ring protons of Tyr 49 were assigned at 6.87 and 7.28 ppm respectively. Because there is no observable J-coupling between the Tyr β protons and its protons on the aromatic ring, NOESY was used to identify the aliphatic spin system. Due to the close proximity of the Tyr β -protons to the 2,6 protons of the ring, NOESY cross-peaks are observed to 3.30 and 3.12 ppm, and these have the same chemical shifts as the β -protons in the TOCSY spectrum, arising from J-coupling to the amide proton at 7.97 ppm (shown in Figure 58). The cross-peak to the α proton was not obvious and so could be under water. From the $^2\text{H}_2\text{O}$ TOCSY spectrum acquired under the same conditions, the α -proton was assigned to 4.75 ppm by J-coupling from the β -protons.(not shown). In the COSY spectrum, three weak cross-peaks were observed between 6.8, 6.9 and 7.1 ppm for the 3,5-protons and

7.4 and 7.6 ppm for the 2,6-protons. Since there is only one Tyr residue, these peaks are assigned to Tyr residue in different conformations. The presence of different conformations for Tyr was observed previously (Breslow et al., 1992).

There are two Ala residues in the helix that are expected to give strong cross-peaks between their α H and β H and to their amide protons. As shown in Figure 59, the methyl in this A_3MX spin system is coupled to an α proton. These residues cannot be assigned unambiguously because the methyl protons are in a very crowded region. An amide proton at 8.50 ppm gave a cross-peak to an upfield-shifted α proton at 3.57 ppm, indicating that this Ala is part of the helix. This cross-peak does not align well with that of the β CH₃. The broad cross-peak at 1.15 ppm is a result of an overlap of β CH₃ of the Ala with protons from other residues. This is expected in a large protein because many protons of Ala, Val, Leu and Ile show cross-peaks to amide protons in this region. Another amide proton at 9.18 is assigned to an Ala because the β CH₃ position is consistent with that of an Ala. The α proton was not observed, but the assignment of this residue as part of the helix was based on the presence of d_{NN} cross-peaks. These cross-peaks will be discussed later.

Arg residues contain side-chain methylene pairs of protons, from the β to the δ positions. The TOCSY spectrum at 55°C showed much more resolved peaks for the β and γ protons than at lower temperature. These protons are distinguishable from the Glu and Gln residues because of their further upfield chemical shift. Figure 60 shows poorly resolved TOCSY cross-peaks from the backbone NH to the δ protons. However, cross-peaks from the ϵ -NH to the same side chain protons confirm the assignment of this spin system to Arg. The cross-peaks from the ϵ -NH to the γ and δ protons are much better resolved than from the NH, as is expected because the coherence transfer is through fewer bonds.

Cys and Asn residues in general are hard to distinguish because they only have α

and β protons coupled in an AMX spin system of nonexchangeable protons. Figure 61 shows the assignment of the helix Cys 44 and Asn 48 residues. The assignment of these residues to the helix is based on the presence of d_{NN} connectivities. No evidence was given from the α -protons because both are close to the water resonance. To distinguish between Asn and Cys, I rely on the exchangeable protons to get an unambiguous set of assignments. Due to the presence of hydrogen bonds, Asn side-chain NH (γ NH₂) protons (at 7.28 ppm) exchange slowly enough to show an NOE cross-peak to one of the β protons at 2.85 ppm, as shown in Figure 61. The rest of the spin system, β H at 2.80 and NH at 7.91 ppm were assigned unambiguously by coherence transfer in the TOCSY spectrum. The β and amide protons of the Cys residue were assigned to 2.70, 2.88, and 8.08 ppm respectively. In both systems the α proton was not assigned unambiguously.

Alignment of Spin Systems to Specific Amino Acid Residues. The second stage of the assignment process was to analyze NOESY spectra that were obtained in ¹H₂O, and align the spin systems to specific amino acid residues. The distance between the NH proton of one residue and the NH proton of the next residue $d_{NN}(i, i + 1)$, is less than 3.6 Å in a helix, where i and $i + 1$ are sequential amino acid residues. This small distance gives rise to NOE cross-peaks between the amide protons of sequential residues and was used to make sequence-specific assignments (Wüthrich, 1986). The 3₁₀ helix formation is characterized by the presence of strong d_{NN} cross-peaks and the α H upfield shift of an average of 0.39 ppm from the random coil value (Wishart et al., 1991). In addition, medium-range distances between the α proton of one residue and the NH proton of another $d_{\alpha N}(i, i + 4)$ and $d_{\alpha N}(i, i + 3)$ were used as will be discussed in more detail later.

Figure 62 shows the complete assignments of the helix in a TOCSY spectrum based on sequential d_{NN} and medium-range $d_{\alpha N}(i, i + 4)$ and $d_{\alpha N}(i, i + 3)$ cross-peaks of residues 39-50. Figure 63 is an illustration of the method of making sequential assignments using d_{NN} cross-peaks. Assignments were started from the N-terminus

toward the C-terminus. The d_{NN} cross-peak that connects NH of the starting residue with NH of the following residue lies in the NOESY spectrum. A horizontal line to the diagonal in the NOESY spectrum gives the position of the NH of the adjacent residue. The NH of an Ala at 9.18 ppm has a strong NOE cross-peak to another NH. This other NH shows a set of TOCSY cross-peaks connected by a vertical line that corresponds to a Leu spin system. Since Ala 41 is the only Ala that is followed by a Leu, it is concluded that the cross-peak was between Ala 41 and Leu 42.

The d_{NN} cross-peaks in a NOESY spectrum recorded at 55°C are shown in Figure 64. Ala 39 has a strong cross-peak to Glu 40, and Ala 41 has a strong cross-peak to Leu 42. Arg 43 has a strong cross-peak to Cys 44, and Gln 45 has a weak cross-peak to Glu 46. Glu 47 has a weak cross-peak to Asn 48 and moderate cross-peak to Leu 50. Asn 48 has a strong cross-peak to Leu 50. Thus, the backbone cross-peaks from NH resonances were discontinued several times. This discontinuity is expected in a 3_{10} -helix because it is less stable than an α helix. 3_{10} -helices are observed in proteins in segments that are most often distorted from the ideal conformation. This instability is because the hydrogen bond geometry is not optimal.

A list of assignments at temperatures 35°C and 55°C of des NP-I is given in Tables VIII and IX respectively. The numbers between parentheses are the chemical shifts for amino acid resonances listed in the literature for a random coil (Wüthrich, 1986). Assignments that could not be determined were left blank. The numbers followed by an asterisk are for assignments obtained from $^2\text{H}_2\text{O}$ spectra. One chemical shift is provided for most of the β proton pairs. Chemical shifts were referenced to the residual water signal at 4.66 ppm for the 35°C spectra and 4.43 ppm for the 55°C spectra. The main differences between these recorded values and the statistical random coil values are in the αH and NH chemical shifts. Both sets of resonances are generally found to be shifted upfield in a helix. The reason for this shift is that α protons are influenced by the peptide

group of preceding residue and hence by the backbone dihedral angle Φ (Wishart et al., 1991).

TABLE VIII

^1H CHEMICAL SHIFTS OF DES NP-I RESIDUES 39-50 AT 35°C, PH 5.8, 30% $\text{C}^2\text{H}_3\text{CN}$

Residue	NH	αH	βH	Others
Ala 39	7.80 (8.25)		1.14 (1.39)	
Glu 40	8.62 (8.37)	3.60 (4.29)	2.13 (2.09;1.97)	γCH_2 2.30 (2.31;2.28)
Ala 41	9.18 (8.25)		1.63 (1.39)	
Leu 42	8.18 (8.42)	4.62*(4.17)		δCH_3 0.90 (0.94;0.90)
Arg 43	9.18 (8.27)	3.64 (4.38)		γCH_2 1.98 (1.70)
Cys 44	8.24 (8.31)	4.10 (4.65)	2.90 (2.96;3.28)	
Gln 45	8.27 (8.41)	3.83 (4.37)	2.22 (2.13;2.01)	γCH_2 2.47 (2.38)
Glu 46	8.37 (8.37)	3.88 (4.29)	2.18 (2.09;1.97)	γCH_2 2.33 (2.31;2.28)
Glu 47	8.89 (8.37)	4.16 (4.29)	2.24 (2.09;1.97)	γCH_2 2.36 (2.31;2.28)
Asn 48	8.02 (8.75)			
Tyr 49	7.97 (8.18)	4.75*(4.60)	3.30;3.12 (3.13;2.92)	3,5H 6.87 (6.86) 2,6H 7.28 (7.15)
Leu 50	7.68 (8.42)		1.72 (1.65)	δCH_3 1.15 (0.94;0.90)

TABLE IX

¹H CHEMICAL SHIFTS OF DES NP-I RESIDUES 39-50 AT 55°C, PH 5.8, 30% C²H₃CN

Residue	NH	αH	βH	Others
Ala 39	8.50 (8.25)	3.57 (4.35)	1.15 (1.39)	
Glu 40	8.14 (8.37)		2.09;2.18 (2.09;1.97)	γCH ₂ 2.43 (2.31;2.28)
Ala 41	9.18 (8.25)		1.55 (1.39)	
Leu 42	8.06 (8.42)	4.62*(4.16)	1.76 (1.65)	δCH ₃ 0.90 (0.94;0.90)
Arg 43	9.15 (8.27)	4.14 (4.38)	1.88 (1.89;1.79) b	γCH ₂ 1.65, 1.70 (1.70), δCH ₂ 3.20 (3.32), εNH 7.36 (7.17;6.62)
Cys 44	8.08 (8.31)	4.08 (4.65)	2.70;2.88(2.96;3.28)	
Gln 45	8.45 (8.41)		2.10 (2.13;2.01)	γCH ₂ 2.45 (2.38)
Glu 46	8.57 (8.37)	3.94 (4.29)		γCH ₂ 2.45 (2.31;2.28)
Glu 47	8.77 (8.37)	4.11 (4.29)	2.18 (2.09;1.97) b	γCH ₂ 2.43 (2.31;2.28) b
Asn 48	7.91 (8.75)		2.80;2.85 (2.83;2.75)	γNH ₂ 7.28
Tyr 49	7.88 (8.18)	4.75*(4.60)	3.10;3.25 (3.13;2.92)	2,6H 7.26, 3,5H 6.98
Leu 50	7.56 (8.42)		1.81 (1.65)	δCH ₃ 1.05 (0.94;0.90)

The values between parentheses are the random coil values. The small letter b stands for broad peak or two peaks too close together to be resolved. The numbers followed by an asterisk are for assignments obtained from ²H₂O spectra at 35°C.

