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HPLC analysis of myoglobin tryptic peptides from selected species of cetaceans

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AN ABSTRACT OF THE THESIS OF David Lawrence Hayteas for the
Master of Science in Biology presented February 2, 1990.

Title: HPLC Analysis of Myoglobin Tryptic Peptides From
Selected Species of Cetaceans.

APPROVED BY THE MEMBERS OF THE THESIS COMMITTEE;

Deborah A. Duffield, Chair

Richard B. Forbes

Malcolm S. Lea

David W. McClure

Due to the large gaps in the fossil record, the evolutionary history of the mammalian order Cetacea is incomplete and controversial. Increasingly researchers are utilizing molecular and biochemical procedures to supplement cetacean paleontology. One of these methods is the comparison of amino acid sequences of myoglobin among species of this

order. Since this method is time-consuming and expensive, an alternative procedure is desirable.

As a candidate for such an alternative, peptide mapping was performed on selected species of cetaceans. This mapping was done utilizing High Performance Liquid Chromatography (HPLC). Myoglobin was extracted from skeletal muscle, purified, digested with trypsin, the digest separated on a C-18 reversed phase column, and eluted with a gradient of increasing acetonitrile concentration. The eluent was monitored with ultraviolet light at 220 nm, and the absorbance of individual peptides was integrated and plotted. The characteristic peptide maps produced for each sample were compared, and the number of differences between samples tabulated in a difference matrix. Based on the amount of peptide similarity, a phylogenetic tree was constructed for the species represented. The identity of each peptide peak was determined through the use of retention coefficients.

This peptide mapping technique produced data showing relationships between species sampled in close agreement with those determined from amino acid sequencing, paleontology, and other biochemical methods. The two species of Kogia form a separate grouping, with differences between the species. The same is true of the delphinids. Lagenorhynchus obliquidens and Lagenorhynchus acutus are grouped with Stenella coeruleoalba, as is Globicephala macrorhynchus and Pseudorca crassidens. Tursiops truncatus exhibits a close relationship to these five

species, as well as to Orcinus orca. Orcinus did not group with Globicephala and Pseudorca as closely as expected, and appeared as a close but distinct group. Phocoena phocoena and Delphinapterus leucas exhibited an extremely close relationship. Differences were apparent between populations of the same species. Samples were identified to genera from comparison of their peptide maps with those of known species, and families exhibited characteristic groups of peak sequences. Unknown samples were also identified through peptide map comparisons. It therefore appears that HPLC peptide mapping of myoglobin tryptic peptides is useful in studying cetacean evolutionary relationships, detecting differences in intraspecific populations, and identifying unknown samples.

HPLC ANALYSIS OF MYOGLOBIN TRYPTIC PEPTIDES
FROM SELECTED SPECIES OF CETACEANS

by

DAVID LAWRENCE HAYTEAS

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

MASTER OF SCIENCE
in
BIOLOGY

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1990

TO THE OFFICE OF GRADUATE STUDIES;

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CHAPTER I

INTRODUCTION

Inexorably tied to the sea, the whales and dolphins, members of the order Cetacea, are one of the most truly aquatic of all the various groups of mammals. Divided into two suborders, nine families, and seventy-eight species (Honacki et al 1982; Gaskin 1982; Corbet & Hill 1986; Vaughan 1986), these animals spend their entire lives in the water, feeding, socializing, and reproducing. Their collective habitat is made up of all the world's oceans, from shallow waters to deep seas, as well as, for some, freshwater riverine environments. Only with the pinnipeds (seals and walruses) and the sirenians (manatees and dugongs) among the mammals do they share this habitat. Largely because of this fact, man's knowledge of the cetaceans has been slow in coming and fragmentary - a mixture of myths, exaggerations, fears, and relatively few facts (Slijper 1962; Haley 1978). Only in the last century has research into these animals begun to present a clearer and more detailed picture of their biology, population dynamics, and distribution. Unfortunately most of this increase in knowledge has come as a byproduct of the almost complete extermination of many species of whales at the hands of modern whalers (Rice 1977). Perhaps this knowledge can now be put to

use in helping to restore populations of these exploited animals.

Included in the recent explosion of knowledge of cetaceans is a picture of the evolutionary history of the order. Traditionally based on paleontological evidence and morphology, this developing outline has recently been supplemented by genetic, molecular, and biochemical findings (Gaskin 1982). Yet the story is far from complete, and a consensus as to its various steps is definitely lacking. Mode of origin, evolutionary pathways, phylogenetic relationships, and taxonomy are constantly the source of debate among authorities in the field (Duffield 1989). Determining the story of cetacean evolution is still an ongoing process.

Increasingly, scientists have turned to the investigation of molecular and biochemical properties of cetaceans in order to shed new light on their evolutionary relationships. For example, chromosomal comparisons have been made among various species (Arnason 1969, 1972, 1974; Duffield 1977, 1983, 1986; Duffield Kulu 1972). Electrophoresis of hemoglobins and other proteins from various species, and from individuals of the same species has been performed for inter- and intraspecific comparisons (Baluda et al 1972; Sharp 1975; Wada & Numachi 1979; Simonsen et al 1982; Andersen 1988; Winans & Jones 1988). Amino acid sequence studies of homologous proteins, namely myoglobin, have been conducted for a small number of species, and the results compared

(Lehman et al 1980; Goodman et al 1982). Protein sequencing determines differences in primary structure between species, which in turn provides information for arriving at an estimate of the relative degree of similarity between these species. It is assumed that the more similarity between two groups the more closely related the groups are; the more closely related they are, the more recently they diverged from a common ancestor (Jukes 1972; Klotz et al 1979). From such comparisons phylogenetic trees can be constructed reflecting relationships based on the sequences of the particular protein being studied (Jukes 1972; Dayhoff & Eck 1972; Klotz et al 1979). An evolutionary history of a group such as cetaceans can therefore be determined utilizing biochemical evidence as well as fossils or morphologies. Most often, however, such lines of investigation are used in combination to arrive at a consensus as to evolution.

One of the earliest and most widely used group of proteins to be sequenced were the globins, namely hemoglobin and myoglobin. Since whale muscle tissue contains an extremely high concentration of myoglobin, it quickly became the protein of choice for sequence studies. By 1980 the amino acid sequence of myoglobin from seventeen different species of cetaceans had been determined (Bogardt et al 1980). Phylogenetic relationships based on the amount of similarity in the sequences among the species were constructed and

evolutionary pathways inferred (Lehman et al 1980; Dwulet et al 1980).

Protein sequencing is very costly and time-consuming (Williams et al 1988). Myoglobin, with its 153 residues, requires expensive equipment and trained technicians for it to be sequenced correctly in a reasonable amount of time (C. Head, personal communication 1986). In addition considerable amounts of tissue are required in order to extract enough myoglobin for sequencing (Hapner et al 1968; Rothgeb and Gurd 1978). This last requirement makes this process less attractive due to lack of supply of whale tissue, resulting from the near extermination and consequent protection of many species (Sigma 1988). Because of these drawbacks, and the fact that the myoglobin amino acid sequence had been determined for at least one species of each modern family, sequencing of this protein in cetaceans virtually ceased after 1980 (Goodman et al 1982). It is apparent, though, that much more can be learned about cetacean evolution from this protein.

Another analytical technique, that of High Performance Liquid Chromatography (HPLC), offers a way to continue this area of research without the drawbacks of sequencing. HPLC is basically a separation process whereby a complex mixture, such as the various peptides resulting from a proteolytic digestion of myoglobin, can be separated, and the various components detected individually. The peptides detected can then be plotted in the form of peaks, producing a characteristic

"peptide map" (Lottspeich & Henschen 1985; Schroeder 1986). Such a map provides much of the same information with regard to evolutionary relationships as that provided by amino acid sequencing, and as micromolar amounts are required for analysis by HPLC, it should be possible using HPLC to develop peptide maps of myoglobin taken from each species of cetacean, and from individuals of the same species, and to do so with a very small amount of muscle tissue (Engelhardt 1979; Yost et al 1980). Relative similarity among species, as well as possible discrete differences between intraspecific populations, could thus be determined. HPLC peptide maps are already used in the identification of hemoglobin variants in humans (Schroeder 1986). Ideally a "peptide atlas" could be developed, containing maps of every species of cetacean, as well as of individuals of various populations. From such an atlas phylogenetic relationships based on myoglobin, similar to those determined from sequencing, could be ascertained. This additional biochemical evidence would then be available for comparison with other phylogenetic data in the ongoing process of elucidating the evolutionary history of Cetacea.

CHAPTER II

REVIEW OF RELATED LITERATURE

The taxonomic organization and phylogenetic relationships of the whales, dolphins, and porpoises has continued to be a source of controversy. Early naturalists considered these animals to be fish, although Aristotle recognized that they had lungs and hair, and nursed their young (Slijper 1962). In the seventeenth century Ray and Linnaeus correctly classified cetaceans as mammals, while Flower in the nineteenth century subdivided their order, Cetacea, into two suborders: Odontoceti, or toothed whales, and Mysticeti, or baleen whales (Haley 1978). Based primarily upon morphological characteristics, most authorities further subdivide the order into nine families and seventy-eight species. This classification is given in Appendix A.

Various modifications of this classification scheme have been proposed in past years, based on evidence provided by paleontological, genetic, and biochemical investigations. Working almost entirely with fossils, Kellogg (1928) proposed dividing Platanistidae into two families: Iniidae, containing Inia, Lipotes, and Pontoporia, and Platanistidae, containing Platanista. He further proposed grouping Monodontidae and Phocoenidae with Delphinidae, separating Kogia into their own

family Kogiidae, and dividing the Balaenidae into the Neobalaenidae as well as Balaenidae (Kellogg 1928). Rice (1977) proposed elevating the two suborders to full orders, and grouping Phocoenidae with the Delphinidae. Haley (1978) and Eisenberg (1981) agreed with Rice in the placement of the porpoises with the dolphins, but not in the elevation of the suborders to ordinal status. Hershkovitz (1966) split Delphinidae into two families: Stenidae, containing the genera Sotalia, Stenella, and Steno, and Delphinidae, containing the rest of the delphinids. Orr (1982) followed the same scheme. A more radical outline has been proposed by Young (1962), who recognized three superfamilies among the odontocetes: Platanistoidea containing the Platanistidae, Physeteroidea containing Physeteridae and Ziphiidae, and the Delphinoidea, containing Delphinidae, Monodontidae, and Phocoenidae. Harrison and King (1978) went even further, proposing five superfamilies, Ziphioidea and Monodontoidea in addition to the above three, with Delphinoidea divided into three families: Delphinidae, Stenidae, and Phocoenidae, with five subfamilies making up the Delphinidae. Kasuya (1973), studying the morphology of the tympano-periotic bone in extant species, favored placing Delphinapterus and Monodon each in their own family, while Duffield Kulu (1972), utilizing karyotype studies, found evidence for placing Delphinapterus with the phocoenids. In general, the classification of the mysticetes enjoys a higher degree of consensus than the odontocetes,

where this disagreement persists about classification at the familial level and below.

It is currently accepted that taxonomic relationships of recent species and genera should reflect evolutionary descent (Dobzhansky et al 1977; Futuyma 1979; Vaughan 1986). The disagreement about cetacean classification mentioned above reflects the confusion and lack of knowledge concerning the evolutionary history of this group. Much of this confusion is due to the paucity of fossil remains, which has been supplemented in the last thirty years with often conflicting molecular and biochemical evidence (Repenning 1976; Gaskin 1982). A general outline, however, has been constructed for cetacean evolution, with much controversy still surrounding the details.

The oldest known remains to be identified as whales have been found in early middle Eocene deposits in equatorial regions of the world. These fossils - Protocetus, Pappocetus, Indocetus, and Pakicetus - have been grouped into the family Protocetidae of the suborder Archaeoceti or Zeuglodontia, the third major subdivision of the order Cetacea (Kellogg 1928; Duffield Kulu 1972; Gingerich et al 1983). All members of this suborder were extinct by the Oligocene (Duffield Kulu 1972). Because of cranial and dental characteristics, both Winge (1921) and, more recently, Van Valen (1968) believed that this family could easily be the form intermediate between the land mammal from which cetaceans evolved and the modern suborders.

Although the subject of much debate, it is now generally thought that whales arose from a terrestrial mammal group, probably the Mesonychidae, which inhabited the regions around the shallow areas of the Paleocene Tethys Sea. It is thought that members of this primitive group found it competitively advantageous to feed in the shallow waters, and natural selection favored a transition to an aquatic way of life (Van Valen 1968; Gingerich et al 1983; Gaskin 1982). Earlier authorities, mainly Winge (1921) and Kellogg (1928), felt that the majority of the characteristics found in the archaeocetes, as well as in primitive odontocetes and mysticetes, pointed to an origin of the whales from a carnivore-like mammal. However, more recent fossil finds, along with molecular and biochemical evidence, indicate a closer relationship between cetaceans and ungulates than between cetaceans and carnivores (Boyden & Gemeroy 1950; Duffield Kulu 1972; Beintema & Lenstra 1982; De Jong 1982; Goodman et al 1982). These data support the view of many authorities, including Van Valen (1968), Eisenberg (1981), and Vaughan (1986), who favor a descent of cetaceans from the Mesonychidae, a family more closely related to the Ungulata rather than the Carnivora. The mesonychids and the ungulates apparently both arose from the same primitive condylarth ancestor (Van Valen 1968). A middle ground has been proposed by Duffield Kulu (1972) who thinks that cetaceans may have evolved from a creodont line just before the divergence

of the carnivore and ungulate groups. The definite origin of the cetacean lineage has yet to be agreed upon.

