Contributions of Individual Amino Acid Residues to the Structural Stability of Cetacean Myoglobins†

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ABSTRACT: The acid-denaturation behavior of eleven cetacean myoglobins has been studied at two ionic strengths, 0.01 and 0.10 M, at 25.0 °C. The myoglobins studied fall into four phylogenetic suborders, representing the sperm whales, dolphins, baleen whales, and beaked whales. The differences in response to acid denaturation among these closely related myoglobins are small but statistically significant. In three cases, free-energy differences between myoglobins can be ascribed to one amino acid difference and in three others to two differences. The differences in response were analyzed in terms of the changes in noncovalent interactions occurring in the native structure. The effects of changes in electrostatic interactions over the whole charge array were calculated for each myoglobin species by using the modified Tanford–Kirkwood theory. The predicted changes in stability correlated well with the experimental observations in most cases. When differences in hydrogen-bonding capability were considered at a first approximation, substantial effects were predicted. When these effects were taken in conjunction with the electrostatic interactions, the correlation with experiment was improved. Additionally, restrictions in motional freedom and packing constraints appeared to be significant in the single-site analysis. The detectable differences in stability due to single amino acid substitutions along with the small differences in stability between the cetacean suborders indicate that compensatory interactions provide the mechanism for the conservation of stability among the myoglobins studied.

A central question in protein chemistry is the definition of the contributions of each individual residue toward the overall structural and functional properties of a given protein. Natural variants containing single amino acid substitutions in the molecule have provided evidence for a range of functional consequences. These can be expressed at different functional levels, for example, in the working of an active site or in the maintenance of stability and resistance to degradation. If the three-dimensional structure of the protein is known, the effect of the substitution may be interpretable as a local change in reactivity or packing shape or as a transmitted change effective at a distance in the protein structure, or both. Because of the gradations in size, shape, and chemical reactivity between residues, any substitution has the potential of causing a measurable change in function at several different levels and in several different chemical or mechanical terms.

Although large sequence variations occur among the myoglobins of the order Cetacea (Bogardt et al., 1980), within the suborders resemblances are close and comparisons can be made between myoglobin species differing in only one or two residue positions. Such comparisons are equivalent to those between allelic variants in a given species (Grütter et al., 1979; Yutani et al., 1982). Other single-step comparisons are provided by semisynthetic substitutions (DiMarchi et al., 1980; Chaiken, 1981).

The function of myoglobin is to act as a reservoir for oxygen or as a carrier to facilitate its diffusion at low pressures, probably both. This function is conserved from one mammalian species to another by selection favoring conservative amino acid substitutions. Restrictions on changes in such characteristics as bulk, polarity, hydrophobicity, and charge type have been recognized, and they are often strongly dependent on the position within the sequence (Bogardt et al., 1980). The oxygen binding function requires a protein scaffold surrounding the heme that is permeable to the O_2 molecule but not to charged species such as the undesirable dismutation product O_2^- (Gurd & Rothgeb, 1979).

The close environment of the heme is conserved in the cetacean myoglobins. Any variations in the reversible combination with O_2 are controlled by substitutions at greater distances. Any given substitution is likely to affect the enthalpic and entropic components of the free energy of O_2 binding in some particular way. When two or more substitutions are involved in the comparison between a pair of myoglobin species, the free-energy value for O_2 binding is largely conserved by compensating adjustments of enthalpic and entropic contributions (Wang et al., 1979).

Among the cetacean myoglobins there is a degree of resistance to denaturation by acid in the pH range distinctly below the isoionic range at room temperature (Theorell & Ehrenberg, 1951; Acampora & Hermans, 1967; Hartzell et al., 1968; Puett, 1973; Friend & Gurd, 1979a). The reversible loss of the native structure can be followed by absorbance or circular dichroism measurements (Friend & Gurd, 1979b; Gurd et al., 1980). The transition from native to denatured state is apparently cooperative at low concentration (Hermans & Acampora, 1967; Shen & Hermans, 1972; Privalov, 1979) and is accomplished without encountering stable intermediate forms, although an initial loosening involving at least the A helix can be observed (Gurd et al., 1980).