Effect of Mononitration. Spectra for mononitrated NP-I were obtained in both ¹H₂O and ²H₂O at pH of 5.8, and 35°C and in 30% C²H₃CN/¹H₂O. Spectra were obtained at the same concentration in ²H₂O but much lower concentration in ¹H₂O

because much of the protein precipitated before obtaining the $^1\text{H}_2\text{O}$ spectra. Apparently the mononitrated NP-I is less stable than the native protein in organic solvents, and perhaps sensitive to changes in pH and temperature.

Figure 65 shows a comparison in the TOCSY spectra in $^1\text{H}_2\text{O}$ between mononitrated and native NP-I. The most obvious difference is the change in chemical shift of the amide proton assigned to be Glu 47. The Glu 47 TOCSY cross-peaks between 2 and 3 ppm in F1 dimension are nonexistent in the mononitrated. Also the d_{NN} NOESY cross-peaks at 7.5 and 8.5 ppm in F1 that connected Glu 47 to Asn 48 and Leu 50 are missing in the mononitrated NP-I. This was an extra evidence that the assignments to Glu 47 were correct, because the residues close to Tyr are expected to be perturbed by the presence of the nitro group.

Assignment of Other Residues. His 80 is the only His in NP-I, and so was relatively easy to assign. The combination of NOESY and COSY cross-peaks shown in Figures 57 and 58 were used to assign the unique imidazole ring protons, and then the rest of the spin system. The ring protons have a spin system of AX, and an observable coupling constants even though these protons are separated by four bonds. The distances between the ring and the β protons are somewhat larger than in Phe and Tyr, and the corresponding NOESY cross-peaks are weak. Weak cross-peaks between the C_4H at 7.45 ppm and the βH at 3.20 and 3.24 were observed; this showed closer proximity of C_4H than the C_2H to the β protons. In Figure 58, the horizontal lines connected the NOESY cross-peaks to the TOCSY cross-peaks. The β protons in the NOESY spectrum have the same chemical shift as two cross-peaks in the TOCSY spectrum arising from J-coupling with the amide protons of the same residue. The α proton at 5.10, and NH at 9.15 ppm were consequently assigned.

There are only two other aromatic residues in des NP-I, Phe 22 and Phe 35, and both are part of the amino terminal β sheet. These were difficult to distinguish

unambiguously because of the extensive overlap of the aromatic ring proton resonances, and because of the presence of different conformations (refer to Chapter X). Another reason for this difficulty is the overlap of ring protons within each Phe residue. Phe ring protons in a random coil give rise to three resonances with very small chemical shift differences; 2,6H at 7.30, 3,5H at 7.39 and 4H at 7.34 ppm. In a protein, resolution is often not sufficient to distinguish between these peaks. Figure 66 shows some resolution of two overlapping peaks that might belong to the different Phe residues. A combination of $^1\text{H}_2\text{O}$ NOESY and TOCSY spectra were used to assign the β and the amide protons as shown in Figure 67. The 2,6H sets show NOESY cross-peaks to the β protons of the same Phe. Due to overlap of the 2,6 protons of both Phe residues, three NOE cross-peaks were observed to the β proton region. One might belong to one Phe and two to the other. The peak at 7.35 ppm that gives a NOESY cross-peak to a β proton at 2.9 ppm, could be assigned to the 2,6H of a Phe in a different conformation. The presence of different conformations and unambiguous assignments of the two Phe's will be discussed in Chapter X.

Glu 81 is a residue in the anti-parallel β -sheet conformation. It was assigned by an overlay of the NOESY and TOCSY spectra as shown in Figure 68. TOCSY NH- αH of His 80 and NOESY $d_{\alpha\text{N}}$ connectivities were used. $d_{\alpha\text{N}}$ connectivities are mainly used to assign sequential residues that are part of the β -sheet structure where $d_{\alpha\text{N}}$ distances are less than 3 Å. The α proton of His 80 shows a strong NOE cross-peak to an amide proton at 9.17 ppm. This amide proton was traced through the TOCSY spectrum to the entire spin system. The α proton was assigned to 4.85 ppm from the TOCSY spectrum recorded at 55°C. The other cross-peaks at 2.18 and 2.50 ppm arise from the β and γ protons of a spin system characteristic of a Glu residue. The downfield-shifted α proton (from 4.29 to 4.85 ppm), characteristic of a β sheet residue (Wishart et al., 1991), and the strong NOE cross-peak at 5.2 ppm in F1 dimension between αH of His and NH of Glu

residue, also characteristic of β sheet structure, confirm the assignment to Glu 81. Glu 81 is the only Glu residue that is part of the β sheet.

Another characteristic spin system shown by the TOCSY spectrum at 55°C, with a downfield-shifted α proton, is the long side-chain residue Arg. The downfield shift is from 4.38 ppm in a random coil to 4.68 ppm. Arg 20 and Arg 66 are the only two residues that are part of the β sheet regions. Figure 69 shows the spin system of Arg with an α proton at 4.68, a broad cross-peak for two β protons at 1.88 ppm, a γCH_2 at 1.70, a δCH_2 at 3.20 and an NH at 8.43 ppm. The NH is also downfield-shifted from 8.27 ppm in a random coil to 8.43 ppm characteristic of a β -sheet residue (Zhou et al., 1992). Sequence specific assignments can be determined using $d_{\alpha\text{N}}$ connectivities. The α proton of this Arg shows an NOE cross-peak to an amide proton (not shown). The amide proton couples with the rest of the spin system in the TOCSY spectrum. The spin system traced is characteristic of a Cys with the two β protons overlapping at 3.3 ppm. The α proton of the assigned Cys is not observed, and could be under the water resonance. Therefore, the residue that follows Cys cannot be assigned by $d_{\alpha\text{N}}$ connectivities. Since both Arg 20 and 66 residues are followed by a Cys residue, the assigned spin system could belong to either Arg. The assigned Cys spin system could then be either Cys 21 or Cys 67.

Asp 30, Glu 31 and Leu 32 were assigned as described next. Asp has only α and β protons that are connected by J-coupling. An α proton at 4.85 and a β proton at 3.18 ppm were assigned to an Asp residue, based on their chemical shifts compared to the random coil values and their coupling in the TOCSY spectrum showed in Figure 69. The assignment of this residue to a specific position in the sequence was verified by the consistency of the sequential assignments that followed, based on $d_{\alpha\text{N}}$ connectivities. The α proton shows an NOE to an amide proton at 7.94 ppm (not shown), which when traced in the TOCSY spectrum gives a characteristic Glu spin system of resolved β protons at 2.20 and 2.40 ppm and γ protons at 2.62 ppm. A characteristic Leu spin system with δ

protons at 1.05, β protons at 1.70, α proton at 4.78, and NH at 7.68 ppm was assigned to a Leu residue based on chemical shifts consistency with the random coil and the coupling pattern. There are four Leu residues in des NP-I. Two were already assigned as part of the helix. This residue could be assigned to either Leu 32 or Leu 11 because both are part of the β sheet. β sheet residues as mentioned earlier have downfield-shifted α protons. The $d_{\alpha N}$ connectivity between Glu 31 and the Leu residue is not observed because the α proton of the Glu residue is bleached by water suppression. For this reason the position of this Leu residue in the sequence could not be assigned unambiguously.

Thr 9 was assigned in $^2\text{H}_2\text{O}$ in 30% acetonitrile as follows. Thr residue was characterized by J-coupling between γCH_3 and a β proton that has a chemical shift very close to the α proton. Thr 9 in des NP-I is the N-terminal residue, and hence showed sharp peaks in the TOCSY spectrum due to the increased mobility. In the TOCSY spectrum, coupling between the γCH_3 at 1.45, β at 4.10, and α proton at 4.35 ppm was observed (not shown). The presence of coupling between the β at 4.10 ppm and α proton at 4.35 ppm was a further check of this assignment. The final Thr 32 residue in des NP-1 was assigned by being the only remaining Thr. Both of these assignments are shown in Figure 78 of Chapter X.