Another source of controversy lies in whether or not the whales are monophyletic, biphyletic, or polyphyletic in origin. Early on, Kellogg (1928), utilizing fossil evidence, favored independent beginnings for all three suborders. Yablokov (1964) proposed a separate origin for each of the living suborders, based on studies of osteology and soft anatomy. Slijper (1962) also favored a separate origin, feeling that each suborder should actually be an order. Rice (1977) concurred. Van Valen (1968), however, took exception with Yablokov (1964), finding no reasons for anything but a monophyletic origin, after an examination of the arguments put forth by the latter. This view has received support from both Arnason (1972, 1974) and Duffield Kulu (1972), utilizing karyotypic evidence, as well as from investigators studying protein structure (Dayhoff et al 1972; Lehman et al 1980; Goodman et al 1982). Indeed, the similarities in the various morphological structures and genetic characteristics are too numerous to indicate anything but a monophyletic origin. As a result, in contemporary usage the cetaceans are considered to be a monophyletic order (Eisenberg 1981; Vaughan 1986).

Following the appearance of the Protocetidae, the cetaceans appear to have diverged rapidly (Duffield Kulu 1972). The archaeocetes (zeuglodonts) separated very early from the main evolutionary pathway, developing into two main

lines of descent. One line, comprising the family Basilosauridae, followed a road toward generally larger size, culminating in the huge, reptile-like Basilosaurus (Zeuglodon) of the late Eocene. The other line, the Dorodontidae, was made up of smaller individuals, more porpoise-like in shape. This line lasted until the early Miocene (Kellogg 1928; Duffield Kulu 1972; Whitmore & Sanders 1976). Barnes and Mitchell (1978) postulated that the odontocetes and mysticetes arose from the Dorodontidae, but it has also been proposed that these two archaeocete groups represent specialized lines derived from the main cetacean stem group (Kellogg 1928). Probably adapted only to warm, shallow waters, the Archaeoceti became totally extinct with the marine cooling of the late Oligocene, and the intense competition from better adapted, though primitive, individuals of the two modern suborders (Gaskin 1982).

By the late Eocene, when the archaeocetes were flourishing, the first primitive odontocetes made their appearance. Represented by the fossils Agorophius and Xenorophus, these primitive forms exhibited an early degree of typical odontocete telescoping, the antero-posterior shortening of the skull, which was not present in the archaeocetes (Duffield Kulu 1972). Kellogg (1928) placed them in the family Agorophiidae, and both he and True (1907) felt that they were ancestral to later odontocetes. New dating of the deposits in which these specimens were found, showing them

to be much younger than originally thought, has since disproved this hypothesis. However, the discovery of the fossil odontocete Andrewsiphius in late Eocene deposits, and its possessing of agorophiid characteristics, has once again revived the earlier theory (Gaskin 1982). In either case, this family is thought to represent a stage related to the modern odontocetes, either a side branch or directly ancestral (Miller 1923; Whitmore & Sanders 1976; Gaskin 1982).

In the Oligocene, the odontocete family Squalodontidae appeared. The large number of fossils of this group suggests that they were successful throughout both the Oligocene and Miocene, though they died out in the early Pliocene (Whitmore & Sanders 1976). Early on, these "shark-toothed porpoises" diverged into three relatively distinct lines of descent: a short-beaked line, represented by Prosqualodon, an unspecialized long-beaked line, represented by Squalodon, and a highly specialized long-beaked line, represented by Neosqualodon (Kellogg 1928; Duffield Kulu 1972). Exhibiting a progressive advancement of the telescoping process, as well as varying stages of heterodont dentition, this family is thought by Kellogg (1928) to have descended from the archaic toothed whales, and to be closely related to the Agorophiidae. Kellogg also thought that this family gave rise to the modern families Ziphiidae and Platanistidae. Simpson (1945), on the other hand, thought that the Squalodontidae gave rise to none of the modern families, but rather represented a specialized

side branch of the main cetacean line. Conversely, Slijper (1962) believed that primitive squalodonts, through intermediate families, were ancestral to all six modern odontocete families, while Duffield Kulu (1972) took the same position as Kellogg (1928). Gaskin (1982) opted for ziphiids only as descending from Squalodontidae, but Harrison and King (1978) believed that squalodonts were most closely related to the platanistids. An extinct family, the Kentriodontidae, is postulated by Barnes (1976) to have also descended from the squalodonts. With Slijper (1962), Barnes believed that this family gave rise to the modern dolphins (Delphinidae), while Gaskin (1982) suggested that certain kentriodontids may have given rise to the platanistids. The part played by the Kentriodontidae, as well as by the squalodonts in general, in cetacean evolution is still much in dispute.

When the reign of the Squalodontidae was at its peak, the earliest representatives of the modern odontocete families appeared. In the very early Miocene the first primitive delphinids occurred, probably derived from archaic toothed whales, kentriodontids, or, less likely, early squalodonts (Duffield Kulu 1972; Barnes 1978; Harrison & King 1978). This group quickly began to diverge, with the most successful division first being the extinct family Eurhinodelphinidae. This specialized family of "long-snouted dolphins" flourished in the middle Miocene, became extinct by the early Pliocene, and possibly gave rise to early direct ancestors of the

various modern genera (Kellogg 1928). Ancestors of the modern delphinids, such as Delphinodon, first appeared in the very late Miocene, and rapidly began to diverge into lines that resulted in today's modern forms. Winge (1921) believed that these lines were five in number, with the Monodontidae probably branching off first from primitive eurhinodelphinids. The delphinids proper then evolved, with Steno, Stenella, Sotalia, and Delphinus representing the most primitive line. This was followed by the appearance of Tursiops, Lissodelphis, Lagenorhynchus, Cephalorhynchus, and Feresa, in turn followed by a group containing Orcinus, Orcaella, Grampus, Pseudorca, and Globicephala. This sequence is based on a progressive shortening and broadening of the face, a process which culminates in the family Phocoenidae, probably the last group of modern delphinids to appear (Winge 1921). Karyotypic and morphological evidence from present day forms tend to support this outline, showing delphinids to be a cohesive group, from which Phocoenidae and Monodontidae arose (Duffield Kulu 1972; Mead 1972; Barnes 1978). Kasuya (1973), from his study of the tympano-periotic bone, not only favored separating the monodonts and phocoenids, but also favored splitting Monodontidae into two families, including Orcaella with Delphinapterus, and placing Monodon alone in its own family. Barnes (1978) agreed with this phylogeny, except for the separation of the Monodontidae. Mead (1972) felt that a subfamily status should be given to Cephalorhynchidae, as well

as to other groupings of delphinids. While earlier authorities tended to group monodonts and phocoenids in the family Delphinidae, most experts now agree on their separation into three distinct families (Kellogg 1928; Rice 1977; Barnes 1978; Mead 1978; Gaskin 1982). As mentioned previously, other authors have proposed alternative outlines of these three families, from dividing delphinids into three families (not including monodonts and phocoenids) to establishing a superfamily containing Monodontidae and Phocoenidae (Young 1962; Hershkovitz 1966). This plethora of phylogenetic schemes exemplifies the disagreement surrounding the evolutionary history of modern delphinids, resulting mainly from the lack of fossil evidence and the reliance on the study of similarities in extant species (Duffield Kulu 1972; Gaskin 1982).

Thus by the lower Pliocene the earliest forms of the line which would give rise to the modern dolphins, porpoises, narwhals, and belugas had appeared. The Eurhinodelphinidae, possibly ancestral to this line, were practically extinct, as were the squalodonts. Showing up at this time, however, were forms representative of the other three modern odontocete families: Ziphiidae, Physeteridae, and Platanistidae. Almost certainly descended from the squalodonts, the first primitive members of the family Ziphiidae, represented by the fossils Diochotichus and Squalodelphis, occurred in the lower to middle Miocene (Kellogg 1928). On the basis of skull

modifications, it is thought that this family rapidly diverged into three main lines of development, lines characterized by the modern genera Mesoplodon, Ziphius, and Hyperoodon (Duffield Kulu 1972). The most primitive of these three is probably Mesoplodon, although Tasmacetus shepherdii appears to have retained the primitive characteristic of unreduced dentition (Gaskin 1982). The ziphiids were well established by the end of the Miocene (Kellogg 1928).

Also in the lower Miocene appeared the first physeterids, Idiorophus, Diaphorocetus, and later, Aulophyseter, possibly directly related to the modern Physeter catodon (Kellogg 1928). Winge (1921) originally placed this family together with the ziphiids, but all later authors placed them into separate families; even though recent karyotyping has shown both groups to have the same chromosome number of $2n=42$ (Arnason 1972, 1974; Duffield 1977). The earliest sperm whales had teeth on both the maxillae and mandibles, but loss of the maxillary teeth soon began to occur. In addition, these early individuals also exhibited the cranial development necessary to accommodate the enormous spermaceti organ so characteristic of today's sperm whale (Duffield Kulu 1972). Kasuya (1973), as well as others, felt that Physeteridae probably arose from the squalodonts, and quickly diverged from the main odontocete line (Slijper 1962). Soon thereafter, possibly by the early Pliocene, the family split into two lines leading to the modern genera Physeter and

Kogia. The fossil record of the latter group is extremely poor, and its evolutionary history, as well as its relationship to other cetaceans, is not well known (Kellogg 1928; Duffield Kulu 1972; Haley 1978).

Primitive forms of the last of the six modern odontocete families, the Platanistidae, also first appeared in the Miocene. Kellogg (1928) described two fossil genera, Proinia and Zarhachis, from this epoch as possibly being ancestral to the line of iniids and platanistids respectively. Indeed he divided this modern family into two: Iniidae and Platanistidae. Later extinct forms from the Pliocene, such as Saurodelphis and Ischyrorhynchus, suggest that this family diverged into two different lines; one leading to the modern Pontoporia, Inia, and Lipotes, and the other to the Platanista (Duffield Kulu, 1972). Even though many authorities have attempted to split these genera into two or more groups based on different characteristics, most experts now agree that grouping them as a single family better reflects true phylogenetic relationships (Fraser & Purves 1960; Zhou et al 1978; Barnes 1978; Gaskin 1982). Probably derived from early squalodonts, this most primitive of modern odontocete families apparently arose from a squalodont line which had already diverged from one which was to later give rise to the family Ziphiidae (Duffield Kulu 1972). Gaskin (1982), however, believed that the kentriodontids were ancestral to platanistids, a view not generally held by other authorities

(Kellogg 1928; Slijper 1962; Harrison & King 1978). Due to the lack of fossil evidence and the various specializations and geographical distributions of modern forms, the history of this group is still the source of much controversy.

It is evident from the above discussion that the history of the odontocetes is, at best, confusing. The situation with the Mysticeti is slightly better. Although historically there has been much dispute as to a mono- or diphyletic origin of the two suborders, most authorities now agree that both groups arose from a common stem line (Van Valen 1968; Eisenberg 1981; Novacek 1982; Gaskin 1982; Vaughan 1986; Duffield 1989). Apparently the divergence from a toothed ancestor began in the Eocene, as baleen feeding had fully evolved by the mid-Oligocene (Whitmore & Sanders 1976). Like the odontocetes, the baleen whales more than likely arose from a main evolutionary line, from which the archaeocetes had already split (Duffield Kulu; Gaskin 1982). The earliest fossil mysticete is possibly Archeodelphis from the early Eocene, although the fragments are so incomplete that definite placement is not possible (Kellogg 1928; Whitmore & Sanders 1976). Another early fossil from the upper Oligocene, Patriocetus, was also thought to be an early mysticete; now, however, it appears to show greater similarity to primitive odontocetes (Kellogg 1928; Duffield Kulu 1972; Harrison & King 1978; Gaskin 1982). In the middle Oligocene there appeared the first fossil to be identified as a true mysticete, Mauicetus, from deposits in New Zealand

(Benham 1939; Whitmore & Sanders 1976). Having cranial features reflecting both archaeocete and mysticete characteristics, this genus is thought by Fordyce (1977) and others to be representative of the first baleen whales, which evolved from an archaic toothed whale line in the southern hemisphere, in response to the abundant food resources being provided by the developing circumantarctic current (Benham 1939; Marples 1956). Mauicetus itself was a primitive member of the first successful family of mysticetes, the Cetotheridae, which radiated rapidly in the early and middle Miocene, diverging into at least five genera and many species (Barnes 1976). By the middle Pliocene this group was extinct. Kellogg (1928) believed that the Cetotheridae gave rise to the modern family Eschrichtiidae, the least specialized of the modern mysticete families, which appeared in the Pleistocene. This hypothesis has been disputed (Gaskin 1982).