The present report deals with the comparison of the stability of a series of myoglobins as a function of pH. The results are interpreted within the assumption that the native cetacean myoglobin structures in solution are very similar and can be based in detail on that of sperm whale myoglobin determined in the crystalline state (Takano, 1977). Evidence for similarities in solution structure include shared topographic regions

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recognized with comparable affinity by monoclonal antibodies (Berzofsky et al., 1982; East et al., 1982), conserved individual NMR resonances (Wüthrich et al., 1970; Cohen et al., 1972; Hayes et al., 1975; Botelho & Gurd, 1978; Botelho et al., 1978; Gurd et al., 1982), and the conservation of observed pK values for individual C^{ϵ}-proton resonances (Botelho & Gurd, 1978). The generally close resemblance (Scouloudi, 1978; H. Scouloudi, unpublished results) of the high-resolution crystallographic structures of harbor seal (Scouloudi & Baker, 1978) and sperm whale myoglobin (Takano, 1977) shows that the crystalline architecture itself is well conserved despite the 26 amino acid substitutions that distinguish these myoglobins.

Chemical modification of exposed histidine residues with bromoacetate provides the most direct evidence linking the dissolved and crystalline states of myoglobin (Banazak et al., 1963; Banazak & Gurd, 1964; Hugli & Gurd, 1970a,b; Nigen & Gurd, 1973; Botelho & Gurd, 1978). Patterns of reactivity in the solution and crystalline states were very similar with the exception of His-36, which proved reactive in the crystal, and in concentrated salt solutions, in keeping with the crystallographic analysis, but not under the normal salt conditions in solution (Botelho, 1975). Other limitations on the applicability of the crystalline structure to the dissolved state may exist. Continuity of O_2 binding kinetics is also seen in dissolved and condensed states of myoglobin (Austin et al., 1975). The observed stability differences are partitioned into contributions from different types of noncovalent interactions. The theoretical analysis roughly follows the overall trends of the observed stability differences and in several cases suggests a structural basis for those involving single-site substitutions, although interconvertibility of free energy makes difficult an accurate prediction of the ultimate partitioning.

Experimental Procedures

Protein Samples. Aquoferrimyoglobin samples of the major fraction of myoglobin of the cetacean species listed in Table I were prepared by the method of Hapner et al. (1968) adjusted to the specific chromatographic conditions referenced in the table. The set of 11 myoglobins represents four suborders: the sperm whales or *Physeteridae* and *Kogiidae* (sperm whale and dwarf and pygmy sperm whales), the dolphins or *Delphinidae* and *Globicephalidae* (killer whale, pilot whale, and bottlenosed dolphin), the baleen whales or *Balaenopteridae* (minke, humpback, and sei whales), and the beaked whales or *Ziphiidae* (goose-beaked and Hubb's beaked whales). Included for reference in Table I are the sequence distinctions of a pinniped myoglobin, harbor seal (Bradshaw & Gurd, 1969), for which accurate crystallographic information is available (Scouloudi & Baker, 1978; Scouloudi, 1978; H. Scouloudi, unpublished results). All samples were shown to be greater than 95% free of deamidation products and minor components by cellulose–acetate electrophoresis at pH 6.5 in 0.05 M phosphate buffer and at pH 9.2 in 0.1 M tris(hydroxymethyl)aminomethane (Tris)–ethylenediaminetetraacetic acid (EDTA)–borate (Rosenmeyer & Huehns, 1967).

Acid-Denaturation Profiles. Stock buffers of 0.125 M acetate of varying acidity were made up at constant ionic strength in glass-distilled water. NaCl was used to adjust the ionic strength. Aliquots of the stock buffer solutions were added to 5 nmol of isoionic aquoferrimyoglobin and were brought to 5-mL total volume for a final protein concentration of 1 μM. A data set of 12–24 samples was constructed to span the acid transition of the myoglobin under study.

