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AN ABSTRACT OF THE THESIS OF Robert Edward Derry for the Master of Science in Chemistry presented September 18, 1974.

Title: Characterization of Zinc-Containing Metalloproteins by Resonance Raman Spectroscopy.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

Joann S. Loehr, Chairman

Dennis W. Barnum

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Thomas M. Loehr

Cobalt-substituted carboxypeptidase and carbonic anhydrase were chosen as candidates for resonance Raman spectroscopy because they possess visible absorption due to the Co(II)-protein complex. However, no evidence for resonance-enhanced peaks due to the cobalt-containing chromophore was found with laser excitation near the visible absorption bands (514.5 nm) or closer to the ultraviolet absorption bands (457.9nm).

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Arsanilazocarboxypeptidase and model azotyrosine compounds were selected for a Raman spectroscopic investigation because they have intense absorption bands in the visible region. All of these substances yielded similar resonance Raman spectra. Although there was no evidence for specific zinc-ligand vibrations, shifts in vibrational frequencies of the azotyrosine chromophore could be used as proof of zinc complexation. The protonated azotyrosine model compound was found to have the phenoxy group hydrogen bonded to the azo group, forming a six-membered ring. Replacement of the proton by zinc resulted in the zinc atom being bound only to the phenoxy group. In arsanilazocarboxypeptidase the azotyrosine at position 248 in the polypeptide chain was found to have a structure similar to the protonated model compound, a six-membered ring with zinc bridging the phenoxy group and the azo group. This gave further proof that Tyr 248 is close to the zinc atom in the native enzyme, even in the absence of substrate.

CHARACTERIZATION OF ZINC-CONTAINING METALLOPROTEINS
BY RESONANCE RAMAN SPECTROSCOPY

by

ROBERT EDWARD DERRY

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

MASTER OF SCIENCE

in

CHEMISTRY

Portland State University

1974

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

The members of the Committee approve the thesis of Robert Edward Derry presented 18 September, 1974.

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ACKNOWLEDGEMENTS

I would like to gratefully acknowledge the excellent assistance and time of Dr. J. Loehr, Dr. T. Loehr, Dr. C. Yoshida, Dr. T. B. Freeman, and Dr. R. Lutz. This work was supported by a grant to Dr. J. Loehr from the Research Corporation.

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INTRODUCTION

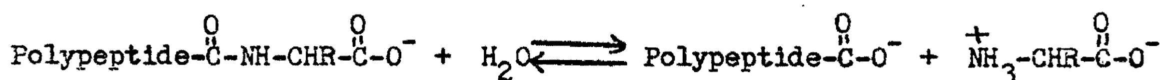
Of the many zinc containing enzymes, two are of special interest because their three dimensional structures are known. These enzymes are carbonic anhydrase and carboxypeptidase.

Carbonic anhydrase catalyses the hydration of carbon dioxide, which is of great physiological importance to animals:

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$$

The enzyme has a molecular weight of 30,000, and it has one polypeptide chain and one zinc atom per molecule (1).

Carboxypeptidase aids in the digestion of proteins in the small intestine of animals. This enzyme hydrolyzes the carboxy terminal peptide bond in proteins:



$$\text{Polypeptide-}\overset{O}{\overset{\|}{C}}\text{-NH-CHR-}\overset{O}{\overset{\|}{C}}\text{-O}^- + H_2O \rightleftharpoons \text{Polypeptide-}\overset{O}{\overset{\|}{C}}\text{-O}^- + \overset{+}{N}H_3\text{-CHR-}\overset{O}{\overset{\|}{C}}\text{-O}^-$$

The enzyme has a molecular weight of 34,600, and it has one polypeptide chain and one zinc atom per molecule (1).

The zinc atom is essential for these enzymes to function normally. Removal of the zinc atoms results in these enzymes becoming inactive; both of these enzymes can be restored to activity by the addition of zinc or cobalt (2).

The active sites of both of these enzymes have been determined by

X-ray crystallography. Carbonic anhydrase has zinc in a distorted tetrahedron in which it is bonded to imidazole nitrogens of His-93, His-95 and His-117, and one water molecule (3). Carboxypeptidase, also, has zinc bonded to four ligands, which are imidazole nitrogens of His-196 and His-69, carboxylate oxygen of Glu-72, and one water molecule when the enzyme is in the crystalline state (4).

Although the crystal structures are known, the structures of the enzymes in solution have not been determined. The structure of an enzyme in the crystalline state may not be identical to the structure of the biologically active form in solution. For this reason, we used vibrational spectroscopy, particularly resonance Raman spectroscopy to observe the structure of the active site of carbonic anhydrase and carboxypeptidase in solution.

Raman Spectroscopy

Raman spectroscopy, like infrared spectroscopy, detects energies of vibrational transitions. However, Raman spectroscopy is concerned with light inelastically scattered by molecules in its path, rather than radiation which is absorbed. In infrared spectroscopy, one sees absorptions due to vibrations involving a change in dipole moment; while in Raman spectroscopy, one sees scattering due to vibrations involving a change in polarizability (5).

Water molecules undergo large changes in bond dipole moments during their normal modes of vibration and are very strong absorbers of the infrared, but are poor scatterers in the Raman effect. Water, thus, produces relatively little interference in Raman studies in aqueous solutions.

Resonance Raman Spectroscopy

Resonance Raman spectroscopy is a special form of Raman spectroscopy in which Raman spectral intensities are enhanced as a result of the coupling of electronic and vibrational transitions of a chromophore (6). Raman scattering intensity is inversely proportional to $(\nu_e^2-\nu_o^2)^4$ where ν_e is the frequency of the electronic transition and ν_o is the frequency of incident radiation (7). Thus, as ν_o approaches ν_e, the Raman intensity increases. The resonance Raman spectrum is strongest when the wavelength of exciting light corresponds to an electronic absorption maximum of the chromophore and gets weaker as one goes away from this wavelength. Generally, lasers are used as light sources because they provide intense, monochromatic lines of various wavelengths.

Resonance Raman spectroscopy is an important tool for investigating metalloproteins that absorb light in the visible region. When the excitation wavelength falls in an electronic absorption band of the sample, then certain Raman bands can be greatly enhanced. These resonance Raman bands correspond to vibrational modes which involve motions of the atoms in the chromophore, that part of the molecule in which the electronic transition occurs (6). The amount of vibrational structure observed in the resonance Raman effect depends on the extent of electron delocalization in the chromophore. Thus, one sees only the metal-ligand chromophore and not the rest of the protein molecule. An additional advantage of resonance Raman spectroscopy is that one only needs to use very low concentrations of the protein (often as low as 10^{-7} molar).

Resonance Raman spectroscopy has been very useful in gaining information about metalloproteins. The heme groups of heme proteins give resonance Raman spectra in which the porphyrin ring vibrations are

enhanced. Studies of heme-iron proteins indicate that certain Raman bands are sensitive to either or both the valence and spin state of the iron (8, 9). The frequency of the strongest band in the spectra for reduced heme-proteins occurs between 1356 and 1361 cm^{-1}, whereas for oxidized proteins it occurs between 1370 and 1378 cm^{-1}. Chemical modifications of heme proteins resulting in the conversion of iron from a low spin state to a high spin state have been correlated with a 1584 to 1566 cm^{-1} frequency shift, which is independent of oxidation state and which is ascribed to the movement of the iron atom out of the heme plane upon increasing its spin state. Oxyhemoglobin has its strongest peaks at 1375 and 1584 cm^{-1} and has thus been identified as a low spin ferric structure (8).

The resonance Raman spectrum of hemocyanin has provided evidence about the binding of oxygen to the active site copper. Upon oxygenation, hemocyanin gives a resonance Raman peak at 742 cm^{-1}, and this peak shifts to 704 cm^{-1} when $^{18}O_2$ is substituted for $^{16}O_2$. The frequency of the O-O vibration is most characteristic of a peroxide-containing molecule in a non-aqueous environment (738 cm^{-1} for Na_2O_2 as a solid) (10).