The modern family Balaenidae first appeared in the early Miocene, and then diverged into two lines, one leading to the modern genera Balaena and Eubalaena, the other to Caperea. This most primitive of the modern mysticete families already had advanced forms ancestral to modern genera by the Pliocene (Duffield Kulu 1972). Primitive balaenopterids first appeared in the middle Miocene, apparently derived from cetothere stock. This family diverged quickly into two lines of descent, one leading to the modern genus Balaenoptera, the other to the

more highly specialized Megaptera (Duffield Kulu 1972). As mentioned previously, the modern family Eschrichtiidae possibly arose also from cetotheres, but remained a monotypic group.

Fossil remains of modern mysticete lines are more widespread than those of the odontocetes, making the reconstruction of phylogenetic relationships easier and more definite (Gaskin 1982). The baleen whales appear to be a cohesive group, while the odontocetes have diverged into many different lines. However, the lack of a continuous fossil record makes the elucidation of the evolutionary history of both groups, as well as their precursors, a matter of conjecture and controversy (Kellogg 1928; Duffield Kulu 1972; Harrison & King 1978; Duffield 1989). An illustration of this final point is shown in the comparative phylogenies reproduced in Figure 1.

Because the paleontological record fails to adequately represent the descent and relationships of the order Cetacea, investigators in recent years have turned increasingly to molecular, chromosomal, and biochemical data to improve their understanding of cetacean evolution. Such data has become available in the last three decades largely as a result of the development of techniques and procedures for the detection of chromosomal banding, DNA base pair composition, and amino acid sequencing of proteins (Ninio 1983). These techniques produce data that characterizes living individuals, species, genera,

Figure 1. Comparative cetacean phylogenies derived from the fossil record. Source: Kellogg (1931), Slijper (1962), Romer (1966).

etc., enabling the relationships between various levels of classification to be determined based on the degree of similarity or difference (Minkoff 1984). Since these relationships are thought to reflect evolutionary descent, the history of a particular order or family can be ascertained by a study of molecular and biochemical traits of present day forms (Novacek 1982; Minkoff 1984). Dayhoff and Eck (1972) put the above principle as follows:

'Relics' of ancient organisms can be found in the biochemical systems of their living descendants. The exceedingly conservative nature of the evolutionary process has preserved such 'relics' in all living species . . . This dynamic preservation of the biochemical components of living cells is often quite as rigorous as the preservation of sedimentary fossils . . . Unlike fossil evidence, all of the biochemical information pertains to direct ancestors.

Thus biochemical evidence provides another avenue of evolutionary investigation. It would be expected to supplement and reinforce fossil evidence, and such has been the case, although complete congruence has been lacking in many instances (Novacek 1982; Wyss et al 1987).

One of the first biochemical techniques to be used to examine the evolutionary relationships of cetaceans was that of comparative serology or "precipitin tests" by Boyden and Gemeroy in 1950. Comparing mammalian orders, they found that the order Cetacea had a correspondence of approximately eleven percent with the Artiodactyla, while exhibiting less than two percent correspondence with the Carnivora (Boyden & Gemeroy

1950). This finding supported the premise of a closer affinity of the cetaceans to the ungulates than to the carnivores (Duffield Kulu 1972). More recently Arnason (1972, 1974), Duffield Kulu (1972), and Duffield (1986) have utilized cytogenetics to elucidate phylogenetic relationships. In this procedure karyotypes from individuals of each living species are compared, and amount of similarity determined. The more similar the karyotypes between two species (or genera, families, etc.), the more closely related they are assumed to be (Arnason 1974; Duffield 1986). These studies indicated that the karyotypes of most species were highly conserved, even between the two suborders, lending support to the premise of a monophyletic origin of the order (Duffield 1986). Among subgroups, the mysticetes exhibit a cohesive chromosomal grouping, with delphinids somewhat split into three divisions. In terms of chromosome banding, Phocoenoides and Delphinapterus show a close relationship, as do Physeter and Mesoplodon (Duffield 1977). A few species, such as Orcinus orca, show a marked divergence from the basic conserved pattern, which Duffield (1986) considered was a result of a difference in the rate of chromosomal evolution rather than reflecting a more distant phylogenetic relationship. Karyotyping, as well as the identification and use of chromosomal markers, is currently being used to detect population differences in delphinids, with the aim of discovering the discreteness of breeding units, a major step

along the pathway of speciation (Minkoff 1984; Duffield 1986). Protein electrophoresis and hematologic profiling has already been used by Duffield and her co-workers to distinguish offshore and onshore varieties of Tursiops truncatus (Duffield et al 1983). It is possible that this type of ecological separation was one way in which various cetacean species arose in the aquatic environment (Duffield 1989).

A biochemical technique which has been used many times in the study of cetacean phylogeny has been comparison of the amino acid sequences of homologous proteins (Lehman et al 1980; Goodman et al 1982). This procedure involves the determination of the amino acid sequence of a protein, such as myoglobin or cytochrome c, from various living species, determining the degree of similarity between the sequences, and from this estimating the relationships among the species. As with karyotyping, the greater the similarity in the sequences between two species, the more closely related the species are presumed to be phylogenetically (Jukes & Holmquist 1972). Evolutionary trees can be constructed based on sequence similarity, since the phylogenetic difference between groups is to a certain extent reflected in the number of accumulated differences in amino acid residues between two homologous proteins (Jukes 1972; Klotz et al 1979; Goodman et al 1982). Such trees are generally constructed using the "maximum parsimony" method, in which the distance between two species is based on the minimum number of base changes (including, as

well, gene duplication and gene expression events) necessary to convert the protein chain of one species into that of another (Jukes & Holmquist 1972; Fitch 1976; Goodman et al 1982). The greater the number of changes needed, the greater the distance in relationship between the two species being compared, and the more distant in evolutionary history they diverged (Goodman et al 1982). Due to the fact that more than one evolutionary event may be required to change an amino acid codon, sequence similarity allows for the construction of a "relative" phylogenetic tree, with only a rough estimate of divergence distances, or real evolutionary time (Jukes & Holmquist 1972; McLaughlin & Dayhoff 1972; Fitch 1976). It has been postulated that protein sequence similarities can be used as a molecular "clock" to time evolutionary events, a theory based on the hypothesis that proteins evolve at a constant rate (Zuckerkindl & Pauling 1962; Wilson 1977). More recent research has indicated that this is not the case, that amino acid substitutions can occur at non-consistent rates, and that the evolution of molecules, as well as organisms, is guided as well by natural selection (Bogardt et al 1980; Goodman et al 1982). Yet even if sequencing and corresponding degrees of similarities cannot be used as evolutionary "clocks", they can be used to help establish closeness of relationship, ancestral lineage, order of appearance, and relative, but not absolute, time of divergence - in short, the relative evolutionary

history of a group (Jukes 1972; Dayhoff & Eck 1972; Fitch 1976; Klotz et al 1979; Goodman et al 1982).

Beginning with the determination of the sequence of sperm whale myoglobin by Edmundson (1965), homologous proteins of cetaceans have been sequenced by many workers for a variety of species, as illustrated in Table I. Although hemoglobin has been extensively sequenced for many terrestrial mammals, especially primates, it has not been the protein of choice for cetacean study. Myoglobin, instead, has been used, probably because it was much easier to obtain in large quantities, and its single chain of 153 residues proved to be quicker and easier to sequence than the four chains of hemoglobin (Lehninger 1982). Myoglobin itself is an oxygen-binding protein with a single heme group tightly bound to the polypeptide chain. Like hemoglobin, it reversibly binds oxygen, and functions mainly in the storage and transfer of oxygen from the blood to the tissues, specifically to the mitochondria of the cell (Dayhoff et al 1972; Kagen 1973; Lehninger 1982). It is found in exceedingly high concentrations in the muscle tissue of marine mammals, enabling these animals to remain submerged for long periods of time (Stryer 1975; Haley 1978). Extraction and purification of myoglobin from muscle is relatively easy, and sequencing is usually done through automatic Edman analysis, following enzymatic digestion (Rothgeb & Gurd 1978; Jones et al 1979a,b).

TABLE I
CETACEAN SPECIES AND THEIR RESPECTIVE
SEQUENCED PROTEINS

Myoglobin

Gray Whale	-	<u>Eschrichtius robustus</u>
Humpback whale	-	<u>Megaptera novaeangliae</u>
Fin whale	-	<u>Balaenoptera physalus</u>
Minke whale	-	<u>Balaenoptera acutorostrata</u>
Sei whale	-	<u>Balaenoptera borealis</u>
Amazon river dolphin	-	<u>Inia geoffrensis</u>
Common dolphin	-	<u>Delphinus delphis</u>
Bottlenosed dolphin	-	<u>Tursiops truncatus</u>
Spotted dolphin	-	<u>Stenella attenuata</u>
Pilot whale	-	<u>Globicephala melaena</u>
Killer whale	-	<u>Orcinus orca</u>
Dall's porpoise	-	<u>Phocoenoides dalli</u>
Harbor porpoise	-	<u>Phocoena phocoena</u>
Goose-beaked whale	-	<u>Ziphius cavirostris</u>
Hubb's beaked whale	-	<u>Mesoplodon carlhubbsi</u>
Sperm whale	-	<u>Physeter catodon</u>
Dwarf sperm whale	-	<u>Kogia simus</u>

α Crystallin

Minke whale	-	<u>Balaenoptera acutorostrata</u>
Harbor porpoise	-	<u>Phocoena phocoena</u>

Cytochrome c

Gray whale	-	<u>Eschrichtius robustus</u>
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Pancreatic Ribonuclease

Minke whale	-	<u>Balaenoptera acutorostrata</u>
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Source: Bogardt et al (1980) and Goodman et al (1982).

Since 1965, the sequences of myoglobin from a total of seventeen different species of cetaceans have been determined (see Table I). Comparisons have been made among the various sequences, and a similarity matrix, shown in Figure 2, has been constructed (Lehman et al 1980). From this matrix a phylogenetic tree for some of these seventeen species, shown in Figure 3, has been developed (Lehman et al 1980). A more complete tree utilizing this same data has been developed by Goodman et al (1982) (see Figure 4). Based on myoglobin sequencing, it appears that the phocoenids and delphinids form a closely related group, with the delphinids split into two separate lines. Both families are in turn more distantly related to Inia of the Platanistidae. In addition, the physeterids form a cohesive group, as do the ziphiids, both of which apparently diverged from the same stem group which in turn split earlier from the common line which later gave rise to the cohesive groupings of the mysticetes (Jones et al 1979a; Lehman et al 1980; Dwulet et al 1980; Goodman et al 1982). From cetacean myoglobin research, Jones et al (1979a) observed that the phylogenetic relationships determined from sequence similarity agreed in principle with that derived from paleontological studies, but that there were variations with respect to the relative timing of the many divergences. In addition, they noted that the residues close to the heme group are the ones most conserved, and that practically all the changes are compatible with the three dimensional structure

C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	A	
2	2	2	4	6	6	7	12	12	11	11	14	15	13	14	1	B
	0	0	4	4	4	7	14	14	13	13	16	17	15	16	1	C
		0	4	4	4	7	14	14	13	13	16	17	15	16	1	D
			4	4	4	7	14	14	13	13	16	17	15	16	1	E
				8	8	10	13	13	12	13	15	17	13	15	3	F
					0	7	14	14	13	13	16	15	15	16	5	G
						7	14	14	13	13	16	15	15	16	5	H
							7	7	8	10	10	13	14	17	8	I
								0	1	3	4	12	11	15	13	J
									1	3	4	12	11	15	13	K
										2	3	12	12	14	12	L
											3	14	14	16	12	M
												15	15	17	15	N
													16	15	16	O
														5	14	P
															15	Q

Key: A = killer whale, B = pilot whale, C = spotted dolphin
D = common dolphin, E = bottlenosed dolphin,
F = Black Sea dolphin, G = common porpoise,
H = Dall porpoise, I = Amazon River dolphin,
J = sei whale, K = gray whale, L = humpback whale
M = minke whale, N = finback whale, O = goosebeaked whale
P = sperm whale, Q = dwarf sperm whale

Figure 2. Cetacean difference matrix obtained by adding the number of different amino acids between pairs of proteins. Source: Lehman *et al* (1980).

Figure 3. Phylogenetic tree representing the possible relationship between three cetacean families as determined from myoglobin amino acid sequence data. Source: Jones *et al* (1979).

Figure 4. Phylogenetic tree of cetaceans determined from myoglobin amino acid sequences using the maximum parsimony method. Source: Goodman *et al* (1982).

of the protein as determined by X-ray crystallography of myoglobin (Kendrew 1961; Jones et al 1979a). Although it would appear that most of the responsible mutations are random and neutral, Bogardt et al (1980) have shown through multivariate statistical analyses that selection has probably acted to favor residue changes that maintain the conformation of the original myoglobin.