The reversible disruption of the native aquoferrimyoglobin structure at low pH was followed for each species according to the decrease in absorbance at 409 nm (Breslow & Gurd, 1962; Acampora & Hermans, 1967; Puett, 1973; Friend & Gurd, 1979a). The absorbance at 280 nm was monitored to ensure that there was no loss of protein material concurrent with the loss of absorbance at 409 nm. Absorbance was measured after equilibrium was attained (15 min) in a thermostated 1-cm cuvette at 25.0 °C with a Perkin-Elmer Model 552 UV–vis spectrophotometer. The pH value of each solution was then measured at 25.0 °C on a Radiometer PHM 4c pH meter with a combination electrode. The precision of the pH measurements was better than ±0.005 pH unit.

An initial data set was constructed for each cetacean myoglobin to define the pH region of the acid transition, followed by a second data set to provide eight points between 12 and 88% denaturation. The more closely spaced data set was repeated if the difference in midpoint of the transition between the first two data sets exceeded 0.01 pH unit. In general, the acid transitions measured at an ionic strength of 0.10 M were more difficult to reproduce.[1]

The spectral change in each case could be fit to an apparent two-state model (Gurd et al., 1980) with a Newton–Gauss non-linear-regression algorithm.[2] The percent native structure retained at each pH value was calculated as

$$\% \text{ N} = \frac{A_{409} - A_{409}(100\% \text{ D})}{A_{409}(100\% \text{ N}) - A_{409}(100\% \text{ D})} \quad (1)$$

The equilibrium constants favoring the native state at each pH value was

$$K = \% \text{ N}/(100 - \% \text{ N}) \quad (2)$$

The difference in the number of bound protons, $\Delta\bar{\nu}_{H^+}$, between native and denatured states was determined with the relationship (Hermans & Acampora, 1967; Tanford, 1970; Privalov, 1979)

$$\text{d log } K/\text{d pH} = \Delta\bar{\nu}_{H^+} \quad (3)$$

Integration of eq 3 allows the estimate of log K at any pH value (Hermans & Acampora, 1967). log K values from all data sets of a particular species were plotted as a function of pH to obtain the slope, $\Delta\bar{\nu}_{H^+}$. pH_{mid}, the pH of half-denaturation, was read directly from the acid-transition curves for individual data sets and was read from the log K vs. pH plots at log K equal to zero for the compiled data. The method for the comparison of several slopes of Snedecor[3] (Snedecor, 1956; Volk, 1958) was used to determine if the log K vs. pH data for closely related species were distinguishable.

In comparing two different myoglobins in the region of the acid transition, the difference in their log K values at some given reference pH was found to be most useful. This value defines the difference in free energy of stabilization:

$$\delta\Delta G = -2.303RT\delta(\log K) \quad (4)$$

When the difference in free energy is referenced to sperm whale myoglobin, the quantity is designated $\delta\Delta G_{sw}$ and is positive if the particular myoglobin is less stable to denatu-

[1] The distinctions between species at μ = 0.10 M are not identical with those seen at the lower ionic strength, reflecting both the difference in the pH at which the transition occurs and the effects of the increased ion cloud. It has been postulated that aggregation effects may be significant at ionic strengths above 0.05 M (Hartzell et al., 1968; S. H. Friend, unpublished results) and may have some bearing on the reproducibility of the data at μ = 0.10 M.

[2] The program used, BMDX85 (revised April, 1971), was developed at the Health Sciences Computing Facility, UCLA (sponsored by NIH Special Resources Grant RR-3), as modified by Dr. R. D. England (Indiana University).

[3] A Basic program (K. Owens, Indiana University) was used in a form modified for the Tektroniks 4051 computer to calculate the error values necessary to complete statistical analysis.