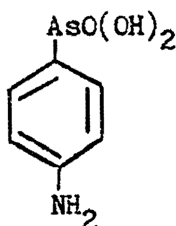
Resonance Raman spectra have also provided evidence about the coordination of the metal ion at the active site of metalloproteins. The resonance spectrum of rubredoxin has shown that the tetrahedral coordination about the iron atom in the crystalline state appears to be maintained in solution (11).

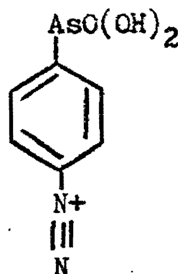
While zinc containing carboxypeptidase and carbonic anhydrase have no chromophores in the visible region, cobalt substituted carboxypeptidase and carbonic anhydrase do have visible absorptions due to the Co(II)-protein complex (12, 13). It was conjectured that the cobalt chromophores of these enzymes might give resonance Raman spectra. From

this one could gain evidence about the structure of the active sites of these enzymes.

Carboxypeptidase can be modified by reaction with an azo reagent to make a derivative in which tyrosine at the active site (Tyr-248) is modified to an azotyrosine (14). This results in a chromophore with an intense absorption in the visible region. The modified carboxypeptidase has a yellow color in the crystalline state, but turns red upon going into solution (15). This is due to a change in the electronic composition of the chromophore. In the crystalline state, azotyrosine-248 is protonated at pH 8.2. When the modified enzyme is dissolved, azotyrosine-248 loses its proton and complexes with zinc. If one removes the zinc atom, the modified enzyme turns yellow in solution because azotyrosine-248 becomes protonated. Thus, carboxypeptidase appears to have a different structure in the crystalline state and in solution. In the crystalline state, the tyrosine near the active site (Tyr-248) has its hydroxyl group pointed away from the active site zinc, and toward the hydrophilic exterior (4). It has been proposed (15) that the tyrosine-248 swings in toward the zinc and its hydroxyl group forms a bond to the zinc when the enzyme goes into solution. With resonance Raman spectroscopy, one might gain information about the active site of carboxypeptidase in solution.

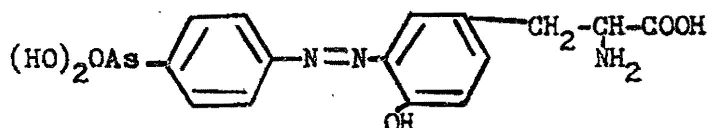
For these reasons, we decided to investigate chromophore containing derivatives of zinc enzymes by resonance Raman spectroscopy. These include cobalt carboxypeptidase, cobalt carbonic anhydrase, and azo derivatives of carboxypeptidase and model tyrosine compounds. Structures for the azo compounds are shown in Figure 1.



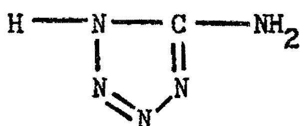


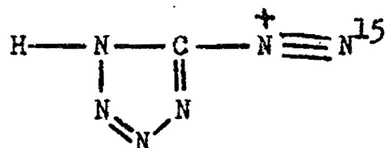
Arsanilic Acid

Diazoarsanilic Acid



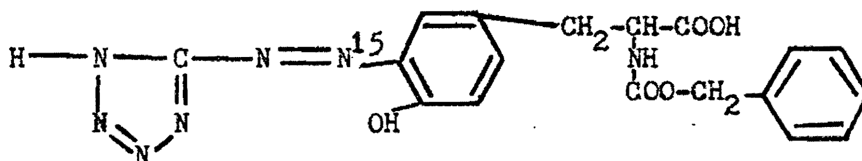
Arsanilazotyrosine (Ars-AzoTyr)





5-Amino-1H-tetrazole

5-Diazo-1H-tetrazole (DHT)



Tetrazolylazo-N-carbobenzoxytyrosine (Tetr-AzoTyr)

Figure 1. Azo derivatives of tyrosine and related compounds (N^{15} designates position of N^{15} substitution).

MATERIALS AND METHODS

Materials

All the reagents were Reagent grade. Hippuryl-L-phenylalanine and p-arsanilic acid were obtained through Sigma Chemical Company. 5-amino-1H-tetrazole was obtained from J. T. Baker Chemical Company. $NaNO_2$, $ZnSO_4$, $CoCl_2$, and ortho-phenanthroline were obtained from Mallinckrodt Chemical Works. Tris (hydroxy methyl) amino methane (Enzyme and Buffer grade) and N-carbobenzoxy -L-tyrosine were obtained from Schwarz/Mann. The cobalt and zinc atomic absorption standards were obtained from Fisher. $NaN^{15}O_2$ (97% N^{15}) was obtained from Stohler Isotope Chemicals.

Carboxypeptidase was obtained from Sigma Chemical Company and Worthington Biochemical Corporation. Although the source of both of these enzymes was the same (bovine pancreas), these two enzymes were different forms of carboxypeptidase A as a result of different methods of purification. The Worthington carboxypeptidase, the γ form, is the less soluble form (16) and it was used in the preparation of arsanilazocarboxypeptidase. The Sigma carboxypeptidase, the δ form (17), was used to make cobalt carboxypeptidase. The γ and δ forms differ in the number of C-terminal amino acids removed in converting the zymogen, procarboxypeptidase, to active enzyme. They differ in their solubilities but show the same pattern of structural differences between crystal and solution as determined from the color of the azo enzymes (18).

Carbonic anhydrase was obtained from Sigma Chemical Company. The

source of the enzyme was bovine erythrocytes.

Enzyme Assays

Carboxypeptidase activity was determined by the rate of hydrolysis of the substrate hippuryl-L-phenylalanine as measured by the increase in absorbance at 254 nm at room temperature. The substrate was allowed to warm up to room temperature before addition of the enzyme. A unit of activity is equal to one micromole of hippuric acid (ϵ_{mM}=0.36) hydrolyzed per minute (19). Specific conditions were substrate concentration of 0.001 M, pH 7.5 buffer of 0.025 M Tris with 0.5 M NaCl, and an enzyme concentration of 1-10 μg/ml.

Carbonic anhydrase activity was measured by the rate of production of hydrogen ions (20). The time in seconds required for a saturated CO_2 solution to lower the pH of 0.012 molar veronal buffer from 8.3 to 6.3 was determined. Activity is proportional to "$\frac{T_o - T}{T}$" where "T_o" is the time without the enzyme and "T" is the time with the enzyme.

Protein Concentration

The concentration of carboxypeptidase was determined using the optical density of a 0.1% solution as 1.94 at 278 nm (21). The concentration of carbonic anhydrase was determined using the optical density of a 0.1% solution as 1.80 at 280 nm (22).

Preparation of Cobalt Enzymes

To minimize trace metal contamination, all glassware was cleaned with a 1/1 mixture of concentrated HNO_3 and H_2SO_4, and then rinsed ten times with tap water and distilled water.

Cobalt carbonic anhydrase was prepared using a modification of the

procedure described by Coleman (23). The enzyme, 5-30 mg/ml, was depleted of zinc by dialysis against 0.01 M acetate buffer (pH 5.5) containing 4×10^{-3} M ortho-phenanthroline for a period of two weeks with changes of dialysis solution every two days. The enzyme was then dialyzed against distilled water for one day to remove traces of ortho-phenanthroline (which forms a colored complex with cobalt), then against 0.025 M Tris-Cl (pH 7.0) containing 0.001 M $CoCl_2$ for one day, then against 0.01 M Tris-Cl (pH 7.0) for one day to remove excess cobalt, and lastly against 0.005 M Tris-Cl (pH 8.5), which is the optimal pH for color formation (24). The dialysis solutions contained 100 ml of solution per ml of enzyme solution.