Lys spin systems can be generally distinguished from the other long side-chain systems if there are enough cross-peaks associated with them visible in the TOCSY spectra. The number of cross-peaks is greater than the number of possible chemical shifts from other residues. A long side-chain spin system is assigned to a Lys residue with γCH_2 at 1.32, δCH_2 at 1.48, βH at 2.4, ϵCH_2 at 3.2, αH at 4.84 and NH at 7.86 ppm. The downfield-shift of the α proton from 4.36 to 4.84 ppm suggested that this residue is part of the β -sheet. There are two Lys residues in des NP-I. Since Lys 59 is the only Lys residue that is part of the β sheet, this spin system is assigned to Lys 59. The chemical shifts of these residues are shown Table X.

TABLE X

¹H CHEMICAL SHIFTS OF DES NP-I β-SHEET RESIDUES AT 55°C, PH 5.8, 30%
²CH₃CN

Residue	NH	αH	βH	Othera
Arg 20*	8.43 (8.27)	4.68 (4.38)	1.88 (1.89;1.79) b	γCH ₂ 1.70 (1.70) b δCH ₂ 3.20 (3.32) b
Cys 21*	8.02	4.98	3.38	
Leu 32*	7.68 (8.42)	4.78 (4.17)	1.72 (1.65)	δCH ₃ 1.05 (0.94)
Lys 59	7.86 (8.41)	4.84 (4.36)	2.40 (1.85)	γCH ₂ 1.32 (1.45) δCH ₂ 1.48 (1.70) εCH ₂ 3.25 (3.02)
His 80	9.12 (8.41)	5.10 (4.63)	3.37;3.47 (3.26;3.20)	C2 8.64 (8.12) C4 7.43 (7.14)
Glu 31	7.94 (8.37)	4.84 (4.29)	2.10;2.40 (2.09;1.97)	γCH ₂ 2.62 (2.31;2.28)
Glu 81	9.17	4.85	2.18	γCH ₂ 2.15
Asp 30	8.03 (8.41)	4.85 (4.76)	3.18 (2.84)	
Thr 9		4.35 (4.35)	4.10 (4.22)	γCH ₃ 1.45 (1.23)

The assignments of the residues that are followed by an asterisk are preliminary. They could be Arg 66, Cys 67 and Leu 11.

Observation of Medium-Range Distances. The common secondary structures contain medium-range ¹H-¹H distances that are sufficiently short for observation by NOEs. These distances are statistically reliable for verifying the presence of secondary structure. This statistical analysis was done by Wüthrich and coworkers to determine the extent and uniqueness to which helical secondary structure is identified by such distances.

(Wüthrich, 1986). A 3_{10} -helix is characterized by strong d_{NN} , weaker $d_{NN}(i, i+2)$, and the presence of relatively strong medium range NOEs, $d_{\alpha N}(i, i+3)$ connectivities. Figure 70 shows the medium range $d_{\alpha N}(i, i+3)$ for some of the residues, which provided a further check of the assignment of the helix residues.

Figure 70 shows the fingerprint region, that is the amide proton to α proton region, of the 55°C NOESY. The α proton of Ala 39 shows an NOE cross-peak to the amide proton of Arg 43, 4 residues away. Arg 43 α proton shows an NOE cross-peak to the amide proton of Glu 46, 3 residues away. Cys 44 α proton shows a cross-peak to the amide proton of Glu 47, also 3 residues away. No cross-peaks were observed for residues Gln 45 and Glu 46. The $d_{\alpha N}(i, i+4)$ is observed primarily in an α helix secondary structure. The $d_{\alpha N}(i, i+3)$ is observed in both the 3_{10} -helix and the α helix. As a result, NP, like most globular proteins, contains somewhat distorted secondary structure elements, which in this case most likely corresponds to a 3_{10} -helix.

DISCUSSION

Effect of Acetonitrile

To determine to what extent the native protein tertiary structure was preserved in the presence of acetonitrile, 150 ms NOESY spectra of des NP-I in the presence and absence of acetonitrile were compared. As can be seen in Figure 71, the pattern of sequential connectivities in the amide region for the fragment 39-50 is identical under both solvent conditions. The relative chemical shifts within each spectrum is conserved, although there are chemical shift differences between spectra due to the presence of the polar organic solvent. The preservation of the relative chemical shift positions under both solvent conditions is an indication that the basic fold of the protein is preserved (Werner and Wemmer, 1992). The d_{NN} cross-peaks between Ala 41 and Leu 42, Ala 39 and Glu 40, and also Glu 47 and Asn 48 are preserved, as shown in Figure 71. Another difference

between the spectra is the larger number of cross-peaks observed in the spectra in the presence of cosolvent, which are thought to be largely due to des NP-I being a monomer. The effect of acetonitrile on dimerization will be discussed in more detail in Chapter X.

Proton Assignments

Of the 93 residues in the primary sequence of bovine NP-I, 24 were assigned sequence-specifically. Other assignments can be made at this time, but not to specific residues in the sequence. The broad α proton peaks, even in the presence of acetonitrile made the sequential assignments very difficult, in addition to all the limitations induced by some degree of dimerization and the large size of the protein, as discussed previously. It was observed that the residues in the helix show little chemical shift dispersion in the α protons, whereas β -sheet domains show much better dispersion, as expected (Bax, 1989). Fortunately, these assignments were sufficient to be a starting point to probe the binding site and the monomer-monomer interface in ways heretofore not possible. This is the subject of the next chapter.

Chemical Shifts and Protein Structure

The assigned helix residues, except for Leu 42, showed α proton chemical shifts consistent with the general trend of upfield shifted α protons. Another trend that was observed was that the amide protons at the N-terminal residues of the helix were shifted downfield compared to those found at the C terminus. Ala 39 and Ala 41, the N-terminal residues amide protons showed a downfield shift of 0.25 and 0.93 ppm from random coil positions. Most of the other residues amide protons were either upfield-shifted or showed no significant difference from the random coil values. The C-terminus residues showed a significant upfield shift. Asn 48, Tyr 49 and Leu 50 showed upfield shifts of 0.84, 0.3, 0.86 ppm from random coil values. The N-terminus/ C-terminus difference in amide proton shifts, is consistent with the trend observed for helices of many different sequences

(Wishart et al., 1991). The downfield shift indicates proximity to a positive charge, and the upfield shift indicates proximity to a negative charge. This net charge formation is consistent with the helix dipole property (Hol, 1985) which explains the presence of net negative charge at the C-terminus, and net positive charge at the N-terminus. The net charge is a result of alignment of the amide groups with hydrogen bonded carbonyl groups.

Concerning the β -sheet residues, all the residues assigned showed downfield shifts of the α proton resonances. α protons were easier to assign in the β -sheets because they were shifted downfield from the solvent line. This downfield trend was not observed for the amide protons since they showed both upfield and downfield shifts.

Figure 48. NOESY (top) and TOCSY (bottom) spectra of 5 mM NP-I dimer. Spectra were obtained in $^2\text{H}_2\text{O}$ with a 150 ms and 50 ms mixing time respectively. Notice the extensive overlap and lack of resolution.

Figure 49. The crystal structure of NP-II bound to dipeptide hormone analog. (Top) The C α backbone (thick lines) showing the position of the cysteine residues (thin lines) and disulfide bridges (dotted lines). (Down) Crystal structure of dimer NP-II. This Figure is adapted from Chen et al., 1991.

Figure 50. Effect of acetonitrile on the spectral resolution of NP-I. 1D spectra were obtained without acetonitrile (A), and with 30% $\text{C}^2\text{H}_3\text{CN}$ (B); at pH 5.8, 35°C and 2 mM protein. Note the increase in spectral resolution manifested by narrower peaks and the presence of downfield shifted α protons in the region of 4.8 to 6.2 ppm.

Figure 51. Temperature effect on 1D spectral resolution of native NP-I. ^1H NMR set of 1D spectra obtained for 2 mM of NP-I at pH 5.8 and 30% $\text{C}^2\text{H}_3\text{CN}$, 90% $^1\text{H}_2\text{O}$ /10% $^2\text{H}_2\text{O}$. Starting from the bottom, the spectra were obtained in $^\circ\text{C}$ at 40, 42, 47, 52, 57, 60, 65 and another 65°C spectrum after one hour. Note how the peaks sharpen and the coupling constants are more easily seen with the increase in temperature.

Figure 52. Temperature effect on 2D TOCSY spectral resolution of des NP-I. (A), (B), and (C) are Clean TOCSY spectra obtained at pH 5.8 and 30% C²H₃CN, 90% ¹H₂O/10% ²H₂O at 25°C, 35°C, and 55°C respectively.

Figure 53. 1D NMR comparison of des (A) and native NP-I (B). Spectra were obtained at pH 5.8 and in 30% C^2H_3CN , 99% 2H_2O , at 35°C.

Figure 54. 2D Clean TOCSY comparison of des (A) and native (B) NP-I. Spectra were obtained at pH 5.8 and 30% C^2H_3CN , 99% 2H_2O , at 35°C with 50 ms mixing time. Notice the more resolved cross-peaks in des NP-I.

Figure 55. Example of a Glu residue in a TOCSY spectrum at 55°C. (A) Glu spin system. (B) TOCSY spectrum at 55°C in 30% C²H₃CN, 90% ¹H₂O/10% ²H₂O obtained under the same conditions discussed before. The position in the sequence was verified from NOESY.