Myoglobin sequence analysis has been applied to other mammals besides cetaceans. Indeed Romero-Herrera et al (1973) have constructed a phylogenetic tree of the major orders of mammals, based on a synthesis of myoglobin sequence results and the fossil record. However, such myoglobin research does have its disadvantages, especially when done with cetaceans. It is time consuming unless automated, and when automated, it is quite expensive (Lehninger 1982; C. Head, personal communication 1986). It requires fresh muscle tissue, a limiting factor because the obtaining and/or importation of cetacean tissue from many species is prohibited in the United States (Sigma 1988), or available only by access to stranded animals. Unfortunately these carcasses are often considerably decayed when found. A substitute process which would make use of small amounts of partially degraded tissue, such as that obtained from many stranded cetaceans, would be desirable. A possible candidate is High Performance Liquid Chromatography (HPLC). This technique is a very sensitive, very fast, and relatively inexpensive separation process, which requires

micromolar amounts of sample, and which furthermore can contain a certain amount of impurities (Engelhardt 1979; Hancock 1986). HPLC separates a sample into its various constituents on the basis of their relative retention on, and elution from, a stationary phase, while being transported in a mobile phase. Detection and identification of the constituents occurs as they are being eluted (Engelhardt 1979; Yost et al 1980; Hupe 1985; Hancock 1986; Harris 1986). Theoretically, if myoglobin could be broken down into a mixture of its constituent amino acids, the mixture separated by HPLC, and these constituents identified, then sequencing of the protein could be pursued without the preceding drawbacks.

This, however, proves not to be as easy as it first appears. There is no enzyme or process which breaks all the peptide bonds of myoglobin, and produces its 153 separate residues in a mixture suitable for introduction into HPLC. This requires many enzymes and cleavage processes, resulting in products which may not all be compatible with a particular HPLC system (Jones et al 1979a; Lehman et al 1980; Bhowen & Bennett 1986). Only Edman degradation allows for the cleaving of a single amino acid residue at a time, but then only from small protein fragments, or peptides (Stryer 1975; Lehninger 1982). Even if a mixture of the 153 residues of myoglobin could be completely separated by HPLC, the residues would not be eluted in the order in which they are arranged in the

polypeptide chain; rather they would be eluted on the basis of their relative hydrophobicity. Therefore the results obtained from such a separation would be more of an amino acid composition comparison, rather than an amino acid sequence (Lottspeich & Henschen 1985). However, it has been demonstrated that HPLC is very effective in separating and purifying peptides, such as those necessary for Edman degradation, and that these peptides can be identified on the basis of their amino acid composition, and their corresponding relative retention times (Browne et al 1982; Sasagawa et al 1982; Lottspeich & Henschen 1985; Bhowm & Bennett 1986; Prestidge 1986). Therefore it should be possible to separate and identify a complex mixture of myoglobin peptides, and use this data to characterize the protein for the individual or species from which it came. Indeed such a technique utilizing HPLC has been practiced for a decade in the identification of hemoglobin variants in humans (Schroeder 1986). In this procedure hemoglobin is taken from the blood, and enzymatically digested by trypsin for a certain length of time. This enzyme cleaves the globin chains on the carbonyl side of each lysine and arginine residue, producing a complex mixture of tryptic peptides (Zubay 1983). This mixture is then passed through an HPLC system, where each fragment is selectively retained on a column, based on its relative hydrophobicity (Engelhardt, et al 1985). An organic polar solvent is then passed through the column, eluting the

peptides in the order in which they were retained. The fragments are each detected by an ultraviolet detector, and plotted on an integrator-plotter (Lottspeich & Henschen 1985). The resulting trace shows a series of peaks, each of a certain defined area and retention time. Such a trace, referred to as a "fingerprint" after Ingram (1958), who first separated peptides with paper chromatography, is also known as a "peptide map" for that protein. This map can be compared to one for hemoglobin taken from another individual, or to a reference map. Hemoglobin variants, which may demonstrate pathologic conditions, can thus be identified through such "peptide mapping" (Schroeder et al 1979; Hancock 1986; Schroeder 1986). In addition, it has recently become possible to predict the relative retention times of peptides of less than twenty residues by applying a mathematical equation, which utilizes retention coefficients, based on the hydrophobic contribution to retention of each individual amino acid of the peptide (Meek 1980; Meek & Rossetti 1981; Browne et al 1982). Thus not only can a peptide map be generated by HPLC, but each peptide which is represented by a peak can be identified with its constituent amino acid composition and sequence to a great degree, especially if the sequence of the protein has been determined for that particular species (Sasagawa et al 1982).

As with hemoglobin, it should be possible to utilize such peptide mapping in the study of cetacean myoglobin.

Muscle tissue from an individual recognized as a cetacean could be digested with trypsin, processed through HPLC, and a map obtained. With many such maps a "peptide atlas" of myoglobin could be constructed, made up of the peptide maps taken from as many cetacean species as possible. Such a mapping process could be used to construct similarity matrices and phylogenetic trees for the order. If relative peptide map relationships prove to match those estimated from myoglobin sequence analysis for those species for which the myoglobin sequence is known, then the use of peptide mapping opens the possibility of considerably expanding phylogenetic myoglobin analysis to a number of species for which it has not been feasible to do sequence analysis due to cost or availability of adequate samples. As peptide mapping is relatively quick and inexpensive, more than one animal per species could be evaluated, a significant improvement on sequence analysis, where sequences for a species are often based on only one animal. Peptide mapping could also be used to help answer the question as to the existence of population differences in myoglobin characteristics, similar to those found for hemoglobin, and whether any differences vary consistently with differences in diving behavior (Hedrick et al 1986). In addition, peptide mapping could provide a very quick and effective screening method for unknown cetacean muscle samples, as is needed in forensic investigations, rapidly and inexpensively identifying an animal to family, and possibly

genus and species, based on its comparison to the maps of known individuals already present in the peptide atlas. In short, HPLC and peptide mapping should prove to be a useful technique for the further elucidation of the order Cetacea.

CHAPTER III

GOALS OF THE RESEARCH

There are two goals of this research. The first is to use HPLC to generate chromatograms from as many cetacean species as possible for the construction of a myoglobin peptide atlas. The second goal is to use this peptide atlas to answer the following questions:

1. What are the phylogenetic relationships of the cetacean species sampled as determined from myoglobin peptide mapping?
2. How do these relationships compare with those determined from myoglobin amino acid sequence analysis, paleontology, and other biochemical techniques?
3. Are there any intraspecific population differences indicated from the myoglobin peptide maps?
4. Is myoglobin peptide mapping a valuable technique for the screening of cetacean muscle samples, and one useful in the study of cetacean evolution?

CHAPTER IV

MATERIALS AND METHODS

SAMPLES

Frozen muscle tissue of various species of cetaceans was obtained from the collection of Dr. D. Duffield of Portland State University; Portland, Oregon. These tissue samples had been collected by her over the previous ten years, and had their origin in various localities, including the eastern North Pacific, the Gulf of Mexico, and the western North Atlantic. Frozen muscle tissue of the Hawaiian monk seal was obtained from the U.S. National Marine Fisheries Service; Honolulu, Hawaii, and that of the harbor seal was obtained from Washington Park Zoo; Portland, Oregon. Purified, lyophilized horse myoglobin was obtained from Sigma Chemical Co.; St. Louis, Missouri. A list of the samples tested, and their sources is given in Table II.

MATERIALS AND EQUIPMENT

Extraction and purification of myoglobin was performed with the use of reagent grade chemicals from J.T. Baker Chemical Co.; Phillipsburg, New Jersey. These included ammonium sulfate, sodium phosphate monobasic and dibasic,

TABLE II

SAMPLES TESTED, AND THEIR RESPECTIVE SOURCE, SITE
OF ORIGIN, AND STATUS WHEN COLLECTED

Sample	Species	Source	Origin	Status
Horse	<u>Equus caballus</u>	Sigma	St. Louis	
HS1	<u>Phoca vitulina</u>	Chemical	Mo.	
MS1	<u>Phoca vitulina</u>	Wash. Park Zoo	Oregon Coast	Stranded
	<u>Monachus schauinslandi</u>	NMFS	Hawaii	Captive
K1	<u>Kogia simus</u>	Sea World	Florida	Stranded
K2	<u>Kogia breviceps</u>	Sea World	Florida	Stranded
K3	<u>Kogia breviceps</u>	Sea World	Florida	Stranded
K4	<u>Kogia simus</u>	Sea World	Calif.	Stranded
K5	<u>Kogia breviceps</u>	Sea World	Florida	Stranded
P3	<u>Phocoena phocoena</u>	PSU	Oregon	Stranded
D3	<u>Delphinapterus leucas</u>	Sea World	Canadian Arctic	Captive
PW1	<u>Globicephala sp.</u>	Sea World	Calif.	Captive
PW2	<u>Globicephala macrorhynchus</u>	Okeanos Foundation	New York	Stranded
KW1	<u>Orcinus orca</u>	Sea World	British Columbia	Captive
KW2	<u>Orcinus orca</u>	OSU Marine Sci.Center	Oregon Coast	Stranded
PC1	<u>Pseudorca crassidens</u>	Sea World	Japan	Captive
U1	Unknown	Unknown	Unknown	Unknown
T1	<u>Tursiops truncatus</u>	Sea World	Gulf of Mexico	Captive
T2	<u>Tursiops truncatus</u>	Sea World	Gulf of Mexico	Captive
L1	<u>Lagenorhynchus obliquidens</u>	Sea World	Calif.	Captive
L2	<u>Lagenorhynchus acutus</u>	Okeanos Foundation	New York	Stranded
S1	<u>Stenella coeruleoalba</u>	Okeanos Foundation	New York	Stranded
LT1	<u>Lagena-Turs. hybrid</u>	Sea World	Calif.	Captive born
C1	<u>Cephalorhynchus commersonii</u>	Sea World	Peru	Captive
C2	<u>Cephalorhynchus commersonii</u>	Sea World	Peru	Captive

EDTA, and potassium ferricyanide. Purification also involved the use of a 6mm x 45mm glass ion exchange column packed with CM Sepharose CL-6B from Pharmacia, Inc.; Piscataway, New Jersey, while concentration of the purified solution was accomplished with the use of polyethylene glycol from EM Science; Gibbstown, New Jersey. Tryptic digestion of the purified myoglobin utilized TPCK-treated bovine trypsin from Sigma Chemical Co., along with reagent grade ammonium bicarbonate, sodium hydroxide, and acetic acid from J.T. Baker Chemical Co. HPLC analysis of the tryptic digest was performed using a Beckman-Altex gradient liquid chromatograph system (Beckman Instruments, Inc.; Berkeley, California). This consisted of two Model 110A solvent metering pumps, gradient mixing chamber, Model 210A sample injection valve with 25 μ l loop, Model 165 variable wavelength detector, Model C1-A integrator plotter, all controlled with a Model 420 system controller. Injections were performed using a Hamilton 100 μ l syringe (Hamilton Co.; Reno, Nevada) with a blunt tip removable needle. The actual HPLC separations were performed on a Vydac Model TP54 reversed phase C-18 protein/peptide column from The Separations Group; Hesperia, California, preceded by an Upchurch Model 1602 Uptight guard column (Upchurch Scientific Co.; Oak Harbor, Washington). The buffer system employed utilized trifluoroacetic acid, acetonitrile, water, and methanol, all HPLC grade from J.T. Baker Chemical Co.

EXPERIMENTAL PROCEDURE

Myoglobin was extracted from each muscle sample following the method of Rothgeb and Gurd (1978) with some modifications. Each sample was thawed for 1-2 hours, until soft enough to slice. It was then cut into cubes approximately 1 cm on each side, and placed into a solution of 70% ammonium sulfate, 20% 0.1M sodium phosphate buffer (pH 6.5), and 10% EDTA. This muscle mixture was then ground in a Waring Blendor at 4°C until a homogenous suspension was obtained. The suspension was then centrifuged for one hour at 4°C at 12,100g. The supernatant, which consisted mainly of crude oxymyoglobin, was then collected and filtered. The oxymyoglobin was converted into crude ferrimyoglobin by the addition of a molar excess of potassium ferricyanide for ninety minutes at 4°C. This solution was then dialyzed against distilled water for 3-4 days at 4°C, with many changes of water. The preparation was then centrifuged again at 12,100g for thirty minutes at 4°C, followed by collection and filtration of the supernatant. The supernatant was then loaded into a Sepharose ion exchange column for purification. The column had been equilibrated with 0.1M sodium phosphate buffer (pH 6.3-6.5), and was developed with the same buffer over a period of 4-5 hours at 4°C. The major fraction (dark band IV) was collected, usually amounting to about 5 ml. This was then concentrated against polyethylene glycol for one hour at 4°C.

The result was a concentrated reddish-brown solution of purified ferrimyoglobin of about 1 ml volume.

This solution was then subjected to tryptic digestion using the method of Schroeder (1986) for hemoglobin. First, 0.1 ml of 0.8M ammonium bicarbonate was added to the solution, and the pH adjusted to 8 through the addition of 1M sodium hydroxide. Trypsin was then added at a concentration of 3 mg/ml of solution. The solution was then incubated at 37°C for 24 hours, at which time another 2 mg/ml of trypsin was added. Incubation was then continued for another 48 hours, for a total digestion time of 72 hours. Digestion was halted with the addition of 3 drops of 5M acetic acid per ml of solution.