Cobalt carboxypeptidase was made using a modification to the method of Vallee et al. (25). The enzyme solution, 5 mg/ml, was dialyzed against 0.1 M Tris-Cl (pH 7.0) containing 1 M NaCl and 2×10^{-3} M ortho-phenanthroline for a period of two days. The dialysis solution was changed to water for one day to remove traces of ortho-phenanthroline, and was then changed to 0.1 M Tris-Cl (pH 7.0) containing 1×10^{-3} M $CoCl_2$ and 1 M NaCl for one day. Next, the enzyme was dialyzed against 0.1 M Tris-Cl (pH 7.0) containing 1 M NaCl for one day, and the solution was changed twice.

Cobalt and zinc carbonic anhydrase solutions were concentrated using an Amicon ultrafiltration cell (model 52) with an Amicon PM-10 membrane, which excludes proteins with molecular weights above 15,000.

Preparation of Diazoarsanilic Acid

The diazonium salt of arsanilic acid was prepared from arsanilic acid and $NaNO_2$ as described by Fairclough and Vallee (26) with the

following modifications: NaBr (0.2 moles per mole of arsanilic acid) was added at the start of the reaction to prevent self-coupling of arsanilic acid and to catalyze the reaction (27). Also, the pH of the mixture was adjusted to 6.5-7.0 instead of 5.0-5.5 in order to improve the specificity of the diazotization reaction (personal communication from Ivan Legg).

Preparation of Arsanilazotyrosine (Ars-AzoTyr)

One hundred ml of 2 x 10^{-4} M tyrosine dissolved in 1 M NaOH (pH 11) was reacted with 0.05 ml of 0.02 M diazoarsanilic acid in an ice bath. The reaction was very rapid and went to completion as judged by formation of a colored product with maximum absorbance at 485 nm.

Preparation of Arsanilazocarboxypeptidase (Ars-AzoCP)

Arsanilazocarboxypeptidase was prepared using a modification to the method of Johansen and Vallee (15). The crystalline carboxypeptidase (40-50 mg) was washed once by suspension in distilled water, centrifuged in a Sorvall centrifuge (10 min. at 12,000 x g), and then suspended in 18 ml of distilled water. To this solution were added 3 ml of 0.02 M diazoarsanilic acid and 0.3 ml of 0.11 M $NaHCO_3$. The pH was quickly adjusted to 8.5 with 0.1 M NaOH and then to 8.7 with 0.01 M NaOH. The mixture was continually adjusted to pH 8.7 for 10 minutes and then left in an ice bath for about two hours. (Note that the pH meter was standardized at room temperature, although pH measurements were made at low temperature.) The crystals were then centrifuged as above and washed twice with 0.05 M phosphate buffer (pH 7.0) to stop side reactions by lowering the pH, followed by a final washing with water. The crystals were dissolved in 5 M NaCl and then dialyzed versus buffer containing 2 M NaCl to the desired pH (0.05 M $NaHCO_3$ for pH 11.5, 0.05 M Tris-Cl for

pH 8.8). The solution of modified enzyme was concentrated by ultrafiltration using an Amicon ultrafiltration cell (model 52) with a PM 10 membrane.

The concentration of the modified enzyme was estimated by the absorbance at 278 nm using an extinction coefficient of 7.32×10^4 M^{-1} cm^{-1} (28).

Preparation of 5-Diazo-1H-tetrazole (DHT)

The preparation of 5-diazo-1H-tetrazole was done using a modification to the method of Sokolovsky and Vallee (29). Care must be taken to use cold solutions and low concentrations when making this reagent since it is highly explosive. Ice cold 10% $NaNO_2$ (0.7 ml) was added slowly with stirring over a period of 5 minutes to 0.1 grams of 5-amino-1H-tetrazole dissolved in 3.3 ml of ice cold 2 M HCl. After 10 minutes, the pH was adjusted to 5.0-6.0 and ice cold water was added to give a final volume of 100 ml.

Preparation of Tetrazolylazo-N-carbobenzoxytyrosine (Tetr-AzoTyr)

Three ml of 0.01 M 5-diazo-1H-tetrazole were combined with 30 ml of 2×10^{-3} M N-carbobenzoxy-L-tyrosine (pH 9.0) and 67 ml of water and left at room temperature for 30 minutes (29).

The metal complexes of Tetr-AzoTyr were made using a 1/1 molar ratio of Tetr-AzoTyr (pH 9.0) and $ZnCl_2$, $Cd(NO_3)_2$, or $Hg(NO_3)_2$. $Hg(NO_3)_2$ had dissolved only in acid solution. Final pH (ca. 8.5) for the mercury complex was determined by maximum color formation.

Atomic Absorption Spectroscopy

Atomic absorption values were obtained with a Varian-Techtron AA-5

Atomic Absorption Spectrophotometer. Enzymes were run at 0.2 to 0.5 mg/ml and the standards were run at 5 to 50 micromolar.

Ultraviolet/Visible Spectroscopy

Ultraviolet and visible spectra and assay kinetics were taken on a Cary 14 Recording Spectrophotometer.

Raman Spectroscopy

Raman spectra were obtained with a Jarrell-Ash 25-300 Raman Spectrophotometer using either a Coherent Radiation Laboratories model 52 argon ion laser (514.5, 488.0, and 457.9 nm) or a Spectra-Physics model 125 helium-neon laser (632.8 nm). Neutral density filters (20%-50%) were used with protein samples to attenuate the laser power so that the sample would not boil. K_2SO_4 was added as an internal standard. The concentration of K_2SO_4 was generally 0.5 M.

RESULTS

Properties of Cobalt-Substituted Zinc Enzymes

The results of cobalt substitution for carboxypeptidase (CP) and carbonic anhydrase (CA) are shown in Table I. The zinc atom of carboxypeptidase was readily removed and replaced with cobalt. After 17 hours, 74% of the zinc was removed from carboxypeptidase. Carbonic anhydrase releases its zinc atoms much less readily. After one week, only 47% of the zinc was removed from carbonic anhydrase. After two days for carboxypeptidase, and two weeks for carbonic anhydrase, almost all of the zinc atoms had been removed. Reconstitution with cobalt was complete in both cases although carbonic anhydrase contained excess cobalt. This was later corrected by including the pH 7.0 rinse to remove cobalt prior to raising the pH to 8.5 (see Materials and Methods). At pH 8.5, excess Co(II) is oxidized to Co(III) which binds more strongly to the protein.

The relative activities of the cobalt and zinc enzymes agree with those of other workers. Cobalt carboxypeptidase with a relative activity of 144% is very close to the value of 150% reported by Vallee et al. (25). Cobalt carbonic anhydrase with a relative activity of 50% is quite similar to the reported value of 45% (30).

TABLE I

PROPERTIES OF COBALT-SUBSTITUTED ENZYMES

Enzyme	Dialysis[a] Time	Specific Activity (units/mg)	Relative Activity	Moles Co[b] / Moles Protein	Moles Zn / Moles Protein
CP	0	45	100	-	0.93
CP	17 hours	-	-	-	0.26
Co-CP	48 hours	65	144	0.95	0.02
CA	0	4160	100	-	1.06
CA	1 week	-	-	-	0.53
Co-CA	2 weeks	2090	50	3.1	0.06

a. Dialysis versus ortho-phenanthroline to remove zinc.
b. Excess cobalt bound to carbonic anhydrase molecule could be removed by more extensive dialysis versus .01 M Tris-Cl (pH 7.0).