Table I: Amino Acid Sequence Differences of Cetacean Myoglobins Studied[a]

	1	4	5	8	12	13	15	19	21	22	27
sperm whales											
sperm whale[b]	Val	Glu	Gly	Gln	His	Val	Ala	Ala	*Val*	Ala	Asp
dwarf sperm whale[c]	Val	Glu	Gly	Gln	His	Val	Ala	Ala	*Ile*	Ala	Asp
pygmy sperm whale[d]	Val	Glu	Gly	Gln	His	Val	Ala	Ala	*Ile*	Ala	Asp
dolphins											
pilot whale[e]	Gly	Asp	Gly	Gln	Asn	Val	Gly	Ala	Leu	Ala	Asp
killer whale[f]	Gly	Asp	Gly	Gln	Asn	Val	Gly	Ala	Leu	Ala	Asp
bottlenosed dolphin[g]	Gly	Asp	Gly	Gln	Asn	Val	Gly	Ala	Leu	Ala	Asp
baleen whales											
minke whale[h]	Val	Asp	Ala	*His*	Asn	Ile	Ala	Ala	Val	Ala	Asp
sei whale[i]	Val	Asp	Ala	*Gln*	Asn	Ile	Ala	Ala	Val	Ala	Asp
humpback whale[j]	Val	Asp	Ala	*Gln*	Asn	Ile	Ala	Ala	Val	Ala	Asp
beaked whales											
goose-beaked whale[k]	Gly	Glu	Ala	Gln	His	Val	Ala	Ala	Leu	Ser	Glu
Hubb's beaked whale[l]	Gly	Glu	Ala	Gln	His	Val	Ala	Ala	Leu	Ser	Glu
pinniped											
harbor seal[m]	Gly	Asp	Gly	His	Asn	Val	Gly	Thr	Leu	Ala	Glu

	28	35	45	51	53	54	56	57	62	66	74
sperm whales											
sperm whale[b]	Ile	*Ser*	Arg	*Thr*	Ala	Glu	Lys	Ala	Lys	Val	Ala
dwarf sperm whale[c]	Ile	*His*	Arg	*Ser*	Ala	Glu	Lys	Ala	Lys	Val	Ala
pygmy sperm whale[d]	Ile	*His*	Arg	*Thr*	Ala	Glu	Lys	Ala	Lys	Val	Ala
dolphins											
pilot whale[e]	*Ile*	Gly	Lys	Thr	Ala	Asp	Lys	Ala	Lys	Asn	Ala
killer whale[f]	*Ile*	Gly	Lys	Thr	Ala	Asp	Lys	Ala	Lys	Asn	Ala
bottlenosed dolphin[g]	*Val*	Gly	Lys	Thr	Ala	Asp	Lys	Ala	Lys	Asn	Ala
baleen whales											
minke whale[h]	Ile	Gly	Lys	Thr	Ala	Glu	Lys	Ala	Lys	Asn	Gly
sei whale[i]	Ile	Gly	Lys	Thr	Ala	Glu	Lys	Ala	Lys	Asn	Gly
humpback whale[j]	Ile	Gly	Lys	Thr	Ala	Glu	Lys	Ala	Lys	Asn	Gly
beaked whales											
goose-beaked whale[k]	Ile	Gly	Lys	Ser	Ala	Glu	Lys	Ala	Lys	His	Gly
Hubb's beaked whale[l]	Ile	Gly	Lys	Ser	Ala	Glu	Lys	Ala	Lys	His	Gly
pinniped											
harbor seal[m]	Val	Ser	Lys	Ser	Asp	Asp	Arg	Arg	Arg	Asn	Gly