The tryptic digest of ferrimyoglobin was then subjected to HPLC analysis. This was performed utilizing two buffers, one consisting of 0.1% trifluoroacetic acid and water, and a second consisting of 0.1% trifluoroacetic acid and acetonitrile. The digest was loaded into the sample injection loop, and the chromatography begun. Sample injection occurred at one minute elapsed time, and a gradient of increasing TFA/acetonitrile concentration was started at 5 minutes elapsed time. This gradient was run for 45 minutes, held constant at 100% for 10 minutes, and then decreased to zero in 10 minutes. Flow rate was 1 ml/min. A graphic representation of the gradient is shown in Figure 3. Eluent was monitored at 220 nm and 2.0 AUFS. for 70 min beginning at time zero. The absorbance at 220 nm of peptides, partially

Figure 5. Change in acetonitrile concentration over time.

digested fragments, impurities, and proteins (myoglobin and trypsin) was integrated and plotted, and the resulting chromatograms documented in the following chapter. All eluent was discarded. The entire system was re-equilibrated with the TFA/water buffer prior to each sample run, and stored in methanol when not in use.

The horse myoglobin, purchased already purified, was not subjected to the extraction and purification segments of this procedure, only the tryptic digestion and chromatographic analysis. As a control, trypsin was also subjected to digestion and chromatography, in order to determine the amount of autodigestion of the enzyme in the absence of myoglobin, and the elution position of any resulting peptides. A detailed, stepwise description of the experimental procedure is given in Appendix B.

ANALYSIS OF RESULTS

The peaks of each chromatogram obtained were plotted as bars along a vertical time axis, with a bar placed at the time which matched the elution time of its corresponding peak. All peaks less than 2 mm in height and below a value of 200 area units were considered artifacts, and therefore not included in the analysis. A "bar chart" was thus produced for each sample. The bar charts were individually compared with one another, and the number of differences in retention times tabulated in a difference matrix. The values in the matrix

were then used in the maximum likelihood computer program of Felsenstein (1981) to develop a phylogenetic tree for the species represented by the samples. Visual comparison of the chromatograms was also used to detect overall similarities. The relative order of elution of detectable peptides was determined using the retention coefficients of Sasagawa et al (1982), and an attempt was made to identify respective peaks in the chromatograms with amino acid sequences of those species for which these myoglobin sequences have been determined.

CHAPTER V

RESULTS

CHROMATOGRAMS

Chromatograms for 24 different samples are shown in Figures 7 through 19. The samples consisted of muscle tissue originating from cetaceans identified as Kogia breviceps (N=3), Kogia simus (N=2), Delphinapterus leucas (N=1), Phocoena phocoena (N=1), Globicephala macrorhynchus (N=1), Globicephala of unknown species identity (N=1), Orcinus orca (N=2), Pseudorca crassidens (N=1), Cephalorhynchus commersonii (N=2), Tursiops truncatus (N=2), Lagenorhynchus obliquidens (N=1), Lagenorhynchus acutus (N=1), Lagenorhynchus-Tursiops hybrid (N=1), Stenella coerueoalba (N=1), and one tissue sample of unknown identity (N=1). The samples also included muscle tissue from the harbor seal, Phoca vitulina (N=1) and the Hawaiian monk seal, Monachus schauinslandi (N=1), as well as myoglobin from the horse Equus caballus (N=1). Retention time on the chromatograms increases from left to right. All myoglobin peptide peaks occurred between elution times of 19 to 32 min. Undigested myoglobin eluted at approximately 47 min, while undigested trypsin eluted at approximately 56 min. Any unretained impurities in the sample solution, such as

acetic acid, eluted at the beginning of each run, usually at approximately 3.5 min. Due to the length of digestion of the samples necessary in this procedure, it was very likely that the trypsin used to produce myoglobin peptides was also partially digested. To determine the retention times of any peptides produced by this autodigestion, trypsin alone was digested for 72 hours, and subjected to chromatographic separation. The resulting trace is shown in Figure 6. Arrows placed in the chromatograms are explained in the discussion chapter.

Figure 6. Chromatogram of trypsin autodigestion.

Figure 7. Chromatogram of sample H1, Equus caballus.

Figure 8. Chromatograms of samples HS1, *Phoca vitulina* (top) and MS1, *Monachus schauinslandi* (bottom).

Figure 9. Chromatograms of samples K1 (top) and K4 (bottom), both Kogia sinus.

Figure 10. Chromatograms of samples K2 (top) and K5 (bottom), both Kogia breviceps.

Figure 11. Chromatogram of sample K3, Kogia breviceps.

Figure 12. Chromatograms of samples P3, *Phocoena phocoena* (top) and D3, *Delphinapterus leucas* (bottom).

Figure 13. Chromatograms of samples PW1, *Globicephala* sp. (top) and PW2, *Globicephala macrorhynchus* (bottom).

Figure 14. Chromatograms of samples KW1 (top) and KW2 (bottom), both Orcinus orca.

Figure 15. Chromatograms of samples PC1, *Pseudorca crassidens* (top) and U1, unknown species (bottom).

Figure 16. Chromatograms of samples T1. (top) and T2, (bottom), both Tursiops truncatus.

Figure 17. Chromatograms of samples L1, Lagenorhynchus obliquidens, (top) and L2, Lagenorhynchus acutus (bottom).

Figure 18. Chromatograms of samples S1, Stenella coeruleoalba, (top) and LT1, Lagenorhynchus-Tursiops hybrid, (bottom).

Figure 19. Chromatograms of samples C1 and C2, both Cephalorhynchus commersonii.

BAR CHARTS

In Figures 20 and 21 the myoglobin peptide peaks are represented by a series of bars. Each bar indicates the relative elution position of its respective peak. The composite of bars for each individual represents its bar chart. Retention time increases from the bottom of the bar chart to the top. In order to simplify the analysis of the bar charts, and in accordance with procedures indicated in the literature, (Dalla Libera et al 1983; Schroeder 1986), a bar calibration of 0.3 min was used; i.e., if two peaks on two different sample chromatograms eluted within 0.3 min, they were placed at the same level on the bar chart. If they eluted 0.3-0.6 min apart, they were placed one level apart on the bar chart, and it was assumed that a shift in elution position of the peptide had occurred. If the two peaks eluted at positions greater than 0.6 minute apart, they were placed at their respective levels on the bar chart, and assumed to represent two separate peaks, and two different peptide segments. The bar charts were used for a detailed comparison of the chromatograms, and to compile a difference matrix.

DIFFERENCE MATRIX

The number of differences in retention times of corresponding peaks between the chromatograms of all pair-wise samples were tabulated from comparisons of the bar charts, and arranged in the form of a difference matrix (Figure 22). The absence of any given peak was counted as a difference. Two bars at one level apart were considered to be a single difference. Two bars at more than one level apart were considered to represent two differences; i.e., one peak absent in one chromatogram, and one absent in the other. The difference matrix was then used to construct a phylogenetic tree for the species sampled in this study.

HS 1	MS 1	K 1	K 2	K 3	K 4	K 5	P 3	D 3	PW 1	PW 2	PC 1	KW 1	KW 2	U 1	T 1	T 2	LT 1	L 1	L 2	S 1	C 1	C 2	
8	11	16	13	16	14	13	11	11	10	9	10	9	8	9	13	14	13	14	13	12	11	11	H1
	10	15	14	13	12	12	11	9	10	9	10	10	10	10	9	11	12	10	9	9	11	12	HS1
		12	15	17	12	13	14	11	10	9	10	11	11	11	10	9	11	11	11	12	12	12	MS1
			6	8	6	7	13	11	10	11	10	13	13	13	12	12	13	11	12	10	9	14	K1
				10	4	7	13	13	11	10	11	9	8	9	9	11	11	12	12	13	11	12	K2
					8	8	13	11	9	9	9	12	10	12	11	11	11	9	9	10	9	11	K3
						6	14	11	11	11	11	13	11	13	13	12	10	9	11	10	11	12	K4
							14	12	10	10	10	12	11	12	11	10	12	10	10	11	12	12	K5
								3	7	6	7	5	7	5	5	7	9	9	6	8	8	10	P3
									6	6	6	6	7	6	5	6	8	7	4	5	7	9	D3
										1	0	3	3	3	2	3	7	4	4	3	1	4	PW1
											1	2	3	2	1	1	7	4	3	4	2	4	PW2
												3	3	3	2	2	7	4	4	3	1	4	PC1
													3	0	2	3	7	5	5	5	4	6	KW1
														3	4	4	6	4	6	5	4	3	KW2
															2	3	7	5	5	5	4	6	U1
																2	8	5	4	5	3	5	T1
																	8	5	4	5	3	3	T2
																		4	8	6	8	7	LT1
																			5	3	5	6	L1
																				3	5	7	L2
																					4	7	S1
																						5	C1

Figure 22. Difference matrix compiled by tabulating the total number of differences between the respective chromatograms.

PHYLOGENETIC TREE

A phylogenetic tree for the sampled species was constructed using the "maximum likelihood" genetic distance computer program of Felsenstein (1981). The tree was developed using the values from the difference matrix. Visual comparisons of the chromatograms were also used when relationships produced by the program were questionable. The distance between species represented by samples utilized in this study approximate the magnitude of differences between the samples, and presumably the amount of evolutionary distance between the species represented as determined by peptide mapping.

RELATIVE RETENTION ORDER OF PEPTIDES

The relative order of elution of myoglobin tryptic peptides was determined for samples from species for which the amino acid sequence is known. This was done by adding the retention coefficients as determined by Sasagawa et al (1982) of the amino acids making up each peptide. The exact retention times could not be ascertained for these peptides using the method of the above authors, as the apparatus, flow rate, and segments of the linear gradient in this research were different from those used by Sasagawa et al. The sums of the retention coefficients for the six known species, as well as for Globicephala melaena and Stenella attenuata, are given in Table III. Each peptide is listed by its residue span and by a number. The chromatograms for the samples of each of these species, and for Globicephala macrorhynchus are repeated in Figures 24 through 29, and the probable peptide corresponding to each peak is indicated with the peptide number.

TABLE III

RELATIVE ORDER OF ELUTION OF PEPTIDES FROM
SUMMATION OF RETENTION COEFFICIENTS

Pep- tide	Resi- due	Species							
		A	B	C	D	E	F	G	H
1	1-16	9.65	10.61	11.66	10.80	11.27	11.27	11.27	11.27
2	17-31	7.16	7.11	7.99	6.95	7.95	6.95	7.95	6.95
3	32-34		3.10	3.10	3.10	3.10	3.10	3.10	3.10
4	35-42	{32-42} 3.16	3.05	3.64	3.09	3.09	3.09	3.09	3.09
5	43-45	1.86	2.07	2.07	1.86	1.86	1.86	1.86	1.86
6	46-47	1.76	1.76	1.76	1.76	1.76	1.76	1.76	1.76
7	48-50	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73
8	51-56	1.69	1.76	1.75	1.69	1.52	1.52	1.52	1.52
9	57-62	{58-62} 2.07	2.15	2.07	2.07	2.07	2.07	2.07	2.07
10	64-77	7.58	6.75	7.49	6.75	6.66	6.66	6.66	6.66
11	80-87	2.96	2.96	2.96	2.79	2.79	2.79	2.96	2.79
12	88-96	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13
13	97-98	.39	.39	.39	.39	.39	.39	.39	.39
14	99-102	3.29	3.29	3.29	3.29	3.29	3.29	3.29	3.29
15	103-118	11.73	11.90	12.11	12.11	12.11	12.11	12.11	12.11
16	119-133	{119-132} 4.61	9.65	5.13	4.67	4.76	4.67	4.67	4.67
17	134-139	5.05	5.05	5.05	5.05	5.05	5.05	5.05	5.05
18	141-145	{140-145} 1.34	1.34	1.79	1.78	1.79	1.79	1.79	1.79
19	146-147	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28
20	148-153	4.93	4.91	4.45	4.91	4.91	4.91	4.91	4.91

Residues 63,78,79 in all samples, 140 in cetaceans, and 133 in Phoca are free lysines with order value of .05; residue 57 in Phoca is a free arginine with order value of .26.

TABLE III

RELATIVE ORDER OF ELUTION OF PEPTIDES FROM
SUMMATION OF RETENTION COEFFICIENTS
(continued)

Species: A = Equus caballus
B = Phoca vitulina
C = Kogia simus
D = Phocoena phocoena
E = Orcinus orca
F = Tursiops truncatus
G = Globicephala melaena
H = Stenella attenuata

Figure 24. Chromatogram of sample H1, Equus caballus, indicating probable peptides.

Figure 25. Chromatogram of sample HS1, Phoca vitulina, indicating probable peptides.

Figure 26. Chromatogram of sample K4, Kogia simus, indicating probable peptides.

Figure 27. Chromatogram of sample P3, Phocoena phocoena, indicating probable peptides.

Figure 28. Chromatograms of samples KW1, *Orcinus orca*, (top) and T1, *Tursiops truncatus*, (bottom), indicating probable peptides.

Figure 29. Chromatogram of sample PW2, Globicephala macrorhynchus, indicating probable peptides.