Raman Spectra of Cobalt-Substituted Zinc Enzymes

Raman spectroscopy was performed with excitation at 457.9, 488.0 and 514.5 nm. The latter two lines were chosen because they most closely correspond to the visible absorption maxima observed for cobalt-substituted proteins between 520 and 560 nm (2). Since the molar absorptivities of cobalt-substituted enzymes are only 200-300 liter $mole^{-1}$ cm^{-1} (2), it was not possible to obtain resonance Raman spectra for either enzyme at concentrations up to 15 mg/ml ($A_{510} \simeq 0.1$). Very high concentrations of cobalt and zinc carbonic anhydrase (43-75 mg/ml) did produce Raman spectra. However, there was no indication of resonance-enhanced peaks as the spectra of the zinc and cobalt enzymes were identical. This can also be seen from Table II in which the Raman spectrum of cobalt carbonic anhydrase is compared with that of lysozyme (31). The peaks of the cobalt carbonic anhydrase spectrum can all be assigned by comparison

TABLE II

RAMAN SPECTRA OF CARBONIC ANHYDRASE AND LYSOZYME

Frequency (cm^{-1})		Tentative Assignment [c]
CoCA [a]	Lysozyme [b]	
420	429 (2)	
480	491 (0)	
-	509 (5)	S-S stretch
550	544 (1)	Trp
645	646 (0)	Tyr
756 (S)	761 (10)	Trp
830	836 (1)	Tyr
880 (S)	879 (5)	Trp
960	964 (0)	
1005 (S)	1006 (7)	Phe
1075	1078 (1)	C-N stretch
1125	1128 (3)	C-N stretch
1205	1210 (2)	Tyr & Phe
1235	1240 (4)	Amide III
1258 (S)	1262 (5)	Amide III
1340 (S)	1338 (8)	Trp
1450 (S)	1448 (9)	C-H deformation
1550 (S)	1553 (8)	Trp

a. Excitation at 514.5 nm. (S) signifies a strong peak. Frequencies are in cm^{-1}.

b., c. The values and assignments for lysozyme were obtained from Lord and Yu (31). The numbers "0-10" represent a relative intensity scale with "10" as the maximum peak height.

to lysozyme, and one observes that the whole carbonic anhydrase protein molecule is involved with no evidence for the cobalt-containing chromophore. Lysozyme has four disulfide bonds, which give rise to the moderately strong peak at 509 cm^{-1} in the Raman, while cobalt carbonic anhydrase has no peak at this frequency. This agrees with the amino acid analysis of bovine carbonic anhydrase in which it was found that the enzyme lacks cysteine (32).

Properties of Azotyrosine-Containing Compounds and Arsanilazocarboxypeptidase

The spectral characteristics of arsanilazotyrosine (Ars-AzoTyr), tetrazolylazo-N-carbobenzoxytyrosine (Tetr-AzoTyr), and arsanilazocarboxypeptidase (Ars-AzoCP) are shown in Tables III and IV. The spectral similarity of the azotyrosine derivatives to Ars-AzoCP has led to their choice as models for the active site tyrosine of arsanilazocarboxypeptidase (15). Since Ars-AzoTyr precipitates upon complexation with metal ions, it was necessary to use the more soluble complexes of Tetr-AzoTyr as models for the zinc-containing Ars-AzoCP. The formation of a red-colored complex in the presence of metal ions between pH 8.0 and pH 9.0 has been considered indicative of complex formation between the metal ion and the azophenolate moiety (18).

In the diazotization of carboxypeptidase, tyrosine was the only residue to undergo significant modification (Table V). However, we were not able to reproduce the literature values for the extent of the modification or the specificity of the reaction with Tyr-248. The modified enzyme had about 90% of the activity of the native enzyme, indicating that the apparent lack of reaction with Tyr-248 might be partly due to

TABLE III

SPECTRAL PROPERTIES OF ARS-AZOTYR AND TETR-AZOTYR

Compound	pH	Color	λ max (nm)	Assignment
Ars-AzoTyr	5	yellow	400	azophenol [a]
	11	orange	480	azophenolate [a]
Tetr-AzoTyr	6	yellow	400	azophenol [b]
	11	orange	480	azophenolate [b]
Zn(II)-Tetr-AzoTyr	9	red	510	Zn-azophenolate [b]
Hg(II)-Tetr-AzoTyr	9	violet	495	Hg-azophenolate
Cd(II)-Tetr-AzoTyr	8.5	pink	485	Cd-azophenolate

a. Tabachnick and Sobotka (27).

b. Johansen and Vallee (18).

TABLE IV

SPECTRAL PROPERTIES OF ARS-AZOCP

pH	Color	λ max (nm)	ϵ ($M^{-1}cm^{-1}$)	Assignment [a]
6.0	yellow	400	0.9×10^4 [a]	azophenol
8.5	red-orange	510	0.8×10^4 [a]	Zn-azo-phenolate
11.5	orange	485	1.0×10^4 [a]	azophenolate
7.0	yellow	278	7.3×10^4 [b]	
7.0		330	2.2×10^4 [b]	
8.5		330	1.7×10^4 [b]	

a. Johansen and Vallee (18).

b. Johansen et al. (28).

TABLE V

PROPERTIES OF ARS-AZOCP PREPARATIONS

Molar Ratios	This Laboratory	Literature	(Ref.)
Azotyrosine / Protein	0.60 [a]	1.0	(14)
Azohistidine / Protein	0.01 [b]	0.05	(14)
AzoTyr-248 / Total Azotyrosine	0.80 [c]	0.95	(28)

a. [Azotyrosine] based on A_{330}, [protein] based on A_{278}, both at pH 7.0.

b. Determined from A_{460} and A_{500} at pH 11.0 according to the method of Tabachnick and Sobotka (33). In their equation, X is equal to the μM concentration of azohistidine and Y is equal to the μM concentration of azotyrosine.

$$16.50\,X + 9.60\,Y = A_{460}$$
$$2.65\,X + 10.50\,Y = A_{500}$$

c. [Azotyrosine] based on A_{330} at pH 8.5.
[AzoTyr-248] based on A_{510} at pH 8.5.

denaturation. The best results were obtained by recrystallization of carboxypeptidase (34) prior to the reaction and careful control of pH during diazotization. However, we were not able to obtain a protein preparation in which more than 80% of the azotyrosines were complexed to the zinc atoms at pH 8.8.

Raman Spectra of Azotyrosine Compounds

In Table VI, the Raman spectra of tyrosine (pH 1.0), tyrosine (pH 11.0), and arsanilic acid are compared. There is very little change in the spectrum of tyrosine upon ionization. The major changes are in the ring modes (especially 1620-1604 cm^{-1}) and the ring CH bends (830-834 cm^{-1}). From this one can see that the spectrum is not really sensitive to the phenol ionization, with the possible exception of the appearance of the new bands at 1419 and 1336 cm^{-1}.

The principal tyrosine lines, the 1600 cm^{-1} ring mode and the 816 cm^{-1} bend, are seen in the arsanilic acid spectrum. The 1096 cm^{-1} line can be assigned to the C-As vibration (35). Thus, arsanilic acid has the spectrum of a substituted benzene ring. However, these strong tyrosine and arsanilic acid lines do not appear in the resonance Raman spectrum of arsanilazotyrosine, although most of the Ars-AzoTyr lines appear to correspond to tyrosine lines:

TYR (pH 11)	ARS-AZOTYR (pH 11)
1177	1162
1212	1200
1271	1245
1362	1338

The shifting of ring modes to lower frequencies is consistent with the electron withdrawing effect of an azo substituent on a benzene ring.

The two major peaks of Ars-AzoTyr at 1385 and 1440 cm^{-1} cannot be

TABLE VI

RAMAN SPECTRAL FREQUENCIES OF TYROSINE AND ARSANILIC ACID [a]

Arsanilic Acid[b]	Tyr (pH 11)[c]	Tyr (pH 1)[d]	Assignment
		495 (m)	
	644 (2)	648 (m)	
816 (10)	834 (6)	830 (s)	Aromatic CH bend (e)
	854 (4)	850 (s)	Ring mode (f)
1096 (5)			C - As stretch (g)
1186 (1)	1177 (4)	1175 (m)	Ring mode and OH bend (e)
	1212 (6)	1212 (s)	Ring mode and CO stretch (f)
1276 (1)	1271 (5)	1270 (m)	Ring mode and CO stretch (h)
	1336 (5)		
	1362 (3)	1362 (m)	Ring mode and $-CH_2-$bend (f)
	1419 (3)		
	1446 (3)	1449 (w)	$-CH_2-$bend (f)
1600 (4)	1604 (10)	1620 (s)	Ring mode (f)

a. Frequencies are in cm^{-1}. Intensities are on 1 to 10 relative scale or noted as (s), strong; (m), medium; (w), weak.

b. Excitation at 632.8 nm.

c. Excitation at 488.0 nm.

d. Excitation at 488.0 nm. No sulfate standard.

e. Pinchas et al. (36).

f. Jakobsen (37).

g. Pettit and Turner (35).

h. Pinchas (38).

assigned to tyrosine since one would not expect to find the tyrosine CH_2 vibrations (1449 and 1362 cm^{-1}) in a resonance spectrum since the CH_2 group is not part of the chromophore. Thus, there is very little correspondence between the tyrosine Raman spectral lines and those in Ars-AzoTyr, and the main contribution to the chromophore and the source of resonance enhancement must be the azo group (-N=N-).