Leu

Figure 56. Assignment of a Leu residue in a TOCSY spectrum of des NP-I at 55°C. Spectra were obtained in 30% $\text{C}^2\text{H}_3\text{CN}$, 90% $^1\text{H}_2\text{O}$ /10% $^2\text{H}_2\text{O}$.

Figure 57. Assignment of Tyr and His ring protons in a COSY spectrum of des NP-I. The spectrum was obtained at 35°C, pH 5.8 and in 30% C^2H_3CN , 90% 1H_2O / 10% 2H_2O . Note the three conformations of Tyr.

Figure 58. Assignment of the aliphatic protons of His and Tyr. The TOCSY spectrum (dotted contours) is overlaid with a NOESY spectrum of des NP-I at 35°C, pH 5.8 and 30% C^2H_3CN , 90% 1H_2O / 10% 2H_2O . The 2,6 H of Tyr at 7.28 ppm show an NOE cross-peak to the β protons of at 3.30 and 3.12 ppm. The His C_4 proton at 7.4 ppm shows weaker NOE cross-peaks to the β protons. The whole spin systems are traced as shown.

Figure 59. Assignment of two Ala residues in the TOCSY spectrum at 55°C. The spectrum was obtained in 30% $\text{C}^2\text{H}_3\text{CN}$, 90% $^1\text{H}_2\text{O}$ /10% $^2\text{H}_2\text{O}$. The αH of 41 could be bleached by water suppression. The poor resolution in the βCH_3 is due to considerable overlap in this region resulting in a broad peak.

Figure 60. Assignment of an Arg residue in a TOCSY spectrum at 55°C. The spectrum was obtained in 30% C²H₃CN, 90% ¹H₂O/10% ²H₂O. Cross-peaks are shown from both, the backbone NH proton and from the ε-NH proton, clearly identifying this spin system as an Arg. Cross-peaks from the backbone NH are not obvious in this Figure but the NH position was verified from d_{NN} connectivities.

Figure 61. Assignment of Cys and Asn residues. TOCSY and NOESY overlay spectra at 55°C in 30% $\text{C}^2\text{H}_3\text{CN}$, 90% $^1\text{H}_2\text{O}$ /10% $^2\text{H}_2\text{O}$. The dotted contours belong to NOESY cross-peaks. Notice the connection between γNH_2 and β -protons. There is a slight upfield shift in NOESY cross-peaks relative to the TOCSY because of temperature fluctuation during acquisition.

Figure 62. Assignment of residues 39-50 in the TOCSY spectrum at 55°C. The spectrum was obtained in 30% C^2H_3CN , 90% 1H_2O /10% 2H_2O at pH 6.0 with a mixing time of 50 ms. Cross-peaks from the same spin system are linked by solid lines with sequence numbers near the corresponding cross-peaks.

Figure 63. Sequential assignment using TOCSY/NOESY spectra at 35°C. Spectra were obtained in 30% C^2H_3CN , 90% 1H_2O /10% 2H_2O . The dotted contours correspond to NOESY cross-peaks. The spin systems are traced in the TOCSY spectrum. NH of Ala 41 has a cross-peak to Leu 42 in the NOESY spectrum

Figure 64. NH-NH region of the 200 ms NOESY spectrum of des NP-I. The spectrum was recorded in 30% $\text{C}^2\text{H}_3\text{CN}$, 90% $^1\text{H}_2\text{O}$ /10% $^2\text{H}_2\text{O}$ at 55°C and pH 6.0. Sequential NH(i)-NH(i+1) NOEs are indicated for the segment of twelve residues.

Figure 65. Comparison of TOCSY spectra between native NP-I and mononitrated. Spectra were obtained at 35°C in 30% C^2H_3CN , 90% $^1H_2O/10\%$ 2H_2O , pH 5.8 and a mixing time of 50 ms. Notice the disappearance of Glu 47 in the mononitrated NP-I.

Figure 66. Assignments of Phe residues in TOCSY $^2\text{H}_2\text{O}$ spectrum at 35° C. The spectrum was obtained in 30% $\text{C}^2\text{H}_3\text{CN}$, 99% $^2\text{H}_2\text{O}$. This spectrum shows some resolution between the overlapping peaks. The rest of the spin system is shown in Figure 67.

Figure 67. TOCSY/NOESY (in dotted contours) overlay spectra to assign the Phe β protons. Spectra were recorded in 30% $\text{C}^2\text{H}_3\text{CN}$, 99% $^2\text{H}_2\text{O}$ at 35°C.

Figure 68. Assignment of Glu 81 in an overlay of TOCSY and NOESY (dotted contours) spectra. Spectra were recorded at 35°C in 30% C^2H_3CN , 90% 1H_2O /10% 2H_2O . The α proton of His 80 has a $d_{\alpha N}$ NOE cross-peak to the NH of Glu 81.

Figure 69. Assignments of some β -sheet residues in des NP-I at 55°C in a TOCSY spectrum. The spectrum was obtained in 30% C^2H_3CN , 90% $^1H_2O/10\%$ 2H_2O . The assignments are shown on the spectrum.

Figure 70. Fingerprint region of the NOESY spectrum of des NP-I. The spectrum was recorded in 30% C^2H_3CN , 90% 1H_2O /10% 2H_2O at 55°C, with a mixing time of 200 ms. Medium-range links through $d_{\alpha N}(i, i+3)$ are shown in solid lines. Sequence numbers are indicated at the $CoH(i)-NH(i+3)$ cross-peaks.

Figure 71. Comparison of NOESY spectra of des NP-I with and without acetonitrile. Both spectra were obtained at identical conditions; 5 mM, pH 5.8, 90% ¹H₂O/10% ²H₂O, and a mixing time of 150 ms. (A) NOESY with 30% C²H₃CN, (B) NOESY in pure water. The amide region is plotted; it shows that the d_{NN} cross-peaks are conserved.

Figure 72. Assignments in the TOCSY spectrum at 55°C in $^1\text{H}_2\text{O}$ at pH 6.0 with a mixing time of 50 ms. The spectrum was obtained for des NP-I in 30% $\text{C}_2\text{H}_3\text{CN}$. Cross-peaks from the same spin system are linked by solid lines with sequence numbers near the corresponding cross-peaks.

CHAPTER X

PROTEIN-PROTEIN AND PROTEIN-LIGAND INTERACTIONS

PROTEIN-PROTEIN INTERACTIONS

Introduction

The self-association binding constant of NP is known to increase with binding to its ligand hormones and related peptides (Breslow and Burman, 1990). Other systems behave similarly, including triliganded human hemoglobin (Mills and Ackers, 1979). The physiological importance of NP dimerization is in causing high density storage and stabilization of hormones. NP self-association involves interfacial β -structure / β -structure contact, which is modulated by the presence of organic cosolvent (see Chapter VII). These β -sheets contacts may be related to the plaques of β -amyloid proteins that are deposited on the brain in advanced cases of Alzheimer's disease (AD). These studies may therefore shed the light on the forces that form the plaques of amyloid protein. It is hoped that the fundamental work presented here will eventually contribute to the development of therapy, by either inhibiting contribution of these and other residues to the formation of the amyloid plaques, or in removing them from the brain. Self-association of NP can be used as a model for intermolecular associations involving β -structures because the amyloid protein is water-insoluble, and hence cannot be directly studied by solution state NMR. From a combination of crystal structure results of the homologous protein NP-II (Chen et al., 1991), pH titration, chemical modification, and NMR results (Breslow et al., 1992), the following residues are believed to be among the ones most perturbed by dimerization: His 80, Phe 35, Thr 9, and Tyr 49. To confirm the contribution of these and other

residues to the interface, 1D and 2D NMR methods were used in this study to probe the differences between monomer and dimer.

Materials and Methods

1D spectra were obtained for dilute samples that were at least 80% monomer (Breslow and Burman, 1990). The purest monomer observed was NP-I at 0.1 mM, pH 5.7, 35°C, 30 % C^2H_3CN . A des NP-I spectrum was also obtained under the same conditions. 1D spectra at higher concentrations were obtained for both NP-I and des NP-I in 1H_2O and 2H_2O , in a series of solvent mixtures with an increasing volume ratio of C^2H_3CN starting from 0% and finishing at 30%, as shown in Figure 73 for NP-I in 1H_2O , and Figure 74 for NP-I in 2H_2O .

2D spectra for both through-bond and through-space analyses were obtained for the "true" monomer of NP-I under the following conditions: 0.5 mM concentration, pH 5.8, 25% C^2H_3CN , and 35°C in 2H_2O . At a higher concentration of 5 mM and in the absence of C^2H_3CN , the same set of 2D spectra were obtained for the NP-I dimer. 2D spectra for an intermediate case were obtained under the same conditions of the dimer but in the presence of 5% C^2H_3CN . 2D spectra of des NP-I were obtained at 5 mM concentration, pH 5.8, 35°C for the three cases: at nearly entirely monomer in 30% C^2H_3CN , an intermediate case in 5% C^2H_3CN , the almost entirely dimer in 0% C^2H_3CN . These spectra were recorded in both 1H_2O and 2H_2O solvents under the same conditions.