CHAPTER VI

DISCUSSION AND CONCLUSIONS

In analyzing the results of this research it is important to recognize a limitation in the use of the difference matrix for determining distances and developing relationships. This matrix tabulates all the differences between each sample; however, it makes no distinction as to the magnitude of these differences. For example, there may be seven differences between two samples, and nine between another two. On face value, one could conclude that the first two species are more closely related (fewer differences in myoglobin primary structure) than the second two species. Yet if the nine differences were merely small shifts in relative retention times of peaks, while the seven differences included one or two missing major peaks, then the first two species would in reality have more differences between them, and would be more distantly related than the second two species. Shifts in retention times can occur with minor solvent or mobile phase contamination, aging of the chromatographic column, and other instrumentation problems, as well as with increasing concentration of the organic modifier, e.g., acetonitrile, especially at concentrations above 40%, where retention times begin to increase rather than decrease as expected (Lottspeich

& Henschen 1985; Engelhardt 1986). In fact, it is well understood that retention times, even under identical conditions, are difficult to duplicate (Krstulovic & Brown 1982). Therefore, differences due to shifts, especially minor ones, may not represent peptide differences such as those indicated by missing or extra peaks (or major shifts, if they can be identified).

It should also be recognized that a complete digestion of cetacean myoglobin by trypsin should result in twenty different residues, and therefore twenty separate peaks. The rationale behind this lies in the fact that trypsin cleaves the peptide bond on the carboxyl side of the basic amino acids lysine and arginine. However, it will not cleave at these points if the residue at the amino end of the peptide bond is proline (Wood et al 1981). Cetacean myoglobin has 23 lysine and arginine residues, thus trypsin digestion should produce 24 peptides. Four of these peptides, however, are single lysine residues, which together would produce one peak, while the lysine residue at position 87 is followed by a proline, and no cleavage would take place at this point, resulting in a single polypeptide from residues 80 to 96 (Kagen 1973; Zubay 1983). Even the best protocols usually give a maximum of only sixteen peaks, as the free lysines, as well as the di- and tripeptides are seldom detected (Caprioli et al 1987). Some of the chromatograms produced here, especially from Phocoena, Delphinapterus, and the delphinids, exhibit more than this

number. These extra minor peaks probably represent incompletely digested protein fragments (Rubenstein et al 1979), impurities, and degradation products, the last resulting from the fact that many of the samples came from animals which had stranded and were already in the process of decay. As mentioned previously, trypsin probably also autodigests, but in the presence of such overwhelming amounts of myoglobin (usually in excess of 100 to 1), the amount of trypsin autodigestion products would be relatively small, and any peaks produced by these would be minor. Since minor extra peaks probably represent autodigestion products, impurities, and protein fragments, they have been ignored, although it cannot be absolutely determined from the techniques used what each minor peak does represent. Some major peaks may also represent impurities, which may be the case in sample P3, but because these cannot be identified for certain, they have been counted and tabulated as part of the difference matrix, probably resulting in a greater number of differences than are actually present. For these reasons, comparative analysis of the chromatograms also required a comparison of the general pattern of the chromatogram of one species to that of another. This additional technique can make allowances for minor peaks, and detect major differences. Therefore, the following interpretation of these chromatographic results will make use of both the difference matrix, and the overall pattern of each chromatogram.

Chromatographic comparisons apply only to retention times, as this reflects the amino acid composition of the peptides (Meek & Rosetti 1981). Peak height and area merely indicate the concentration of each peptide in the sample (the greater the amount, the greater the height and area of the peak corresponding to this peptide), although, to a lesser extent, they also can give an indication of the number of aromatic residues in any given peptide (Schroeder et al 1979). In tryptic digests peak height and area can vary depending on the course and completeness of the digestion, and the potential degradation of the sample; this, coupled with the fact that it is rare for any compound to exhibit an exact equal trace or repetitive HPLC analysis, was the reason that only differences in retention times were used in the comparison of these chromatograms, and differences in peak heights and areas were ignored (Yost et al, 1980).

The relative order of elution of the tryptic peptides was tabulated in order to try to determine the identity of each of the chromatographic peaks. If this could be done, then any shift in or absence of a peak could give an indication of the location of the changes which occurred in the primary sequence of myoglobin for those species which had not been sequenced. However, this procedure also has its limitations. The relative order was determined through the addition of the retention coefficients of Sasagawa et al (1982), and not through the use of these coefficients with the necessary

equations for the ascertaining of absolute retention times. The reason for this was that the instrumentation and gradient used in this research were similar to, but not exactly the same as that used by Sasagawa et al. Because of this, the retention times calculated by these authors cannot be exactly correlated with those obtained here (Browne et al 1982; Sasagawa et al 1982). The literature does not appear to contain reports of retention coefficients determined using conditions as in this research (Blackburn 1986). However, addition of coefficients determined from a procedure closest to the one used here should produce a good approximation of the relative order of elution of peptides, and this should provide a means of making a tentative identification of peaks. The fact that sequences are generally determined from only one individual of a species, and from fresh, non-degraded tissue, also puts a limitation on such attempted identification.

As a point of reference and a check on technique, purified lyophilized myoglobin from the horse, Equus caballus, was chromatographed. An examination of this trace (Figure 7), as well as the bar chart and difference matrix, shows a pattern quite different from those of the cetaceans. Apart from the three peaks marked by the lower arrows, which appear in all the traces except two, and apparently represent highly conservative peptides, or ones in which amino acid changes result in very similar retention times, the horse chromatogram exhibits a totally different pattern. This would be expected

from an animal from a different order, *Perrisodactyla*, with nineteen amino acid differences in its myoglobin compared to that of the sperm whale (Kagen 1973). It was initially hoped to use purified sperm whale myoglobin as an additional point of reference; this, however, was not possible due to a ban on the importation of sperm whale products by the U.S. government (Sigma, 1988). In addition to the horse, chromatograms were produced for the myoglobin of the harbor seal, *Phoca vitulina*, and the Hawaiian monk seal, *Monachus schauinslandi* (Figure 8). The patterns produced were also unique, with a large number of differences. This would be expected, since these animals are also from a different order, *Pinnipedia*, with the harbor seal exhibiting twenty-six amino acid substitutions from that of the sperm whale (Kagen, 1973). The chromatograms of all three species provided "out groups" against which comparisons with the cetacean samples could be made.

Figures 24 and 25 show that the attempt at identification of peaks in the chromatograms of the horse and the harbor seal appears to be successful. As an example, peptide 16 in the horse has a relative retention value of 4.61, while in the harbor seal it has a value of 9.65. The peaks so labeled exhibit this change in elution position. Likewise, peptide 1 has a value of 9.65 in the horse, and 10.61 in the harbor seal; this is illustrated by the large peak of a later elution time on the trace of this latter species in comparison to its placement on that of the horse.

All the seventeen peaks on the chromatograms of these two species, with the exception of one early peak on the horse, can be identified with a fair degree of confidence, but characterization of the amino acid composition of each peak eluant would be necessary for certain identification.

It is apparent from an analysis of the general pattern of the chromatograms from the order Cetacea, as well as an examination of the difference matrix, that there are two main subdivisions in the species examined. The five representatives of Kogia, samples K1-K5, have similar general patterns, which in turn are different from the rest of the samples. Indeed, the number of differences almost delineates the Kogia as an "out group" for the rest of the cetacean samples. The three peaks present early in the chromatograms, and marked with the lower arrows (see Figures 9-11), appear to be distinctive in Kogia, and not present in the same place in any of the other samples, including Equus, Phoca, and Monachus. These peaks appear to represent a "signature" for the two species of Kogia, indicating that a chromatogram bearing these distinctive peaks is from an individual of the subfamily Kogiidae. Peptide map analysis apparently can thus be used to screen out this group from the rest of the cetaceans. Table III and Figure 26 indicate that peptides 5, 6, 7, 8, 9, and 19 may be responsible for this signature. In addition, there are fewer total differences between the five Kogia samples than between the Kogia and any of the other samples. It

therefore appears that the Kogia chromatograms form a group, which is different from the rest of the cetaceans tested. This would be expected from the current taxonomical placement of this genus with Physeter in the family Physeteridae, while the rest of the cetaceans are placed in other families. This peptide separation of the Kogia into a distinctive group is further was to be expected from the fact that the amino acid sequence of Kogia myoglobin differs in thirteen to fifteen residues from that of the rest of the cetaceans tested in this research for which sequencing has been done (Dwulet et al 1977; Jones et al 1980).

Even though the samples from the genus Kogia form a separate group, further examination of the results indicate that it is a diverse assemblage, and one in which two lines can be discerned. The difference matrix shows that K2 and K4 appear to be the most closely related, with K1 and K5 more distant. Indeed, the chromatograms show that the pattern of K2 is the most similar to that of K4, and somewhat less so to those of K1 and K5. K5 is missing a peak, indicated by the upper short arrow (Figure 10), which is present in the chromatograms of the other Kogia samples, while at the same time it exhibits an extra peak (upper long arrow) present only in sample K3. Samples K2 and K4 have similar patterns, and this, together with the difference tabulation, suggests that these two samples are more similar in their myoglobin than to the myoglobin of the other samples. This contrasts with the

fact that K2 was identified as Kogia breviceps, the pygmy sperm whale, while K4 was identified as Kogia simus, the dwarf sperm whale. The chromatographic comparison would suggest that both individuals are of the same species. It is interesting that K2 was an Atlantic animal, while K4 was a Pacific whale. The similarity in peptide maps would suggest that if the samples were not misidentified, that these two populations of the two different species may be quite similar. It is also possible that one of the animals may have been misidentified; field determination of Kogia species is very difficult, especially with younger, stranded animals (Haley 1978).

Sample K1 shares the most similarity with K2 and K4. This agrees with the identification of K4 as a simus individual, but not with K2 being a breviceps animal. The case with K1 is further complicated by the fact that this individual was a calf, while the rest of the Kogia samples were adults, with a corresponding lower yield of myoglobin, which in turn would lead to an absence of some peptides detectable in samples with more myoglobin, and more apparent observation of impurities inherent in the tissue of a stranded animal. Sample K5 seems to be less similar to these previous three, especially with the missing and additional peaks. What can be deduced from all this is that K2 was probably a simus animal misidentified as a breviceps, and that the differences between it and K4 are indicative of population differences in the species. The differences between K1 and K2 are probably

due to the differing myoglobin yields. The greater number of differences between these first three and K5 probably corresponds to the differences present between species. Figure 26 indicates that these changes probably reside in part in peptides 1, 10, and 16.

Sample K3, identified as an Atlantic Kogia breviceps, is somewhat of an anomaly (see Figure 11). Although not exhibiting a great number of differences when compared with the other Kogia, its chromatogram does not have the same general appearance. The myoglobin from this sample came from the heart of the animal rather than from the skeletal muscle, and the resulting trace shows some extra peaks, which might possibly be spurious, and which may appear in traces taken from cardiac muscle. The whole pattern seems to be most similar to K4 and K5, and somewhat less so to K2, but it is not possible from this trace to place the animal conclusively as Kogia simus or Kogia breviceps. A chromatogram of myoglobin from skeletal muscle of this individual would be needed to shed more light on this, as it is very possible that direct comparisons between skeletal and cardiac myoglobin are not valid. It is probably safe to say that K3 is in the genus Kogia, but its relationship to the other K samples is questionable.

Of the second main subdivision indicated by the chromatograms, a separation can be made into two groups. Based on both the difference matrix and the general patterns, it

appears that samples P3 and D3 make up one group, while the rest of the samples make up a second group. This second group is characterized by four peaks, indicated by the lower short arrows on Figures 13 through 19, while P3 and D3 have only three (lower short arrows), as indicated on Figure 12. This agrees with the fact that all of the samples of the second group are identified as members of the family Delphinidae; in fact, it appears that the arrangement of these four peaks is characteristic of the delphinids, probably their peptide map "signature". The grouping of P3 and D3, however, is a surprise, given species and family designations. Not only are the general patterns very similar, but the chromatograms are different only in three peaks, two of which are present on that for P3, and absent or small on that for D3, while one is absent on that for P3 and present on that for D3. These three peaks or their absence are indicated by the three upper long arrows on the traces of P3 and D3, respectively (see Figure 12). The peak early in the chromatogram of P3, which is absent on that for D3, probably represents peptide 13, present in too low a concentration in D3 to be detected, while the small peak on D3 which was not counted probably represents peptide 8. The large peak near the end of the D3 trace, and absent on the trace for P3, very possibly represents a trypsin autodigestion peptide, as the relative order of elution does not lead to its identification (see Figure 27). In any event, P3 and D3 are very similar, and closely related. What is surprising is that

P3 came from a harbor porpoise, Phocoena phocoena, while D3 came from a beluga, Delphinapterus leucas. These two species have traditionally been grouped into separate families, Phocoenidae and Monodontidae. The peptide maps of their respective myoglobins indicate that they are much more closely related than two separate families of the same order. They should probably be placed in at least the same family; the chromatograms indicate that they could almost be the same genus. These findings agree with those of Duffield (1978), who found a similar close relationship between the families Monodontidae and Phocoenidae based on karyotypes, and Kasuya (1973), who favored placing the two species together after studying the morphology of the tympano-periotic bone of cetaceans. The myoglobin amino acid sequence of the harbor porpoise has been determined (Meuth et al 1978), but that for the beluga has not. The peptide maps of these two indicate that the total number of different residues between them is very low.