The vibrations that give rise to the lines at 1385 and 1445 cm^{-1} in Ars-AzoTyr were assigned to the azo group (-N=N-) on the basis of N^{15} substitution. The great similarity between the resonance Raman spectra of Ars-AzoTyr and Tetr-AzoTyr can be seen in Table VII. The three major lines of both spectra occur in the region 1338-1445 cm^{-1}. Substitution of one of the azo group nitrogens of Tetr-AzoTyr with N^{15} resulted in frequency changes for only two peaks. There was a shift from 1388 to 1382 cm^{-1} and from 1445 to 1438 cm^{-1}. Thus, these two peaks can be assigned to vibrations involving the azo group (-N=N-). This is in agreement with the azo dye, methyl orange, in which Machida et al. (39) assign 1420 and 1395 cm^{-1} lines to N=N stretches. They also assign the 1155 cm^{-1} line to phenyl-N stretch, but our line at 1149 cm^{-1} was too weak and broad to determine if it had shifted.

The substitution of one N^{15} for N^{14} in Tetr-AzoTyr would be expected to cause the N=N vibration to shift 24 cm^{-1} to lower frequency. The observed shift was only 7 cm^{-1}. This indicates that the benzene ring contributes to the -N=N- vibrations. Similar results were obtained for the C-O stretching vibration (1250 cm^{-1}) of phenol with O^{18} in place O^{16} in CCl_4. Upon substitution, the shift of the 1250 cm^{-1} band was 6 cm^{-1} instead of the expected 30 cm^{-1}. This was ascribed to the involvement of the ring in the C-O vibration (38).

TABLE VII

PRINCIPAL RAMAN SPECTRAL FREQUENCIES OF ARS-AZOTYR, TETR-AZOTYR, AND N^{15}-TETR-AZOTYR[a]

Ars-AzoTyr (pH 11)	Tetr-AzoTyr (pH 11)	N^{15}-Substituted[b] Tetr-AzoTyr (pH 11)	Assignment
1338 (m)	1342 (10)	1342 (10)	Ring mode?[c]
1385 (m)	1388 (10)	1382 (10)	N=N stretch[d]
1440 (s)	1445 (10)	1438 (7)	N=N stretch[d]

a. The spectra were obtained with 488.0 nm excitation. The strongest peak was arbitrarily set equal to 10 for Tetr-AzoTyr. For Ars-AzoTyr no sulfate internal standard was used and intensities are denoted by (s), strong and (m), medium. Frequencies are in cm^{-1}.

b. The diazo reagent was prepared from $NaN^{15}O_2$ and 5-amino-1H-tetrazole. In Tetr-AzoTyr the labeled nitrogen is attached to the tyrosine ring (see Figure 1).

c. Jakobsen (37).

d. Machida et al. (39).

In Table VIII, the Raman spectra of 5-diazo-1H-Tetrazole (DHT) and several forms of Tetr-AzoTyr are compared. The pH 11.0 form of Tetr-AzoTyr and the zinc complex of Tetr-AzoTyr (pH 9.0) are almost identical except for the position of the peak at 1345 cm^{-1} and in the relative intensities of the peaks at 1345, 1389 and 1443 cm^{-1}. From Table IX one can see that both Tetr-AzoTyr (pH 11.0) and the zinc complex of Tetr-AzoTyr have maximum intensity enhancements close to their absorption bands (ca. 500 nm). A theoretical calculation based on the equation below (7) show that both Tetr-AzoTyr (pH 11.0) and the zinc complex have the expected pattern of intensity enhancements for a chromophore having an absorption maximum at 490 nm (Table IX).

$$F_A = \frac{(\nu_o - \Delta\nu)^2 \ (\nu_e^2 + \nu_o^2)}{(\nu_e^2 - \nu_o^2)^2}$$

where ν_o = Incident frequency
$\Delta\nu$ = Observed Raman shift
ν_e = Electronic state responsible for resonance enhancement
F_A^2 is proportional to the intensity of the Raman peak.

The Raman spectrum of the pH 6.0 form of Tetr-AzoTyr (Table VIII) shows some differences from the high pH spectrum of Tetr-AzoTyr. It has new peaks at 1401, 1470 and 1494 cm^{-1}. The peak at 1438 cm^{-1} is at a lower frequency in the pH 6.0 form. The peak at 1351 cm^{-1} corresponds more closely to the peak at 1352 cm^{-1} in the zinc complex than to the peak at 1345 cm^{-1} in the pH 11.0 form. The low pH form of Tetr-AzoTyr has maximum resonance with the 457.9 nm excitation (Table IX) rather than with the 514.5 nm excitation which was observed with the high pH forms of Tetr-AzoTyr. This is to be expected since the absorption maximum for the pH 6.0 form of Tetr-AzoTyr is at 400 nm.

TABLE VIII

RAMAN SPECTRAL FREQUENCIES OF DHT AND TETR-AZOTYR[a]

DHT (pH 6)	Tetr-AzoTyr (pH 6)	Tetr-AzoTyr (pH 11)	Tetr-AzoTyr + Zn (pH 9)
1142 (2)		1149 (2)	1148 (2)
	1191 (1)	1187 (1)	1193 (1)
	1241 (4)	1242 (3)	1240 (2)
	1263 (4)	1260 (2)	1264 (3)
1273 (2)			
1355 (2)	1351 (8)	1345 (10)	1352 (10)
	1386 (6)	1389 (10)	1388 (7)
	1401 (6)		
1425 (2)			
	1438 (10)	1443 (10)	1443 (7)
1478 (10)	1470 (4)		
	1494 (2)		

a. Excitation at 514.5 nm. Frequencies in cm^{-1}. The intensity of the strongest peak was set equal to 10.