	83	109	118	121	122	129	132	140	151	152
sperm whales										
sperm whale[b]	Glu	Glu	Arg	*Gly*	Asp	Gly	*Asn*	Lys	Tyr	Gln
dwarf sperm whale[c]	Glu	Glu	Arg	*Ala*	Asp	Gly	*Ser*	Lys	Tyr	Gln
pygmy sperm whale[d]	Glu	Glu	Arg	*Ala*	Asp	Gly	*Thr*	Lys	Tyr	Gln
dolphins										
pilot whale[e]	*Glu*	Glu	Arg	Ala	Glu	Gly	Asn	Lys	Phe	His
killer whale[f]	*Asp*	Glu	Arg	Ala	Glu	Gly	Asn	Lys	Phe	His
bottlenosed dolphin[g]	*Asp*	Glu	Arg	Ala	Glu	Gly	Asn	Lys	Phe	His
baleen whales										
minke whale[h]	Glu	Asp	Arg	*Ala*	*Glu*	Ala	Asn	nLys	Phe	Gln
sei whale[i]	Glu	Asp	Arg	*Gly*	*Asp*	Ala	Asn	Lys	Phe	Gln
humpback whale[j]	Glu	Asp	Arg	*Ala*	*Asp*	Ala	Asn	Lys	Phe	Gln
beaked whales										
goose-beaked whale[k]	Glu	Asp	*Arg*	Ser	Asp	*Ala*	Thr	Lys	Phe	His
Hubb's beaked whale[l]	Glu	Asp	*Lys*	Ser	Asp	*Gly*	Thr	Lys	Phe	His
pinniped										
harbor seal[m]	Glu	Glu	Lys	Ala	Glu	Ala	Lys	Asn	Phe	His

[a] Entries for sequence positions that vary within the respective phylogenetic suborders are italicized. [b] *Physeter catodon* (Edmundson, 1965; Romero-Herrera & Lehman, 1974). [c] *Kogia simus* (Dwulet et al., 1977). [d] *Kogia breviceps* (M. L. Crowl-Powers, J. L. Meuth, D. E. Harris, and F. R. N. Gurd, unpublished results). [e] *Globicephala melaena* (Jones et al., 1978). [f] *Orcinus orca* (Meuth et al., 1981). [g] *Tursiops truncatus* (Jones et al., 1976a). [h] *Balaenoptera acutorostrata* (Lehman et al., 1977). [i] *Balaenoptera borealis* (Jones et al., 1979). [j] *Megaptera novaeangliae* (Lehman et al., 1978). [k] *Ziphius cavirostris* (Lehman et al., 1980). [l] *Mesoplodon carlhubbsi* (Dwulet et al., 1980). [m] *Phoca vitulina* (Bradshaw & Gurd, 1969).

ration by acid than sperm whale myoglobin.

Theoretical Procedures

Three-Dimensional Structures. The sperm whale myoglobin crystal structure used is that of Takano (1977) with modifications in the orientation of Glu-4 and His-36 (Botelho et al., 1978). A representative species was selected from each of the three remaining phylogenetic groups. Three-dimensional structures were constructed, with the sperm whale myoglobin structure as a foundation. The computer algorithm used builds in the new residue in the shadow of the previous one and then rotates it through a number of positions, selecting a position compatible with the van der Waals radii of neighboring groups (Feldmann et al., 1981). At no time were changes in backbone conformation necessary to allow fit of the new side chain. The microenvironment of each variant position was examined by using stereoviews[4] of the area within 7–10 Å of the replaced

[4] The stereoviews were generated by using NEWCOMB, an interactive version of C. Johnson's ORTEP program (Oakridge National Laboratory). This version was written by Dr. J. Huffman (Indiana University Molecular Structure Center).[5]

side chain and calculations of interatomic distances. The positioning of the new residue was in most cases comparable to that of the old in these computer-generated structures.

Many of the substitutions studied have the potential to exert an influence on the hydrogen-bonding network, electrostatic interactions, packing, or motional constraints of the molecule. There is however limited basis to test for correct placement of substituted residues. The sensitivity of the calculated electrostatic free energy to side-chain placement was tested. As would be expected, large differences arose between alternative structures if a strong electrostatic interaction was substantially eliminated in one. If the strong interaction was maintained in both structures, the results were insensitive to the exact conformations of the affected residues. Without the aid of a macromolecular energy-refinement algorithm, it was elected to limit refinements beyond the van der Waals contact level to those substitutions that affect conserved residues believed to contribute substantially to the electrostatic stabilization of myoglobins (Friend & Gurd, 1979b). On the basis of these criteria, further adjustments were made in only four positions: Glu-4 → Asp, Asp-27 → Glu, Arg-45 → Lys, and Asp-122 → Glu. The rationales for these adjustments are given in the supplementary material.[6]