The rest of the chromatograms are from individuals who are members of the family Delphinidae. As mentioned previously, they exhibit a general basic pattern, but they also exhibit differences which indicate the presence of at least four separate lines. One line is represented by the chromatograms of PW1, PW2, PC1, C1, and C2. Four of these samples are separated by only two differences at most, and their patterns are very similar. C2 is separated by four

differences from PW1, PW2, and PC1, and five from C1, and exhibits a pattern somewhat different from the first four (see Figures 13, 15, and 19). PW1 and PW2 are different only in the presence of a peak on PW2 (indicated by the upper long arrow), which is absent on PW1 (see Figure 13). PW1 was identified as a Pacific Globicephala, while PW2 was listed as an Atlantic Globicephala macrorhynchus, the short-finned pilot whale. PW1 may also be a Globicephala macrorhynchus, with the single difference due to population discreteness. On the other hand, PW1 may be another species of Globicephala, Globicephala scamonii, which some authorities believe is the true classification for the eastern north Pacific pilot whale (Haley 1978). Neither individual was identified as a Globicephala melaena, the long-finned pilot whale, when the sample were obtained, which is the species of Globicephala that has been sequenced (Jones et al 1978). The relative order of elution was determined from this sequence (see Table III), and applied to the chromatogram of sample PW2 (see Figure 29). This indicates that the difference apparently present between PW1 and PW2, as well as between PC1 and PW2, occurs in peptide 1. (The small peak ignored as a probable impurity in PW1 and PC1, and indicated by the upper short arrow, may actually be peptide 1 in a different position.) Samples PC1, Pseudorca crassidens, the false killer whale, and C1, Cephalorhynchus commersonii, Commerson's dolphin, are also very similar to Globicephala, with PC1 being basically identical with PW1 (see

Figures 13 and 15). PC1 differs from PW2 only in the absence of the one peak identified in the comparison of PW2 and PW1, while C1 differs from PW2 also in the absence of this peak, and in the presence of one small peak at the end of the chromatogram (indicated by the upper long arrow on Figure 19). This small peak is the single difference between C1 and PC1 and PW1. The overall patterns of these four samples are practically identical, indicating that they form a cohesive group, and appear to be quite closely related in regard to their myoglobin composition. The somewhat different pattern of C2, the second Commerson's dolphin, indicates that, although it belongs to this group, it exhibits differences which show that it is probably from a different population than that of C1. Differences between C2 and PW1, PW2, and PC1 probably reflect residue changes expected between different species. These differences probably occur in peptides 1, 14, 15, and 17 (see Figure 29).

Another separate line of delphinids is represented by the chromatograms of samples KW1, KW2, and U1 (Figures 14 and 15). KW1 and KW2 were identified as killer whales, with U1 being a sample of unknown origin. These three patterns are very similar to those of the Globicephala line, with most of the differences being minor shifts in retention times. This is also true when this group is compared to the Tursiops group, as will be seen later. KW2, a transient Orcinus orca, appears to exhibit a slightly higher number of differences

from the previous two groups than the rest of the members of this group do, and it exhibits a higher number of differences from even the members of its own group. This would not be expected, as these animals are supposedly from the same species, although recent mitochondrial DNA analysis has revealed distinct differences between resident and transient killer whale populations (Stevens et al 1989). (Resident Pacific killer whales live in a localized area, such as Puget Sound, while transient Pacific killer whales travel along the entire coast of North America.) Sample KW2 came from a stranded animal, and the yield of myoglobin was quite low. In such cases, many of the smaller peptides apparently occur in such small quantities as to be undetected by the chromatographic apparatus. Even in excellent tryptic digests with a large amount of resulting peptides, free lysine residues are seldom detectable (Caprioli et al 1987). The differences between KW2 and the rest of this second delphinid line can possibly also be attributed to this poor myoglobin yield, especially where peaks are missing. A fresh sample from a transient killer whale would be needed to determine if there were any substantive differences in myoglobin peptides between resident and transient Orcinus orca. The results here do not support any definite conclusions.

Myoglobin amino acid sequences have been determined for Globicephala melaena, Orcinus orca, and Tursiops truncatus (Jones et al 1978; Castillo et al 1977; Jones et al 1976).

They show differences of no more than two residues between any two species. Thus, a close similarity in the peptide maps of these three groups is to be expected. However, the peptide maps do indicate one or two more differences than would be expected from the amino acid sequences. Some of this could be due to the fact that the Globicephala in this study was Globicephala macrorhynchus, not Globicephala melaena, as in the sequence study, and the myoglobin sequence of the killer whale was based on Atlantic animals, while Orcinus orca samples used in this investigation were Pacific individuals. In addition, the bottlenosed dolphin samples for this study originated in the Gulf of Mexico, while the sequence of myoglobin was determined from an Atlantic animal. The identification of peaks from the determination of the relative elution order of peptides is not as definite with certain peaks in the comparison of these three species, a result which also may be due to these above reasons, as well as the fact that some of the samples used in this study were from stranded individuals. In any event, all three lines are very closely related, with individuals of each line showing even closer similarity among themselves, with the exception of the Cephalorhynchus individuals.

One of the samples chromatographed, U1, was taken from an unknown animal. The resulting trace proved to be practically identical to KW1, the resident Pacific killer whale. The only difference between these two traces was the

absence of a tiny peak near the end of the chromatogram of U1, indicated by the upper long arrow (see Figure 15). This probably represents a trypsin autodigestion peptide and was ignored. Even the peak height ratios of these two are approximately the same, suggesting that the unknown sample is a second extraction of muscle from the same individual which supplied that for KW1. This finding points to a value of peptide mapping in making possible the identification of a sample through the comparison of its map with that of one which is known. As will be mentioned later, this has important practical applications.

A third distinct delphinid line according to these results is that represented by samples T1 and T2, both Florida bottlenosed dolphins, Tursiops truncatus (Figure 16). The traces are almost identical, with the only differences being a small shift in one peak, and the absence of another on T2 (which may be present, but in too low a concentration to be detected and counted), as indicated by the upper long arrows on each trace (see Figure 16). This delphinid line appears to be equidistant from the Globicephala and Orcinus groups, and slightly more distant from the Lagenorhynchus group. It also appears to be very homogenous, but these samples were both taken from inshore Gulf of Mexico individuals, so it was not possible to test for the presence of any population differences, such as has been found between onshore and offshore populations of Tursiops (Duffield et al 1983).

The homogeneity of the Tursiops line contrasts somewhat with that of the fourth line distinguished from the chromatograms, the Lagenorhynchus line. The two samples, L1 and L2, were taken from individuals of two different species: L1 from a Lagenorhynchus obliquidens, the Pacific white-sided dolphin, and L2 from a Lagenorhynchus acutus, the Atlantic white-sided dolphin. Although the two traces have the same general pattern, they differ by a major shift in retention time of peaks near the beginning and end of the chromatograms, as well as the absence of a peak in L2 as compared with L1. (These are indicated by the upper long arrows in Figure 17.) These differences, along with differences in retention times of two peaks near the end of the chromatograms (upper long arrows in Figure 17), are typical of observed species differences. Myoglobin has not been sequenced from any Lagenorhynchus (Goodman et al 1982), so it is not possible to compare sequence data with these peptide maps. However, when comparing the maps with those of Tursiops and Orcinus and their identified peaks, it appears that the differences in Lagenorhynchus are possibly occurring in peptides 1, 2, 7, 8, and 19. L2 exhibits a closer affinity to the sample taken from Stenella coeruleoalba, the striped dolphin, than to any other of the samples tested. Interestingly, both of these animals were from the Atlantic, and from approximately the same area. This Stenella sample also shows a slightly lesser affinity to the Globicephala group, indicating that it is almost as close

to this group as it is to L2. The amino acid sequence for Stenella attenuata, the spotted dolphin, has been determined (Jones et al 1979), and the sequence places it in a grouping with Tursiops truncatus and Delphinus delphis, and separate from Globicephala and Orcinus. The latter determination agrees with this peptide map analysis, but not the former, as the chromatograms indicate differences between Tursiops and Stenella at least as great as that between Stenella and the killer and pilot whales. The Stenella used in this study was an individual from a different species and from a different ocean than that used in the sequence determination. Presumably only one individual was used in the sequencing study, and these above factors could account for most of the discrepancies. Because of this, no attempt was made to identify the peaks of sample S1 from the relative order of elution in Table III, although when comparing this chromatogram with that of PW2 (Figure 29), differences observed may possibly reside in peptides 1 and 19. The peptide map analysis places Stenella with Lagenorhynchus, although further sampling may show it to be in its own separate group.

Figure 18 shows the chromatogram for the final sample, that taken from the skeletal muscle of a Lagenorhynchus-Tursiops hybrid. These hybrids have occurred in captivity, but none have survived (Duffield 1989). Inheriting the genes for myoglobin of Lagenorhynchus and Tursiops, it would be expected that a peptide map of the myoglobin from such an animal would

combine the maps of the parent species. In other words, the peaks of Lagenorhynchus and Tursiops should both be present in the map of LT1. The LT1 chromatogram indeed does show characteristics of both species, especially L1 and T2. Because these hybrids are so young when they die, and their myoglobin yields are so low, the trace of LT1 exhibits small peaks, while peaks present in the other delphinid traces are practically non-existent in this trace. This factor is the main cause of the generally higher number of differences between LT1 and the other delphinid samples. Therefore, it was not possible to determine if this map actually did combine the maps of both parent species. However, visual analysis of the general patterns shows that LT1 has its closest affinity to L1, Lagenorhynchus obliquidens, and T2, Tursiops truncatus, which is not surprising, as this hybrid was a cross between a Pacific Lagenorhynchus obliquidens, and an Atlantic Tursiops truncatus. The peptide map examination cannot determine that its Tursiops parent was an Atlantic or a Pacific animal, as no Pacific bottlenosed dolphin was tested.

What then can be concluded from the construction and analysis of peptide maps of various cetaceans? As mentioned earlier, the main purpose of this study was to develop phylogenetic relationships between species to add more information to the working out of cetacean evolutionary history. Using the values from the difference matrix, a phylogenetic tree has been constructed from the maximum

likelihood method (Felsenstein 1981). As illustrated, the order can first be split into two distantly related groups, the first being the two species of Kogia (probably the subfamily Kogiidae). The second group is then split into a group containing Phocoena and Delphinapterus, and a group containing the delphinids. The delphinids can then be divided into the Lagenorhynchus, Tursiops, Globicephala, and Orcinus groups, with Stenella grouping with Lagenorhynchus, and Cephalorhynchus with Globicephala, or possibly forming its own separate line. Therefore this research shows the Kogia closely related, Phocoena and Delphinapterus closely related, Globicephala and Pseudorca closely related, and in turn somewhat more distantly related to Cephalorhynchus and Tursiops, then to Orcinus, then to Lagenorhynchus and Stenella. This phylogenetic determination is in fairly close agreement with that provided by myoglobin amino acid sequence analysis, as would be expected (Jones et al 1979; Goodman et al 1982). The only exception is the separation of Stenella from Tursiops, and its placement with Lagenorhynchus, and the closer affinity of Globicephala and Tursiops than Globicephala and Orcinus. The tree also agrees closely with the traditional ones based on morphology, with the exception of the placing of Phocoena with Delphinapterus, and the placement of Stenella with Lagenorhynchus. As mentioned previously, some authorities (Winge 1921; Duffield Kulu 1972) do favor the separation of Stenella from Tursiops. In short, this tree suggests an

evolutionary history quite similar to the ones expounded previously, with a few exceptions.

Another aim of this research was to see if there were population differences in myoglobin from members of the same species. The findings seem to indicate that population and individual differences in myoglobin do exist, and can be detected by myoglobin peptide analysis. There are differences between individuals of Kogia simus. There is a minor difference between the Atlantic and Pacific forms of Globicephala. Tursiops shows a similar minor difference between two individuals from the Gulf of Mexico. Lagenorhynchus shows differences between two supposedly closely related species from different oceans. Orcinus might possibly exhibit myoglobin differences between its resident and transient populations. Even the differences between the two Cephalorhynchus samples could be due to the origin of the animals from two different populations. Therefore it appears that population differences are common in the myoglobin of cetaceans. This calls into question the validity of using an amino acid sequence based on only one individual for phylogenetic analysis. The myoglobin of a member of a cetacean species from one ocean may be different from that of a member of the same species from another ocean. Sequencing of the protein from different individuals of a population and of different populations is needed to answer that question.

A third aim of this study was to determine if peptide mapping could be used to screen cetacean muscle tissue for identification purposes. It appears that the answer is a partial yes. The "signatures" found indicate that tissue can be identified down to at least the family level. Determination of genus and species is not so certain, especially in closely related groups, such as the delphinids. More extensive mapping of these cetacean species may reveal peptide map "signatures" for genera, but "signatures" for species probably do not exist.