TABLE IX

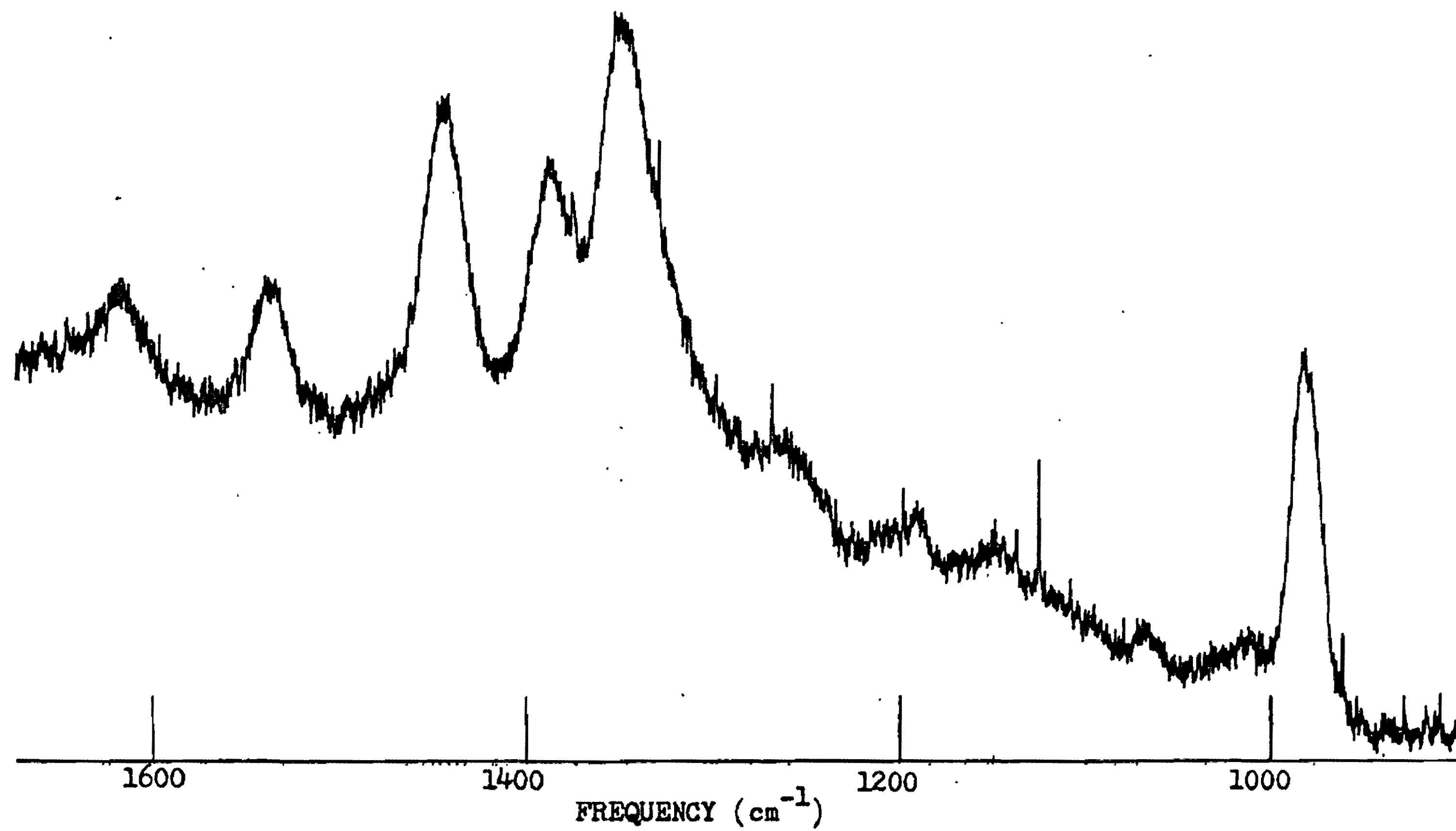
INTENSITIES OF SELECTED RAMAN FREQUENCIES OF AZO COMPOUNDS AS A FUNCTION OF EXCITATION LINE

Compound	Raman Frequency[a] (cm^{-1})	Wavelength of excitation (nm)[b] 514.5	488.0	457.9	λ_{max} (nm)
DHT	1273	10	-	30	ca. 400
	1478	10	-	22	
Tetr-AzoTyr (pH 6)	1352	10	-	13	400
	1386	10	-	17	
	1437	10	-	27	
Tetr-AzoTyr (pH 11)	1343	10	10	5	480
	1389	10	10	4	
	1445	10	10	7	
Tetr-AzoTyr +Zn (pH 9)	1351	10	9	5	510
	1388	10	9	4	
	1444	10	10	6	
Ars-AzoCP (pH 6)	1429	10	20	25	400
Ars-AzoCP (pH 8.8)	1392	10	10	7	510
	1435	10	12	11	
Ars-AzoCP (pH 11.5)	1392	10	12	6	485
	1444	10	16	10	
Predicted behavior[c] for 1392 cm^{-1} Raman peak with electronic transition at 490 nm.		10	-	3	

a. Peak frequency was the average of the peak positions obtained with the different excitation wavelengths.

b. For each frequency, the peak intensity at excitation of 514.5 nm was arbitrarily set equal to 10.

c. Theoretical calculation based on derivation of Albrecht and Hutley (7).



<u>Figure 2</u>. Raman spectrum of Zn(II)-Tetr-AzoTyr (pH 9.0) at 488.0 nm excitation.

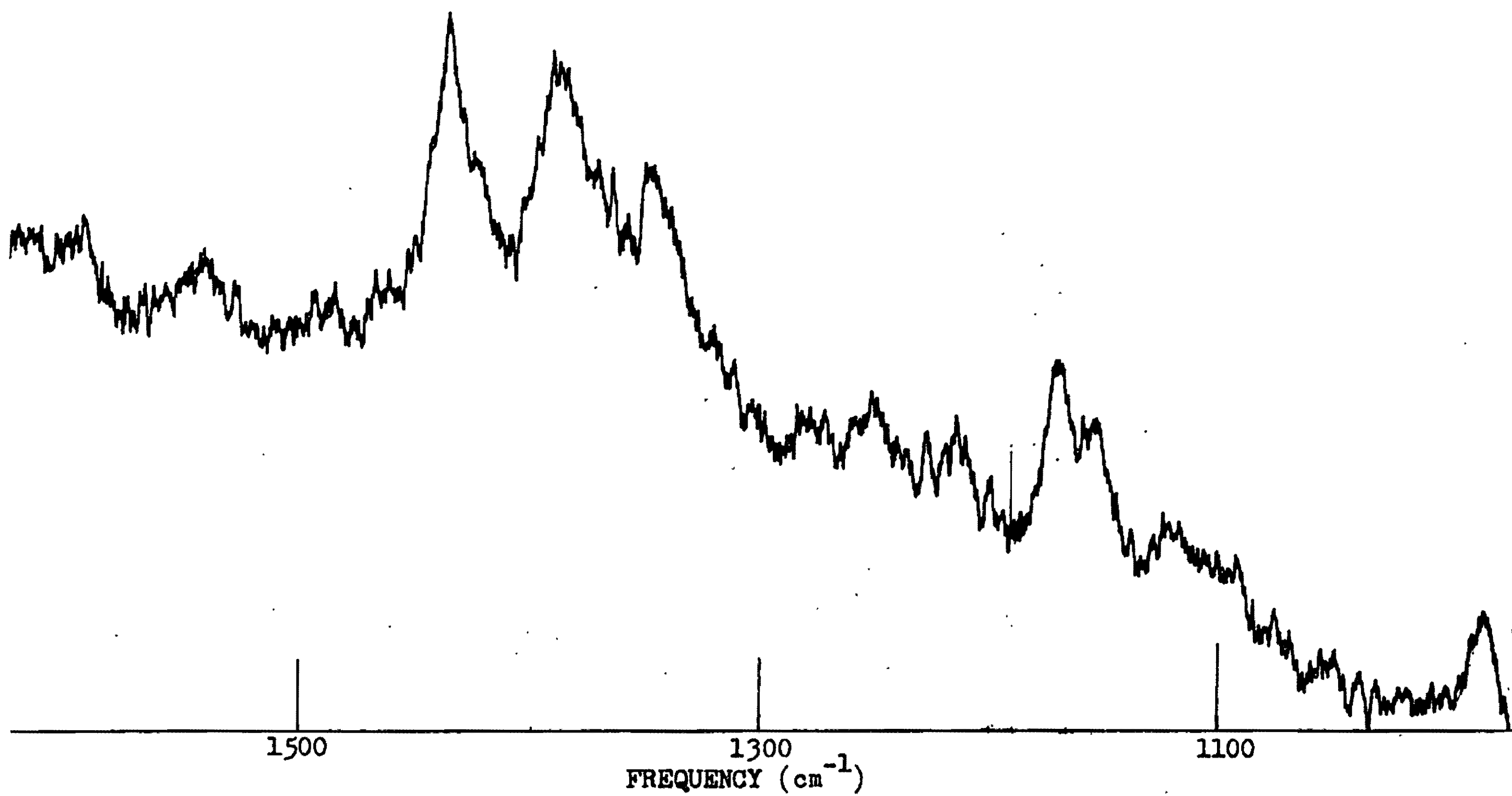


Figure 3. Raman spectrum of N^{15}-Ars-AzoCP (pH 8.8) at 488.0 nm excitation.