RESULTS

Observations From The 1D Spectra

Figures 73 and 74 show the changes in 1D spectra as acetonitrile was added. In both sets of spectra, chemical shift differences and sharper peaks were observed with an

increasing concentration of acetonitrile. In the upfield region between 0 and 1 ppm, there are resonances at around 0.5 ppm. This chemical shift is considerably upfield compared to resonances expected from a random coil. Resonances are generally displaced to higher field if they are near aromatic amino acid residues, above or below the ring plane, experiencing local fields that oppose the external magnetic field. Upon addition of acetonitrile, these resonances are shifted back toward the normal range by almost 0.4 ppm. This indicated that the aromatic ring protons that caused the upfield shift to these positions in the dimer are not in such close proximity to these protons in the monomer. This observation proved that there is at least one aromatic ring at the NP-NP interface in close proximity to residues that have higher field resonances such as Ala, Thr or Val. Another reason for this chemical shift difference could be due to a three-dimensional shift within the monomer interior, involving an aromatic residue.

The increase in resolution is another indication of the formation of monomer in 30% acetonitrile. The region between 4.8 and 6.5 ppm is characteristic of α protons of β -sheet structure (Szilgay and Jardetzky, 1989). The shift of one peak, from 6.5 to 5.8 ppm, may result from increased main chain flexibility in 30% acetonitrile (Wishart et al., 1991). Flexible β -sheets would be expected to have averaged chemical shifts closer to random coil values. Such an increase in flexibility would then be coupled to formation of the monomer in the presence of the organic co-solvent. The increase in resolution in this region especially in the $^1\text{H}_2\text{O}$ spectra of Figure 73, made assigning α protons of certain residues in this protein possible as shown in Chapter IX

In the aromatic region between 6.5 and 8 ppm in the $^2\text{H}_2\text{O}$ spectra, changes indicating averaging of ring flips rotations, exchange between conformations and absence of dimer peaks might be among the effects observed. Slow 180° ring flips caused by restricted rotation of Phe and Tyr rings generally gives rise to broadened, and perhaps extra peaks, due to loss of averaging between 2 and 6, and 3 and 5 ring protons. Phe and

Tyr could exist in different conformations giving rise to extra peaks for the same proton. Phe and Tyr ring protons of the dimer might be in a different environment in the monomer, and so would give rise to resonances with different chemical shifts. The aromatic resonance at 7.6 is shifted to 7.3 ppm in 30% acetonitrile. The broad Tyr peaks with extra small peaks showing at 6.8 indicates the presence of Tyr in alternative conformations. The spectra recorded in the presence of intermediate concentration of acetonitrile exhibit these changes in chemical shifts until the peaks merge, at 20% acetonitrile. All these possible reasons for obtaining less dispersion of peaks were further investigated using 2D NMR.

The amide protons between 8 and 10 ppm observed in $^1\text{H}_2\text{O}$ spectra, show increasing resolution as acetonitrile is added. This increase in resolution is very important for sequence specific assignments because obtaining assignments depends on observing NOE cross-peaks between amide protons, as shown in Chapter IX.

Another observation from the 1D spectra is the higher resolution in $^1\text{H}_2\text{O}$ compared to $^2\text{H}_2\text{O}$, especially in the upfield region at identical and intermediate concentrations of acetonitrile as shown in Figures 73 and 74, the fourth spectrum from the top of both sets. This solvent isotope effect is an evidence of the contribution of hydrogen bonding to dimerization since deuterium forms stronger hydrogen bonds than hydrogen.

Observations From 2D Spectra

Phe 35. Figure 75 shows the NOESY and COSY spectra of des NP-I in $^2\text{H}_2\text{O}$ at 5% acetonitrile. In the COSY spectrum, an αH at 6.4 ppm shows coupling to a β proton at 3.1 ppm. The NOESY spectrum identifies another β proton at 2.6 ppm, and a weak cross-peak at 2.4 ppm. There is a strong cross-peak in the NOESY spectrum between this α proton at 6.4 ppm and another α proton at 5.3 ppm. The αH at 5.3 ppm shows a weak cross-peak to 3.1 ppm, in addition to two strong cross-peaks at 2.6 and 2.4 ppm. The β and α protons of these two spin systems show strong NOE cross-peaks to the aromatic

region at 7.25 ppm. This aromatic peak was assigned to one of the Phe 2,6 ring protons. The two different α positions could, at this stage, be assigned to the same Phe residue exchanging between different conformations, or to one Phe in the dimer close to a different Phe from the alternate monomer. Two minor aromatic peaks at 7.40 and 7.55 ppm that show through bond and through space coupling were observed. These might be either due to a restricted conformation, causing slow ring flips and chemical shift difference between 3 and 5 ring protons and hence these residues can be assigned to 3, 4 and 5 aromatic ring protons, or more likely, to the presence of different conformations.

In the NOESY and COSY spectra in $^2\text{H}_2\text{O}$ with 30% acetonitrile shown in Figure 76, two α protons, at 6.3 and 5.7 ppm show strong cross-peaks between each other in the NOESY spectrum. The α proton at 6.3 ppm showed a very weak cross-peak to a β proton at 2.9 compared to the β proton showed at the same chemical shift from the α proton at 5.7 in the NOESY spectrum. It is clear from the intensity of the cross-peaks that there is more contribution from the one with an upfield α proton. The small contribution from an extra β proton observed at 5% acetonitrile is diminished to nonexistent. The spin system giving rise to α proton at 6.3 ppm and β protons at 3.2 and 2.9 ppm is very diminished, compared to the spin system of α proton at 5.7 and β protons at 2.9 and 2.7 ppm. Another observation is the appearance of a strong peak at 7.38 ppm with strong NOE cross-peak to a β proton at 2.97 ppm. The rest of the spin system is identified by the TOCSY spectrum (not shown) to show a resolved double of doublets for β protons at 2.9 and 3.1 ppm, and an α proton at 5.07 ppm.

The above observations demonstrate the possible effect of the protein environment on the rotational motions of the aromatic ring. In the dimer, the increased complexity of the 1D spectrum in the aromatic region is due to chemical shift dispersion resulting from the shielding of the Phe residue from the solvent. In a phenylalanine ring, the two proton pairs 2,6 and 3,5 are symmetrical, and hence equivalent in an isotropic solvent to show a

spin system of AMM'XX'. In the interior of a protein, or at the monomer-monomer interface, the individual ring protons experience different shielding effects and an AGMPX spin system might be observed. These effects from the protein environment are averaged out by rapid rotational motions as acetonitrile was added, resulting in a shift of the equilibrium towards the monomer. The peaks that were dispersed in the 5% spectra were assigned to a Phe residue. There are two Phe residues in des NP-1. This Phe residue was assigned to Phe 35 based on previous observation of the involvement of Phe 35 at the interface (Breslow et al, 1992; Chen et al., 1991).

A more probable explanation for the appearance of extra peaks is the presence of the Phe residue in different conformations. The strong NOESY cross-peaks between the two α protons discussed above, in both dimer and monomer, were shown to be due to chemical exchange between two different conformations and not due to dipole-dipole relaxation due to closeness in space. This is described in more detail in the following section. The peak at 7.38 ppm was assigned to the 2,6 ring protons of a major conformation of Phe 35 in the dimer different from the other two and is more dominant. This conformation belongs exclusively to the monomer and it is at different chemical shift because of its existence in a different environment. This conformation was shown not to exchange with the other two by saturation-transfer 1D experiments. Another difference between the monomer and dimer is the change in the nearly equal contribution of the two conformations in the dimer to a prevailing one in 30% acetonitrile. This is shown by decrease in intensity in NOESY and TOCSY (not shown) cross-peaks of one conformation relative to the other. This is direct evidence of participation of Phe 35 at the interface.

Phe 22. In a combination of NOESY and COSY spectra in $^2\text{H}_2\text{O}$ at 5% acetonitrile as shown in Figure 75, two conformations for Phe 22 were observed. Similarly to Phe 35, there are two α protons at 5.8 and 5.4 ppm that show strong NOE

cross-peaks to each other. These were shown by saturation transfer to belong to the same Phe residue that is exchanging between two different environments. These two α protons do not show COSY cross-peaks to β protons at the signal to noise ratio plotted in Figure 75. They could belong to two minor exchanging conformations, causing broad peaks and hence diminished COSY cross-peaks. The aromatic region shows overlapping of peaks of these two conformations at 7.25 and 7.24 ppm for the 2,6 ring protons. Two other spin systems might belong to Phe 22, based on the presence of NOE cross-peaks between the aromatic protons and protons in the β protons region. These α protons are observed only in the dimer. The α proton at 5.6 shows NOE cross-peaks to the two β 's at 3.1 and 2.95 ppm. The other α proton at 5.5 ppm showed NOE cross-peaks to the β protons at 3.05, and 3.15 ppm. These α protons are coupled to β protons in the COSY spectrum. The NOE cross-peak between α protons of these two spin systems could be due either to close proximity between two Phe residues in the dimer or to chemical exchange between dimer and monomer. The aromatic ring protons for this Phe residue are not as dispersed but apparently undergo rotational averaging to cause an overlap at 7.25 and 7.1 ppm manifested by the increase in intensity compared to the dispersed Phe 35 ring protons.