The major purpose of this research was to determine the value of myoglobin peptide mapping in the elucidation of cetacean evolutionary relationships. It appears that this technique is a valuable one, but one which can be improved. It is relatively fast, relatively accurate, and makes use of small amounts of fresh or partially degraded samples. It apparently can distinguish between orders and between families. Distinguishing between genera is more difficult, while distinguishing between species is much more difficult. The resolving power of this method can be improved in part by increasing the number of samples tested, generating many peptide maps for each species, from representatives of different populations. This would help to determine if the differences observed in this study were consistent over many individuals. Developing many maps for every species of cetacean in a comprehensive peptide atlas would enable a

determination of phylogenetic relationships among all the species of cetaceans, as well as provide a basis of comparison for trees constructed from protein sequence analysis and morphology. Samples from cetaceans whose identity is unknown could be checked against such an atlas, and their identification determined. Compiling a comprehensive collection of maps would be time consuming and perhaps impossible, but it would be a logical next step.

No component characterization of the individual peptides was carried out in this research, although the attempt was made to identify the peaks on the basis of retention coefficients of the constituent amino acids of each peptide. A definite improvement of this technique would involve the determination of the amino acid composition of each peptide peak, and its subsequent identification on the basis of its relative retention time, using the retention coefficients developed by Meek and Rosetti (1981), Browne et al (1982), or Sasagawa et al (1982). What changes were actually occurring between the peptides of different species or populations could thus probably be more conclusively ascertained. It is also well known that different peptides may elute at the same retention time (Krstulovic & Brown 1982), so a longer gradient, coupled with rechromatography of certain regions, may provide greater resolution of individual peptides, along with their subsequent identification (Bohlen & Kleeman 1981; Schroeder 1986).

An application of this technique presented itself during the progress of this research. The harbor seal and monk seal chromatograms discussed earlier were developed not only for a comparison against the cetaceans, but also as part of a criminal investigation into the possible poaching of an endangered Monachus schauinslandi by an accused individual. Here HPLC peptide mapping proved to be of value in forensic investigation. Samples taken from a monk seal carcass allegedly killed by the individual, and from frozen meat found in his freezer were analyzed with this technique, and found to be identical, due to the almost perfect correlation of their respective chromatograms (see Figure 30). A known monk seal sample, MS1, as well as the harbor seal sample, HS1, were also analyzed, the chromatograms compared, and the carcass and freezer samples identified as monk seal, and not harbor seal. These results were submitted as evidence in the case, and are illustrated in Figure 30. The accused individual later admitted that he had indeed killed the endangered seal. Therefore the procedure and techniques applied in this research have forensic application, as well as containing evolutionary information. They could possibly be used in helping preserve, as well as helping elucidate the evolutionary pathways of the Cetacea, the mammals of the sea.

Myoglobin Peptide Profiles Using HPLC

Profile A (meat from freezer)

Profile B (meat from carcass)

Profile C (meat from known
monk seal)

Profile D (meat from known harbor seal)

Myoglobin Peptide "Fingerprints"

[illegible]

- A. Meat from freezer
B. Meat from carcass
C. Meat from known
monk seal
D. Meat from known
harbor seal

A. B. C. fingerprints
have same bar code
D. fingerprint has
different bar code

Figure 30. HPLC analysis of myoglobin tryptic peptides presented as evidence in Hawaiian Monk Seal poaching case.

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APPENDIX A

CLASSIFICATION OF RECENT WHALES

Order Cetacea

Suborder Mysticeti

Family Balaenidae

Balaena mysticetus

bowhead

Eubalaena glacialis

northern right whale

Eubalaena australis

southern right whale

Caperea marginata

pygmy right whale

Family Eschrichtiidae

Eschrichtius robustus

gray whale

Family Balaenopteridae

Balaenoptera musculus

blue whale

Balaenoptera physalus

fin whale

Balaenoptera borealis

sei whale

Balaenoptera edeni

Bryde's whale

Balaenoptera acutorostrata

minke whale

Megaptera novaeangliae

humpback

Suborder Odontoceti

Family Physeteridae

Physeter catodon

sperm whale

Kogia breviceps

pygmy sperm whale

Kogia simus

dwarf sperm whale

Family Monodontidae

Monodon monoceros

narwhal

Delphinapterus leucas

beluga

Family Ziphiidae

Tasmacetus shepherdi

Shepherd's beaked whale

Berardius arnuxii

Arnoux's beaked whale

Berardius bairdii

Baird's beaked whale

Mesoplodon pacificus

Longman's beaked whale

Mesoplodon bidens

Sowerby's beaked whale

Mesoplodon densirostris

Blainville's beaked whale

Mesoplodon europaeus

Gervais' beaked whale

Mesoplodon layardii

strap-toothed whale

Mesoplodon hectori

Hector's beaked whale

Mesoplodon grayi

Gray's beaked whale

Mesoplodon stejnegeri

Stejneger's beaked whale

Mesoplodon bowdoini
Mesoplodon mirus
Mesoplodon ginkgodens

Mesoplodon carlhubbsi
Ziphius cavirostris
Hyperoodon ampullatus

Hyperoodon planifrons

Family Delphinidae

Orcaella brevirostris
Peponocephala electra
Feresa attenuata
Pseudorca crassidens
Orcinus orca
Globicephala melaena

Globicephala macrorhynchus

Steno bredanensis
Sotalia fluviatilis
Sousa chinensis
Sousa teuszii
Lagenorhynchus albirostris
Lagenorhynchus acutus

Lagenorhynchus obscurus
Lagenorhynchus obliquidens

Lagenorhynchus cruciger
Lagenorhynchus australis
Lagenodelphis hosei
Delphinus delphis
Tursiops truncatus
Grampus griseus
Stenella attenuata
Stenella dubia
Stenella frontalis
Stenella plagiodon

Stenella longirostris
Stenella coeruleoalba
Lissodelphis peronii

Lissodelphis borealis

Cephalorhynchus heavisidii
Cephalorhynchus eutropia
Cephalorhynchus hectori
Cephalorhynchus commersonii

Andrew's beaked whale
 True's beaked whale
 ginkgo-toothed beaked whale
 Hubb's beaked whale
 Cuvier's beaked whale
 northern bottlenose whale
 southern bottlenose whale

Irrawaddy dolphin
 melon-headed whale
 pygmy killer whale
 false killer whale
 killer whale
 long-finned pilot whale
 short-finned pilot whale
 rough-toothed dolphin
 tucuxi
 Indo-Pacific sousa
 West African sousa
 white-beaked dolphin
 Atlantic white-sided dolphin
 dusky dolphin
 Pacific white-sided dolphin
 hourglass dolphin
 Peale's dolphin
 Fraser's dolphin
 common dolphin
 bottlenose dolphin
 Risso's dolphin

spotted
 dolphins

spinner dolphin
 striped dolphin
 southern right whale dolphin
 northern right whale dolphin
 Heaviside's dolphin
 black dolphin
 Hector's dolphin
 Commerson's dolphin

Family Phocoenidae

Phocoena phocoena

harbor porpoise

Phocoena spinipinnis

Burmeister's porpoise

Phocoena sinus

vaquita

Phocoena dioptrica

spectacled porpoise

Phocoenoides dalli

Dall's porpoise

Neophocoena phocoenoides

finless porpoise

Family Platanistidae

Platanista gangetica

Ganges River dolphin

Platanista minor

Indus River dolphin

Inia geoffrensis

Amazon porpoise

Lipotes vexillifer

white flag porpoise

Pontoporia blainvillei

franciscana

Source: Gaskin (1982), Honacki et al (1982), Corbet & Hill (1986), Vaughan (1986).

APPENDIX B

STEPWISE EXPERIMENTAL PROTOCOL

Extraction of Myoglobin

1. Thaw frozen muscle tissue at room temperature for 1-2 hours, or overnight at 4°C.
2. Cut muscle tissue into cubes of 1 cc or less.
3. Place cubes into 1.5 volumes of 70% saturated ammonium sulfate (472 g/l at 0°C), 20% 0.1M sodium phosphate (pH 6.5), and 10% 10 mM EDTA.
4. Homogenize the muscle in solution by grinding in a Waring Blendor at 4°C. This involves high speed grindings for 30-45 sec followed by 4-5 min soaking periods, until solution is uniform in color and consistency.
5. Centrifuge the homogenate at 12,100g for one hour at 4°C.
6. Collect the supernatant and remove the floating debris with filtration using Whatman #1 paper.
7. Add a 50% molar excess of solid potassium ferricyanide to the supernatant with gentle stirring at 4°C for 90 min.
8. Dialyze against distilled water at 4°C for 3-4 days, changing water frequently.
9. Remove any resulting precipitate with centrifugation at 12,100g and at 4°C.
10. Concentrate supernatant with polyethylene glycol. (Put

supernatant into a dialysis bag, place this in a jar of polyethylene glycol for about 30 min, rinse bag with distilled water, and retrieve concentrated supernatant.)

11. Load concentrated supernatant into a Sepharose ion exchange column at 4°C. (Use a glass column, preferably 4.5 x 40 cm, with a porous disc at the outlet, fill the column with a slurry of 2/3 CM-Sepharose CL-6B and 1/3 0.1M sodium phosphate buffer, allow the Sepharose to settle, then equilibrate the column by passing 500 ml of 0.1M sodium phosphate buffer, pH 6.5, through it. Drain the phosphate buffer down to the level of the Sepharose, and load the concentrated myoglobin sample.)
12. Drain in the myoglobin sample.
13. Develop the column with 0.1M phosphate buffer at a flow rate of about 100-200 ml/hr for 4-5 hours. The major ferrimyoglobin component, the darkest band, will separate visibly from the other slower or faster moving bands.
14. Collect the major fraction, the darkest band (IV), in a beaker, and discard the remaining portions.
15. Concentrate the major fraction with polyethylene glycol.
16. Subject the concentrate to digestion, or store as a frozen solution.
17. Clean Sepharose column by passing 0.1M sodium phosphate buffer through it, and pipetting any collected debris from the surface of the Sepharose. Store in phosphate buffer at 4°C.

Tryptic Digestion of Myoglobin

1. If extracted myoglobin was stored frozen, unthaw and remove any debris by centrifugation at 12,100g and at 4°C.
2. For every 1 ml of concentrated myoglobin solution, add .1 ml of ammonium bicarbonate (0.8M = 1.2g in 20 ml).
3. Adjust the pH to 8 with the addition of 1M sodium hydroxide.
4. Add 3 mg of TPCK-treated trypsin for every 1 ml of myoglobin solution and stir gently.
5. Cover, and incubate the solution at 37°C for 24 hours.
6. Add another 2 mg of trypsin at the end of 24 hours, and incubate the solution for 48 hours at 37°C, for a total digestion time of 72 hours.
7. Reduce pH to 2 with the addition of 3 drops of 5M acetic acid.
8. Subject to HPLC analysis.

HPLC Analysis of Myoglobin

1. Prepare buffer A - 0.1% TFA (trifluoroacetic acid) - 1 ml of TFA in 999 ml of water. Use only HPLC grade reagents!
2. Prepare buffer B - 1 ml of TFA in 999 ml of acetonitrile.
3. Turn on power to pumps.
4. Place flask with methanol on top of one of the pumps, place inlet filters to pumps in the methanol, dial in a flow rate of 0.5 ml/min, turn switch at back of pumps to pump, and flush entire system with methanol. (This assumes

that precolumn, analytical column, UV detector, and integrator-plotter have all been connected to the system.) Flush system first with drain valve open, then closed. (Remember to prime pumps if necessary.)

5. Check entire system for leaks.
6. Flush sample injection valve with methanol.
7. Flush entire system with water.
8. Flush entire system with buffer A.
9. Each flushing involves pumping 1 ml/min of solvent through system with drain valve open for 5 min, and with the valve closed for 10 min.
10. Program controller as per instructions in manual as follows:

time	buffer A	buffer B
0.0	100	0
5.0	100	0
15.0	80	20
30.0	65	35
40.0	50	50
45.0	20	80
50.0	0	100
60.0	0	100
70.0	100	0
85.0	100	0

flow rate = 1 ml/min to time 85, then 0 ml/min at time 86
chart speed = 1 cm/min, alarm at time 86, end at time 86

11. Turn on UV detector, and integrator-plotter.
12. Program UV minicontroller as per instructions in manual as follows:
 $\lambda = 220 \text{ nm}$, AUFS = 2.0
13. Turn switch at back of pumps from pmp to pro. The system is now controlled by the controller.
14. Place inlet filter from pump B into flask containing buffer B. Place inlet filter from pump A into flask containing buffer A.
15. Turn on mixing chamber.
16. Flush sample injection valve with buffer A.
17. Load myoglobin sample into sample injection loop through injection port with microliter syringe (100 μl blunt tip).
18. Set integrator-plotter as per instruction sheet as follows:
 min. area = 100, is wt = 0, all other values as in method 41
19. Make sure UV light is on, pumps are set to pro, mixing chamber is on, integrator-plotter is on, and nothing is leaking.
20. Start controller program and integrator-plotter.
21. At 1 min elapsed time, inject sample by moving sample injection valve to inj.
22. Remove microliter syringe and clean with water, then methanol.
23. Run entire program.

24. At end of chromatographic run, flush sample injection valve with water in preparation for another run.
25. Discard all eluant.
26. When finished for the day, flush entire system with water, then with methanol.
27. Store entire system in methanol.