1432-1442 cm^{-1} (Table X). This peak is lower in the pH 8.8 and pH 6 forms (1435 and 1432 cm^{-1}) than in the pH 11.5 form (1442 cm^{-1}). The 1345 cm^{-1} line is too broad to assign its position accurately. Substitution of N^{15} for N^{14} in Ars-AzoCP (Table X) causes the peaks at 1390 and 1440 cm^{-1} to shift to lower frequencies and identifies these peaks as -N=N- vibrations. This is similar to the shifts observed for Tetr-AzoTyr (Table VII).

TABLE X

RAMAN SPECTRAL FREQUENCIES OF ARS-AZOCP AND N^{15}-ARS-AZOCP [a]

N^{14}-Ars-AzoCP			N^{15}-Ars-AzoCP [b]	
pH 6.0	pH 8.8	pH 11.5	pH 8.8	pH 11.5
(1345)	(1345)	(1345)	(1343)	(1350)
1389 (10)	1394 (10)	1392 (10)	1383 (10)	1383 (10)
1432 (14)	1435 (14)	1442 (13)	1430 (10)	1433 (10)

a. The intensity of the peak at 1380-1390 cm^{-1} was arbitrarily set equal to 10. Excitation at 488.0 nm.

b. The diazo reagent was prepared from $NaN^{15}O_2$ and arsanilic acid.

DISCUSSION

Cobalt-Substituted Enzymes

Cobalt-substituted carboxypeptidase and carbonic anhydrase were chosen as candidates for resonance Raman spectroscopy because they possess visible absorption spectra due to the Co(II)-protein complex. Since histidine provides two and three cobalt ligands, respectively, it was hoped that the visible absorption spectra would be due to the Co(II)-histidine chromophore as well as Co(II) d-d transitions.

Studies of model Co(II)-imidazole and Co(II)-histidine complexes show that although Raman intensities were low, there was a small amount of resonance enhancement associated with a 250 nm charge-transfer band in the ultraviolet for tetrahedral cobalt-imidazole and with a 560 nm absorption band in the visible for tetrahedral cobalt-histidine (40). The peaks most susceptible to resonance enhancement were imidazole ring modes at 1165, 1210 and 1255 cm^{-1}. In these spectra, the total enhancement was only three-fold, where enhancement is defined as the ratio of sulfate standard concentration to sample concentration required for equal Raman peak intensities. In more favorable cases the enhancement could be 1,000 fold (heme proteins) to 10,000 fold (carotenoids). The lack of resonance enhancement was ascribed to lack of much involvement of ligand orbitals (charge transfer) in the electronic transitions giving rise to visible and ultraviolet absorption. Thus, resonance enhancement of ligand vibrations was not observed.

In the case of the cobalt-substituted enzymes, we found no evidence

of resonance-enhanced peaks with excitation near the visible absorption bands (514.5nm) or closer to the ultraviolet (457.9 nm). Although our spectra were not of high quality, we would have been able to observe enhancements of 10 fold or greater. In this respect, the cobalt-substituted zinc enzymes appear to be similar to the model cobalt complexes. Cobalt-substituted carbonic anhydrase and carboxypeptidase also have absorption spectra, magnetic properties, and crystal structures similar to those of tetrahedral low spin Co(II) complexes (2).

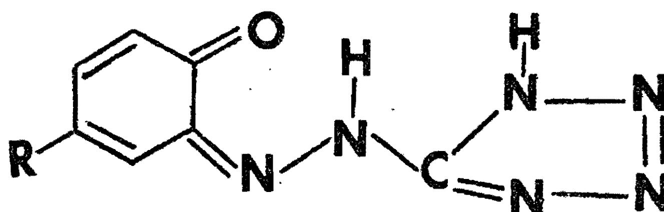
Azotyrosine Compounds

All the azotyrosine-compounds that we made are similar to each other. All these compounds have resonance Raman spectra in which the three major peaks lie in the range 1340-1450 cm^{-1}. An examination of these peaks in the model system can tell us something about the protein arsanilazocarboxypeptidase.

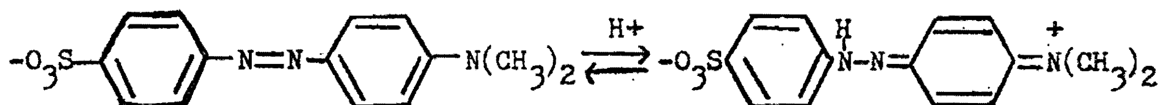
In the pH range studied (pH 6.0-11.5) the tetrazole and carboxylate moieties of Tetra-AzoTyr remain unchanged; the amino group ionization at pH 9 should have little effect as this is probably not part of the chromophore. The observed spectral changes which are related to the ionization state of the azo phenol moiety have been characterized by Johansen and Vallee (18). Tetr-AzoTyr and Ars-AzoCP have similar titration curves. Tetr-AzoTyr has a pK of 9.0 for azo phenol ionization. The zinc complex of Tetr-AzoTyr has two pK values of approximately 7.0 for zinc complexation and concomitant loss of a proton and 9.5 for dissociation of zinc and azophenolate. Thus, Tetr-AzoTyr is protonated at pH 6.0 and ionized at pH 11.0. The zinc complex of Tetr-AzoTyr is at a maximal concentration at pH 9.0. Apoarsanilazocarboxypeptidase has a pK of 9.4. Arsanilazocarboxypeptidase has two pK values (7.7 and 9.5), one for the formation

and one for the destruction of the zinc azotyr-248 complex (18). Thus, azotyr-248 is protonated at pH 6.0 and ionized at pH 11.0. At pH 8.8, the zinc azotyr-248 complex is at a maximal concentration.

There are at least two possible places of protonation on the azophenol moiety of Tetr-AzoTyr: either the phenoxy group or the nitrogen double bond that links the two rings together. Protonation of the nitrogen double bond that links the two rings would destroy the aromaticity of the benzene ring and result in a quinoid type structure:

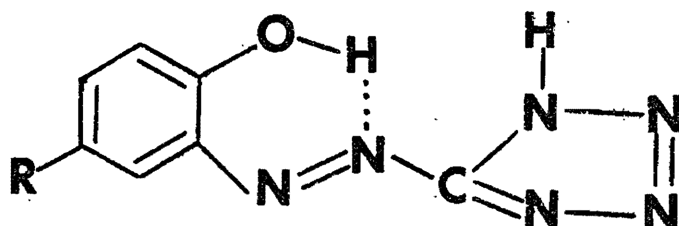


Such a marked change in structure would be expected to give rise to a change in the Raman spectrum. Indeed this does occur in many azo dyes such as methyl orange. The resonance Raman spectrum of methyl orange changes greatly upon protonation. The peaks at 1420 and 1395 cm^{-1} which have been ascribed to N=N stretch vanish and a new peak at 1183 cm^{-1} due to N-N appears (39). This is proof that a quinoid structure is formed:



However, when Tetr-AzoTyr is protonated, there is no loss in the peaks that we assigned to N=N vibrations at 1445 and 1389 cm^{-1} (Table VIII) and no new band near 1183 cm^{-1}. But there is a shift in the position of the peak from 1444 to 1437 cm^{-1}. There is also a shift in the position of a peak at 1343 to 1352 cm^{-1}. It seems likely that this latter peak is due

to the tyrosyl group since this peak was unaffected by N^{15} substitution and yet it is involved with the protonation of Tetr-AzoTyr. Thus, it appears that the phenoxy group of the Tetr-AzoTyr (low pH) is protonated. It also seems probable that there is hydrogen bonding between the hydroxyl group of Tetr-AzoTyr and the nitrogen double bond that links the rings together, since the N=N vibration (1445 cm^{-1}) is affected. This would result in a thermodynamically favorable six-membered ring:



In the zinc complex of Tetr-AzoTyr the peak for the tyrosine ring vibration is at 1352 cm^{-1} (Table VIII). This makes it similar to the low pH form of Tetr-AzoTyr. But the positions of the peaks assigned to the N=N group (1445 and 1389 cm^{-1}) are the same as in the high pH form of Tetr-AzoTyr. Thus, zinc appears to affect the N=N group very little. This implies that there is only a bond formed between the zinc atom and the phenoxy group to Tetr-AzoTyr.