In the NOESY and COSY spectra at 30% acetonitrile shown in Figure 76, the aromatic protons overlap at 7.48 ppm and show three NOE cross-peaks. These were traced to two β protons giving rise to two different spin systems; one with a β proton at 3.18 ppm and an α proton at 5.7 ppm and the other with α proton at 5.7 and β proton at 2.9 ppm assigned previously to a conformation of Phe 35. The α protons at 6.1 and 5.7 ppm show strong NOE cross-peaks to each other and to β protons. These α protons were shown by saturation transfer to belong to the same Phe residue that exists in different environments (see below). The presence of two spin systems was verified by observing the β protons in the TOCSY spectrum (not shown). The two major contributions

remaining in the monomer show that there are two conformations for Phe 22 one with an α proton at 6.1 ppm and the other with its α proton at 5.7 ppm.

Saturation Transfer by Chemical Exchange. NOESY spectra show not only cross-peaks arising from incoherent transfer by cross relaxation but cross-peaks arising from chemical exchange as well (Wüthrich, 1986). The different pathways can be distinguished by temperature. The presence of exchange was detected by saturation transfer method (McConnel and Thompson, 1957). Figure 77 shows 1D spectra resulting from saturation of peaks in 30% acetonitrile at 45°C for the monomer, and in 0% acetonitrile at 25°C for the dimer. In the monomer at 45°C, the peak at 6.3 ppm showed saturation transfer to the peak at 5.75 ppm. The effect on the α proton at 5.75 ppm as saturated spins exchange between the two α protons proved the presence of exchange between two conformations of the same spin system assigned to Phe 35.

The peak at 6.1 ppm in the monomer in Figure 77 shows saturation transfer to 5.7 overlapping with the α proton of Phe 35. The strong intensity of the peak at 5.75 in the 1D spectrum supports the argument of the two overlapping peaks at 5.7 ppm. The peaks at 6.1 and 5.7 ppm were assigned unambiguously to two different conformations of Phe 22. Due to the lower resolution in the dimer, no data about Phe 22 was observed by saturation transfer. The overlapping peaks at 5.7 ppm were assigned to one conformation of Phe 35 and to one conformation of Phe 22.

Upfield Shifted Residues. In the COSY spectrum at 5% acetonitrile shown in Figure 78, two very high upfield resonances were observed. One at 0.40 and the other at 0.45 ppm show cross-peaks to 3.95 and 4.55 ppm respectively. The peak at 3.95 shows a cross-peak very close to the diagonal at 4.15 ppm. This spin system is characteristic of threonine with the γCH_3 shifted upfield from 1.23 ppm in a random coil to 0.4 ppm in the dimer, indicating very close proximity to an aromatic ring. The peaks at 3.95 and 4.15 ppm were assigned to the β and α protons respectively. Since there are only two Thr

residues in des NP-I, this spin system was assigned to Thr 38 because Thr 9 was assigned unambiguously in Chapter X. The other spin system, consisting of α proton at 4.55 ppm and β proton at 0.45 ppm was assigned to an alanine residue because Ala is the only spin system other than Thr that has such upfield β protons and no γ protons. This residue could be assigned to Ala 69 due to its proximity to a Phe ring at the interface in the crystal structure. These results were verified by the NOESY spectrum, in showing cross-peaks between Phe 35 aromatic protons and both β and γCH_3 of Ala and Thr respectively.

In the NOESY and COSY spectra at 30% acetonitrile, the farthest upfield peak is at 0.93 ppm which was assigned to Leu 42 residue with an α proton at 4.64 ppm. There is considerable overlap in this region in the monomer, and the shifted Ala could not be assigned unambiguously. The downfield shift is due to the disappearance of the ring current shift caused by Phe 35. From the NOESY spectrum of Figure 76, no proximity of the Ala to a Phe was observed. Thr 38 also experienced the same downfield shift of γCH_3 to 1.55 ppm and the β at 4.27 and α at 4.40 ppm constitute the rest of the spin system determined from the TOCSY spectrum in $^2\text{H}_2\text{O}$ (not shown).

Table XI is a summary of the possible conformers for the residues investigated and their chemical shifts. Phe 35 exists in three conformations in the monomer. Phe 35(1) and (2) show exchange cross-peaks between their α protons where Phe 35(1) is more dominant in the dimer. The assignment of Phe 35(3) is tentative because it does not show exchange cross-peaks with the other conformers. This conformer is obvious only in the monomer. Phe 22 exists in two conformations in the monomer. Thr 38 exists in multiple conformations in the dimer as shown by the complexity of the COSY cross-peak between β and γ protons of Figure 78. Tyr 49 is not listed in this table but it also shows multiple conformations that are affected upon change from dimer to monomer. One Ala residue is greatly affected upon change to monomer.

TABLE XI

ASSIGNMENTS OF DIFFERENT CONFORMATIONS IN THE MONOMER

RESIDUE	α H	β H	OTHERS
PHE 35(1)	6.3	3.2, 2.9	2,6; 7.48
PHE 35(2)	5.7	2.9, 2.7	2,6; 7.48
PHE 35 (3)	5.1	2.9, 3.1	2,6; 7.38
PHE 22(1)	6.1	3.2, 2.8	2,6; 7.48
PHE 22(2)	5.7	3.2	2,6; 7.48
THR 38	4.4	4.27	γ CH ₃ 1.55

Some of the residues that are shown to contribute to the monomer-monomer interface are Phe 35, Thr 38 and probably Ala 69. The extra peaks observed for Phe 35 in the dimer result from hindered ring rotations, and perhaps non-equivalence of the two protons in a single conformation. The two conformations of Phe 35 in the dimer are in very different environment than when they are in the monomer indicated by the larger chemical shift difference between the two α protons of the exchanging conformers. Also the two conformers of Phe 35 exchange between environments that are very different compared to the two conformers of Phe 22. This shows that Phe 35 is more affected by the environment than Phe 22 suggesting proximity to the monomer-monomer interface.

NP-II Studies For Comparison

Bovine NP-II is the vasopressin-associated NP. NP-I and NP-II are very homologous proteins. They both bind peptide hormones and dimerize at high concentrations. Comparison of the two proteins gives a starting point for the subsequent work on assigning NP-II. However, the dimer interface between molecules of NP-II is significantly more hydrophobic than that of NP-I (Breslow et al., 1992). To ascertain the

reasons for the NMR spectral changes which occur during addition of acetonitrile, it was important to compare the effect on NP-I to that of NP-II.

NP-II Studies

A 3 mM NP-II sample in 90 % $^1\text{H}_2\text{O}$ at pH 7.55 was titrated with an increasing volume ratio of $\text{C}^2\text{H}_3\text{CN}$ to 22%. Figure 79 shows the effect of increasing acetonitrile on the resolution. The same downfield shift as seen for NP-I in the region between 0 and 1 ppm was observed upon addition of acetonitrile, indicating the contribution of an aromatic ring to the interface. The peak at 7.5 ppm also was shifted upfield, perhaps due to rotational averaging of the ring flips upon changing to a monomer. The same concentration of acetonitrile in both proteins produced more monomer in NP-I than NP-II as shown in Figure 80. This was concluded from the better resolution in NP-I, specifically at 0.8 ppm, and at 7.0 and 7.2 ppm for the Tyr ring protons. The upfield shift of the α proton of the β -sheet residue at ~6 ppm assigned to Phe 35, is further shifted in NP-I than NP-II, indicating a more monomer character in NP-I.

PROTEIN-LIGAND INTERACTIONS

The binding between the peptide hormone and NP is positively cooperative with NP dimerization. Thus the monomeric complex NP-peptide hormone does not exist, at least at a concentration accessible by NMR. With our efforts to obtain more resolved spectra of NP-I under the optimized conditions discussed in Chapter IX, we could obtain assignments and secondary structure information for the helix, which is close to the binding site. Other residues that contribute to hormone binding are Ser 52, side chains of Pro 24, Pro 53, and Cys 54, as shown by the X-ray structure of NP-II (Chen et al., 1990). By comparing spectra of free NP-I to hormone-bound NP-I, information was obtained

about the residues that contribute to binding and to the conformational change induced by the peptide.

Methods and Materials

The only additional chemical required for this work is the peptide hormone analog PheTyrNH₂, which was provided by our collaborator. Solution studies have established the use of small dipeptides analogs of the hormone amino termini as models for the hormones in their interaction with NP (Breslow et al., 1973).

Conventional 2D spectra were obtained for native NP-I, 5 mM, pH 5.8, 30% C²H₃CN in 90 % ¹H₂O, 10 % ²H₂O. To the same sample 5 µl of 550 mM peptide in 30% C²H₃CN in 90 % ¹H₂O, 10 % ²H₂O were added, and spectra were obtained under identical conditions to those described in Chapter VIII.

RESULTS

NMR Assignments of Native NP-I

More extensive overlap of peaks is expected for a protein with an extra ten residues. For this reason the assignments were taken from des NP-I by comparing the amide to amide cross-peaks of native NP-I and then tracing the spin system looking for the peaks that resemble those of the des NP-I peaks. The assignments for des NP-I could be easily correlated to native NP-I because there were only small differences in the relative chemical shifts. The native NP-I showed stronger NOE cross-peaks in the amide-to-amide region which made assignment of the helix residues easier than the other residues. This further indicated that the helix is less distorted in the native. Table XII lists these assignments.

TABLE XII

¹H CHEMICAL SHIFTS OF NATIVE NP-I RESIDUES 39-50 AT 35°C, PH 5.8, 30%
C²H₃CN

Residue	NH	αH	βH	Others
Ala 39	8.51 (8.25)		1.64 (1.39)	
Glu 40	8.14 (8.37)	3.83 (4.29)	2.14 (2.09;1.97)	γCH ₂ 2.32, 2.56 (2.31;2.28)
Ala 41	9.25 (8.25)		1.65 (1.39)	
Leu 42	8.21 (8.42)	3.77 (4.17)		δCH ₃ 0.90 (0.94;0.90)
Arg 43	8.80 (8.27)	3.64 (4.38)	1.83 (1.89, 1.79)	δCH ₂ 3.26, 3.38 γCH ₂ 1.82, NH 7.4
Cys 44	9.29 (8.31)		3.14 (2.96;3.28)	
Gln 45	8.0 (8.41)		2.16 (2.13;2.01)	γCH ₂ 2.36, 2.52 (2.38)
Glu 46	8.69 (8.37)		2.19 (2.09;1.97)	γCH ₂ 2.35, 2.52 (2.31;2.28)
Glu 47	8.90 (8.37)		2.25 (2.09;1.97)	γCH ₂ 2.52 (2.31;2.28)
Asn 48	8.05 (8.75)		2.71 (2.83,2.75)	
Tyr 49	8.05 (8.18)		3.30;3.19(3.13;2.92)	3,5H 6.87 (7.05) 2,6H 7.28 (7.35)
Leu 50	7.74 (8.42)		1.80 (1.65)	δCH ₃ 1.16 (0.94;0.90)

The spaces left blank are due to reduced spectral resolution in native NP-I. The amide-to-α fingerprint region is very crowded in the native protein and so some of the peaks could not be assigned unambiguously. I therefore focus on the helix residues, observing chemical shift differences and conformational changes from des NP-I to native NP-I, and then from NP-I to the bound PheTyrNH₂-native NP-I. Figure 81 shows a portion of the 35°C TOCSY spectrum with the assignments of the helix residues.

Secondary Structure Determination of Native NP-I

The helix segment in NP-I is expected to contribute to peptide binding (Breslow and Burman, 1990) due to its proximity to the peptide binding site (Chen et al., 1991). Figure 82 shows the amide-to-amide region in native NP-I. Ala 39 shows a strong NOE cross-peak to Glu 40. Glu 40 shows an NOE cross-peak to Arg 43, three residues away. Glu 47 also shows an NOE cross-peak to Leu 50, three residues away. NOE cross-peaks between amide protons three residues away are not very common in helices. Cross-peaks two residues away, such as Asn 48 and Leu 50, are common in 3_{10} -helices (Wüthrich, 1986). Ala 41 shows a strong cross-peak to Leu 42. Glu 45 shows a strong cross-peak to Glu 46 that was not observed in des NP-I. A very weak NOE cross-peak was observed between Glu 47 and Asn 48. This cross-peak was observed to be much more intense in des NP-I. These two observations suggest there is in fact, some conformational difference between des NP-I and native NP-I.

Comparison of Free NP with NP-Peptide Complex

Chemical Shift Difference. The native free NP-I and peptide bound NP-I NOESY and TOCSY spectra were compared in order to observe changes in chemical shifts that occurred upon binding. Several striking differences were observed. First, the Glu 47 amide proton shifted from 8.9 to 8.75 ppm. The β protons were slightly shifted, from 2.25 to 2.20 ppm, while the γ protons were not affected, as shown in Figure 83. The reason the characteristic Glu spin system was assigned to Glu 47 at 8.75 ppm in the bound spectrum was based on the amide-to-amide NOE cross-peaks. Figure 84 compares the positions of Glu 47 in the free and bound protein. In the free, the Glu 47 NH at 8.9 ppm shows a weak cross-peak to Asn 48 and a stronger cross-peak to Leu 50. In the bound spectrum, the NOE cross-peak still exists between Glu 47 and Asn 48 but with the Glu

NH at 8.7 ppm. This verifies that Glu 47 is the residue that is shifting. Amide proton shifts of this magnitude are associated with formation of strong NH H-bonds.

Conformational Change. The NOE cross-peak of the amide protons of both Glu 47 and Asn 48 to Leu 50 disappeared, suggesting a conformational change upon binding that placed Leu 50 at a farther distance than what it was in the free. This conclusion is supported by the disappearance of the NOE cross-peaks of β and δ Leu protons to the aromatic protons of a Phe ring in the presence of the peptide as shown in Figure 85. The horizontal lines connect the NOE cross-peaks with the TOCSY cross-peaks. In the free protein, the δCH_3 peak of Leu 50 at 1.2 ppm shows an NOE cross-peak to an aromatic ring proton at 7.5 ppm. In the bound, extra NOE cross-peaks were observed indicating, a change in conformation, and the alignment is lost between the NOESY and TOCSY cross-peaks.

Stabilization of the Helix. Another very important observation is the stabilization of the helix by the peptide. This is deduced from observing a complete set of stronger d_{NN} connectivities throughout the helix. Spectra of the amide region are shown in Figure 84 of both bound and free NP-I. Figure 86 shows a comparison of d_{NN} cross-peaks with their relative intensities of des NP-I at 35°C, des NP-I at 55°C, native NP-I at 35°C, and the NP-I-bound peptide at 35°C. All other conditions were kept constant. Notice the extra connectivity between residues 46 and 47 in the bound NP-I that did not exist in any of the others. Also the intensity of the NOE between residues 47 and 48 is quite remarkable in the bound compared to the free NP-I.

Chen and coworkers (1991) have recently reported the crystal structure of the PheTyrNH₂ dipeptide-bound NP-II. This determination was followed by a preliminary report of crystallographic analysis of tripeptide-bound NP-II and oxytocin-bound NP-II (Benatan et al., 1991; Rose et al., 1991). No crystal structure has been determined for a free NP yet. The residues 39-49 comprise a 3-turn 3_{10} -helix in the peptide-bound NP-I

complexes. According to our NMR observations of free protein, there is an evidence of a distorted helix, as shown by data from short-range and medium-range NOE cross-peaks. These observations suggest that the helix is stabilized and probably shifted towards an ideal structure in the presence of the peptide.

Figure 73. 1D spectra showing acetonitrile titration of NP-I in $^1\text{H}_2\text{O}$. Spectra were obtained at 35°C and pH 5.8 from 0% acetonitrile (bottom), 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, 25% to 30% (top). Notice the increase in resolution between 0 and 1 ppm, 4.8 and 5.6 ppm, and 7 and 10 ppm.

Figure 74. 1D spectra showing acetonitrile titration of NP-I in D_2O . Spectra were obtained at $35^\circ C$, pH 6.0 in increasing concentration of acetonitrile from 0% (bottom), 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, 25% to 30% (top). The peaks in the aromatic region shift upon addition of co-solvent.

Figure 75. NOESY and COSY spectra of des NP-I in the presence of 5% acetonitrile in $^2\text{H}_2\text{O}$. Spectra were recorded at 35°C, pH 5.8 on 5 mM sample concentration. Notice the assignments of two major and one minor conformation of Phe 35, and two conformations for Phe 22 in the NOESY spectrum (top).

Figure 76. NOESY and COSY spectra of des NP-I in the presence of 30% acetonitrile in $^2\text{H}_2\text{O}$. Spectra were recorded at 35°C, pH 5.8 on 5 mM sample concentration. Notice the assignment of three conformations of Phe 35 and two for Phe 22 in the NOESY spectrum (top).

Figure 77. Saturation transfer between different conformations of the Phe 35 and Phe 22 residues in native NP-L. (A) Reference; (B) and (C) are difference spectra of the monomer in 30% acetonitrile at 45°C. (D) and (E) are reference and difference spectra of the dimer in 0% acetonitrile at 25°C. The arrow points to the peak that was saturated. Both samples were obtained in $^2\text{H}_2\text{O}$.

Figure 78. Portions of COSY spectra of NP-I des in 5% (A) and 30% (B) acetonitrile at 35°C. Notice the complexity of the COSY cross-peak in (A) at ~4 ppm indicating multiple conformations of Thr 38 in the dimer.

Figure 79. Acetonitrile titration of NP-II in $^1\text{H}_2\text{O}$. Spectra were obtained at 35°C from 2%, 11%, 19% to 22% acetonitrile (bottom to top).

Figure 80. Effect of acetonitrile on both NP-I (A) and NP-II (B). Spectra were obtained in 20% acetonitrile. Notice the more resolution in (A) between 0.5 and 1 ppm.

Figure 84. Comparison of the amide region between the peptide bound (A) and the free (B) NP-I. Notice the disappearance of the cross-peak between 47 and 50 and between 48 and 50 residues in the bound. Notice also the increase in intensity of the cross-peaks between 46 and 47, and between 47 and 48.

Figure 82. Amide-to-amide region in native NP-I at 35°C. The cross-peaks show sequential residues in the helix segment. This is a NOESY spectrum obtained with 150 ms mixing time in 30% acetonitrile in $^1\text{H}_2\text{O}$ at 35°C.