Arsanilazocarboxypeptidase

In contrast to the zinc complex of Tetr-AzoTyr, the pH 8.8 form of Ars-AzoCP has its N=N peak at 1435 cm^{-1} which is lower than the value of 1442 cm^{-1} in the pH 11.5 form of the enzyme (Table X). The low value of the 1435 cm^{-1} peak is similar to that of 1432 cm^{-1} for the pH 6.0 form of the enzyme, indicating that the azo group is involved in zinc binding in arsanilazocarboxypeptidase. Although the 1345 cm^{-1} peak is too broad to assign its exact frequency, it is most likely that Ars-AzoCP (pH 8.8) has

a structure similar to the protonated form of Tetr-AzoTyr, a six-membered ring in which the zinc atom is coordinated to both the phenoxy group and the nitrogen double bond. Thus, the protonated form of Tetr-AzoTyr appears to be a better structural model than the zinc compound for the pH 8.8 form of Ars-AzoCP.

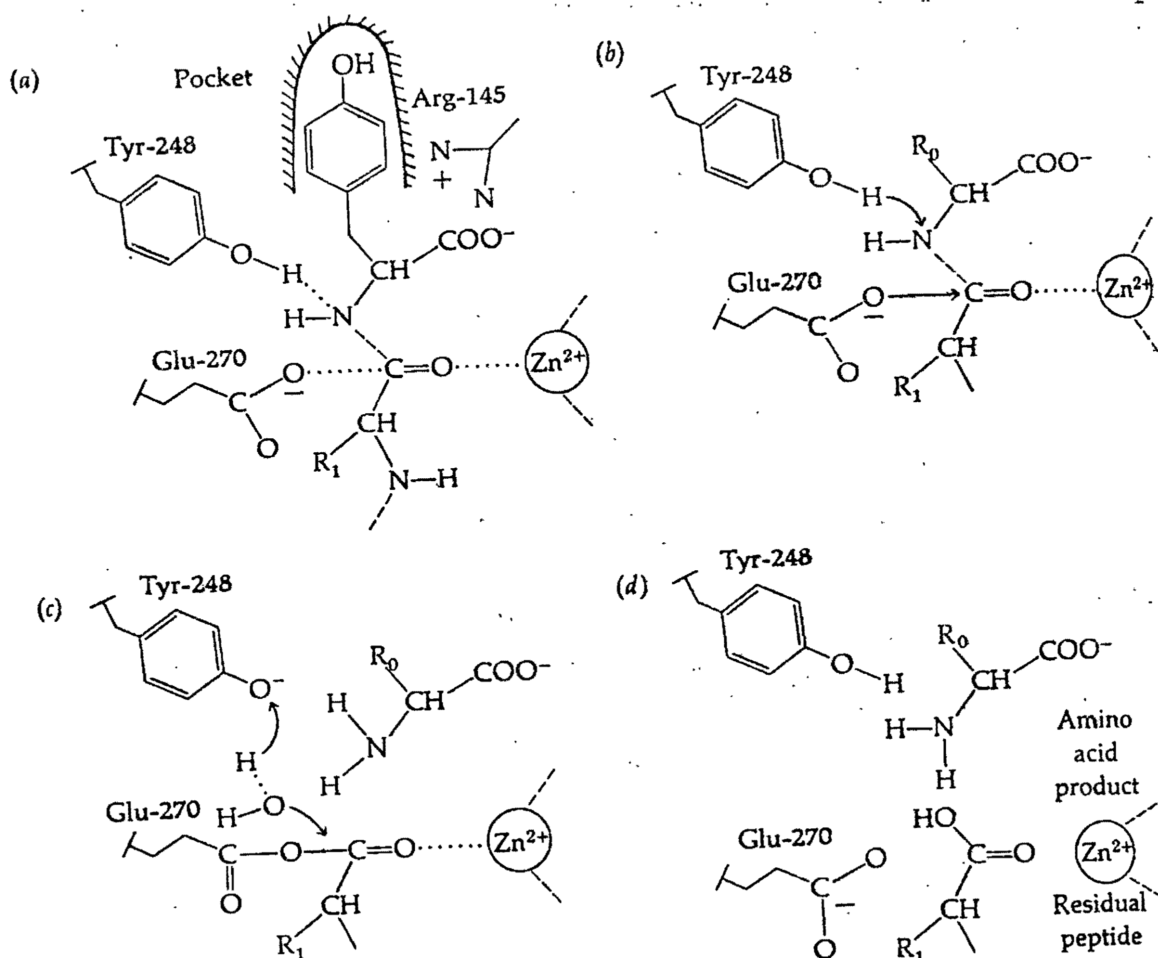
The involvement of zinc in a six-membered ring in Ars-AzoCP implies that the zinc atom is five-coordinate, since it has three protein ligands (2 His and 1 Glu) in addition to the two to azotyrosine. Such a coordination geometry appears to be quite feasible for the metal atom in carboxypeptidase. Cobalt-substituted carboxypeptidase appears to be five-coordinate on the basis of magnetic and spectral properties (41).

Yet one must wonder if this is an adequate model of carboxypeptidase since the presence of the azo group may have altered the structure of the enzyme. By putting the azo group in the protein we have set up the protein for formation of a thermodynamically favorable six-membered ring. Thus, we must question the physiological significance of the modified carboxypeptidase and whether or not zinc is bound to Tyr-248 in the native form of the enzyme.

The pH of intestinal juice varies from 7.0 to 8.0 (42). The pK value for formation of a zinc complex of Ars-AzoCP is 7.7 and maximum complex formation occurs at pH 8.8; the pK for dissociation of zinc is 9.5 (18). In Ars-AzoCP from which the zinc has been removed, Tyr-248 has a pK of 9.4. Thus, diazotization of tyrosine lowers its pK value which is normally close to 10.0. This means that in the native enzyme the pK for complexation with zinc is probably greater than 7.7 and there is less chance for Tyr-248 to bind to the zinc atom in the physiological pH range of 7.0 to 8.0. At the pH optimum of the reaction which is 7.5 (43),

no more than 20% of the Tyr-248 at the active site would be bound to zinc. In the proposed reaction sequence for peptide hydrolysis by carboxypeptidase (Figure 4), the protonated form of Tyr-248 is believed to be important in initiating the reaction (4).

Although zinc does not appear to be complexed with Tyr-248 in the active form of carboxypeptidase at pH 7.5, the demonstration that the zinc can complex with Tyr-248 at higher pH values has interesting structural implications. The carboxypeptidase crystals which were used in the X-ray structure determination have a yellow color when Tyr-248 is diazotized (18). The crystal structure of the unreacted enzyme indicates that Tyr-248 is 17 Å away from the zinc atom and that the substrate must induce a conformational change in the protein in order for Tyr-248 to move in towards the active site (4). However, when yellow crystals of Ars-AzoCP are dissolved without a change in pH they turn red. Thus, it is likely that in the native state of carboxypeptidase, Tyr-248 and zinc are considerably closer to one another than in the crystalline state.



<u>Figure 4.</u> Proposed mechanism of action of carboxypeptidase.

a. Tyr-248 acts as proton donor to susceptible bond of substrate.
b. The carboxylate oxygen of Glu-270 attacks the carbonyl of the peptide bond with the zinc atom assisting by polarizing the carbonyl group. This results in a tetrahedral intermediate. Breakdown of the tetrahedral intermediate is assisted by the donation of a proton from Tyr-248. The C-terminal amino acid is released.
c. The resulting acyl-enzyme is attacked by water with the assistance of the phenolate moiety of Tyr-248.
d. This frees the polypeptide chain of the substrate being hydrolyzed.

Figure taken from reference 44, page 274.

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