Figure 83. TOCSY spectra of both the free (A) and the peptide bound (B) native NP-I. The chemical shift difference from 8.9 to 8.75 ppm in Glu 47 upon binding is shown. Spectra were obtained in 30% acetonitrile at 35°C as 1.1:1 complex of PheTyrNH₂ to native NP-I.

Figure 84. Comparison of the amide region between the peptide bound (A) and the free (B) NP-I. Notice the disappearance of the cross-peak between 47 and 50 and between 48 and 50 residues in the bound. Notice also the increase in intensity of the cross-peaks between 46 and 47, and between 47 and 48.

Figure 85. TOCSY / NOESY overlay spectra to show the change in Leu 50 upon binding. NOESY cross-peaks were plotted as dotted contours. Notice the loss of alignment between the NOESY and TOCSY cross-peaks in the bound (B) compared to the free NP-I (A).

Figure 86. Sequential connectivities observed for the helix segment of NP-I. Des NP-I at both temperatures is compared to the native NP-I free and peptide bound at 35°C. Thick bars indicate strong NOEs, medium thickness indicates medium intensity NOEs, and thin bars indicate weak NOEs. Notice the appearance of bars in the bound NP-I that were absent in the free indicating a more stable helix.

CHAPTER XI

CONCLUSION

NMR was used in this work to give answers to some questions about ligand-macromolecule and macromolecule-macromolecule interactions. For this purpose, structure determination and conformational analysis were done on ligands ranging in size from small organic species to medium-sized proteins. The interactions of these ligands were then studied with Group II antibodies. Understanding the binding site of Group II antibodies will shed the light on the mechanism of the maturation of the immune response to memory and the contribution of the carrier. Understanding the interactions of antibodies with ligands leads to a model of interaction that is consistent with a lock-and-key mode but with some component of induced-fit.

SUMMARY

Antibody-Antigen Interactions

NMR is the only technique that has given structural information about Group II-PC specific antibody-antigen complexes. No X-ray structures have been determined which would provide detailed three-dimensional information of these antibodies. The X-ray structure work on related antibodies was for a Group I antibody. Since there are large changes between Group I to Group II due to somatic mutation and clonal recruitment, this X-ray structure is not a suitable model for Group II antibodies.

A set of ligands differing in size and charge was designed to probe the Group II antibodies binding site. The PC-analog haptens used as ligands were NPPC, NPDBP, FPPC, TFPPC. These haptens differ either in the choline moiety, or in the p-phenyl group, which was either nitro, fluoro, or trifluoromethyl.

A set of amino acids, peptides, and proteins coupled to phenyl-PC were used to evaluate the contribution of the carrier molecule. The amino acids and peptides used were linked to phenyl-PC by diazophenyl linkage. His-PC, Tyr-PC, GlyHis(PC)Gly, GlyTyr(PC)Ala, and an octapeptide-PC were used. The medium-sized protein NP-II was also homogeneously coupled to PC, and used as the ultimate model.

Sample Preparation Methods

Immunochemical techniques including tissue culture, hybridoma technology for monoclonal antibodies production, and binding assays were used in the course of this work. Production of smaller fragments of antibodies that still bind antigens was important for studying interactions within the NMR limits.

Small molecules models were synthesized using standard organic synthesis techniques. Proteins were prepared and isolated by standard biochemical techniques, including chemical modifications of amino acid residues using organic reagents. Proteins were successfully homogeneously linked to phenyl PC.

Results

Lock-and-Key Hypothesis. The question as to whether there are significant conformational changes upon binding in either antibody or antigen, or do they interact passively in a lock and key mode is still generally unresolved. TRNOE studies have shown conformational changes in a number of ligands. Changes were observed in Tyr-PC, His-PC, GlyTyr(PC)Ala. These conformational changes could be either due to the antibody binding inducing a conformational change, or the antibody selecting one conformation that already exists (perhaps as a small fraction) but is undergoing averaging with other conformations.

A single conformation was observed upon binding, even when there is a mixture of conformations in free solution, as shown for NPDBP and octapeptide-PC. This

observation is similar to other works on antibodies (Clare et al, 1986). From the study of the various ligands, there is evidence that the combining site is restrictive since only one conformation was tolerated. A single conformation for a bent structure of phenyl-PC was observed in all the bound ligands. The latitude that the antibody combining site has for a ligand suggests the presence of a mechanism by which antibodies can still recognize different structures as long as they can access a particular structure which they prefer to bind. The interaction of an antibody to its antigen with no conformational change is consistent with the “lock and key” hypothesis where specificity arises from geometrically complementary shapes.

Recognition of Flexible Regions. Flexibility and structural fluctuation play important roles in antibody activity. In the M3C65 Fv, more flexible regions were recognized in the form of narrower linewidths than the rest of the peaks in the spectra. The conformation of the antibodies has been studied as a function of binding to antigen. Change in flexibility that is induced by antigen binding was observed in the more resolved Fv spectrum, in the appearance of extra NOE cross peaks showing an increase in the effective correlation time and / or restriction to a single local conformation. This observation supports the induced fit mechanism, where a ligand induces a conformational change in a protein that results in its increased ligand binding affinity. The free energy released upon binding could compensate for the energy required to induce changes in the ligand. The ability to bind analogs of different sizes is an evidence that the binding site has substantial flexibility, since the binding pocket can accommodate a number of ligands of different size, shape, and electronic nature.

Initial Data on Antibody Binding Site. The relatively high concentration of the antibody was supplied as sFv, a single chain Fv where VH and VL are connected by a polypeptide linker. Initial data showed that the sFv is functionally active, with binding site properties similar to those of the parent antibody or other antibodies of the same group.

Initial data on antibody-to-ligand transferred NOEs were presented which showed that at least one aromatic amino acid residue is closely involved in the binding of the ligand. Other amino acids that are important for binding, such as Thr 55(L), were assigned. The data presented provides a basis for future work.

Antigenicity Versus Immunogenicity. Antigenicity is the ability of antigens to bind to antibodies. Immunogenicity is the ability of antigens to generate immune responses. Immunochemical binding studies were correlated with the NMR results. Both showed that within the series of ligands studied, the relative binding affinity (I50 values) decreased as the size of the carrier molecule increased. Only macromolecules can generate immune responses. This shows that strong binding of the antigen is not correlated with immunogenicity.

Contribution From The Carrier. The effect of the carrier was demonstrated by comparison of Tyr-PC to octapeptide-PC. In Tyr-PC, the conformations are averaged in solution which shows that the ring rotations are not restricted. In the peptide where there is a longer chain carrier, averaging between conformations is restricted giving rise to at least three different conformations. The carrier therefore contributes to restriction of conformations, perhaps making the task easy for the antibody to choose between three conformations that differ in energy in the octapeptide-PC. When the octapeptide PC binds to the antibody, the antibody stabilizes one conformation over the others so that they diminish to nonexistent.

Drug Design. Understanding more precisely the chemical features of antibody-antigen interactions would aid in designing antibodies with improved properties for use as drugs and as diagnostic reagents. The same methods used in this work to determine the conformations of antigens can be used for conformational analysis of drug molecules that are recognized by drug receptors.

NP as an Antigen Model

To analyze the conformation of NP in solution, sequential assignments were determined for key parts of the protein. Understanding the forces involved in the dimerization of NP could also contribute to the understanding of antibody-protein complexes. Studying the interactions with the peptide hormone, besides being important physiologically, is also a useful model for understanding antibody-peptide interactions, and more generally protein-peptide interactions.

NP Assignments. ^1H NMR spectra obtained on proteins the size of NP that dimerize at high concentration are very complicated and difficult to analyze. To resolve these spectra, an organic cosolvent was used that lowered the dimerization constant. Several conditions were examined to obtain more resolved spectra. Portions of the protein were then assigned unambiguously. The helix polypeptide segment that contributes to hormone binding, consisting of residues 39-50 was assigned. β -sheet structures are known to contribute to dimerization. For this reason, residues that were part of the β -sheet and could contribute to the monomer-monomer interface were assigned. These residues are Thr 9, Thr 38, Lys 59, Phe 22, Phe 35, His 80, Glu 81, Glu 31, and Asp 30. Other residues were assigned but their position in the sequence was not determined unambiguously. These were Arg, Cys, Ala and Leu residues.

NP Dimerization. The existence of some residues in several conformations, and the change that happens on going from monomer to dimer was a direct evidence of the contribution of the residues to the monomer-monomer interface. There is a major contribution from Phe 35, whereas Tyr 49 and Phe 22 show less perturbation. Chemical shift differences were also monitored and gave information about stability and ring current effect differences between monomer and dimer.

NP-Ligand Interactions. Information about the interaction between NP-I and

PheTyrNH₂ was obtained from observing chemical shift differences that are affected by binding. Conformational changes upon binding were observed from NOE cross-peak differences between certain residues. The disappearance or appearance of NOE cross-peaks reflected this conformational shift. Data was also obtained about stabilization of the helix segment upon binding.

FUTURE SUGGESTIONS

Studies of the Fv-octapeptide PC complex can be done to determine the interaction of the peptide residues with residues in the Fv and hence assign the combining site residues. This would give the orientation of the hapten in the binding site and allow a molecular model of the interaction. Synthesis of tripeptides with different residues around Tyr could be done to evaluate the contribution of the carrier. Specific residues in the Fv sequence could be determined by NMR using mutated and isotope labeled Fvs.

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