The procedure used to adjust the computer-generated coordinates consisted of making rotations about the appropriate bonds to bring the necessary atoms into juxtaposition.[5] Intermediate and final positions were checked against the comparable positions in the sperm whale myoglobin structure to ensure maximum homology. No changes in backbone conformation were necessary, and only rotations about single bonds were allowed. All changes were monitored to ensure that van der Waals contacts were not violated. Substitutions within the phylogenetic suborders were made in the same manner.

The calculated $pK_{1/2}$ values for the His residues were compared to those determined experimentally under slightly different conditions (Botelho et al., 1978). The correlation coefficients were as follows: sperm whale, 0.865; pilot whale, 0.906; minke whale, 0.923. Experimental data were not available for the beaked whales. No changes were made in side-chain conformation to achieve an optimum fit between the computed and observed histidine pK values.

Electrostatic Proton-Binding and Free-Energy Calculations. The procedure is that of March et al. (1982). The energy of interaction between pairs of charges, W_{ij}, is modulated by the fractional accessibility of the charged atoms to the solvent:

$$W'_{ij} = W_{ij}(1 - \overline{SA}_{ij}) \quad (5)$$

where $\overline{SA}_{ij}$ is the average static accessibility of ith and jth groups (Lee & Richards, 1971; Matthew et al., 1978a). This formalism leads to substantially the same result in the iterative procedure as that starting from the form $W'_{ij} = W_{ij}(1 - SA_j)$, which was previously adopted (Friend & Gurd, 1979a,b; Gurd et al., 1980). The newer formalism avoids asymmetry, and the calculations converge more rapidly (Matthew et al., 1982; Matthew & Richards, 1982).

The intrinsic equilibrium constant in the absence of effects from other charged sites, $(pK_{int})_i$, is modulated by the extent of electrostatic interactions of site i with other charged sites j:

$$pK_i = (pK_{int})_i - \frac{1}{2.303kT}\sum_{j\neq i}^{n} W'_{ij}Z_j = (pK_{int})_i + \sum_{j\neq i}^{n} \Delta pK_{ij} \quad (6)$$

where Z_j is the fractional charge on site j. A consistent set of pK_i values is computed at each pH and ionic strength by an iterative procedure (Matthew et al., 1978b; Botelho et al., 1978). The parameter $pK_{1/2}$ is defined as the pH at which a particular residue is half-titrated and is the computed value used in comparison with experimental values. Theoretical protein titration curves are generated by summing the fractional-charge occupancy of each site over all charged sites on the protein as a function of pH.

The overall electrostatic free energy calculated for a protein summed over its n-charged sites is given by

$$\Delta G_{el} = (1/2)\sum_{i=1}^{n}\sum_{j\neq i}^{n} W'_{ij}Z_iZ_j = (2.303kT/2)\sum_{i=1}^{n}\sum_{j\neq i}^{n} pK_{ij}Z_j \quad (7)$$

This form differs from that in which the $(1 - SA_i)(1 - SA_j)$ term was used for each pair of sites instead of the average as employed here. The effect of the alteration in definition is not large in most cases, although the calculated values of ΔG_{el} are larger, and renders the argument consistent (March et al., 1982).

In the following discussion, individual interaction energies are designated $\Delta G_{el,ij}$. These values when summed over all other groups give the free-energy contribution of a particular group i, $\Delta G_{el,i}$. It is these values summed in turn and divided by 2 that give the overall electrostatic free energy of stabilization under the given conditions (eq 7). When free-energy terms are compared among the myoglobins of different species, the difference is designated as $\delta\Delta G_{el}$.

Results and Discussion

Acid-Transition Profiles. Figure 1 shows the dependence of the percent native structure, % N, on pH at μ = 0.01 M for the myoglobins of the sperm, killer, minke, and two beaked whales. These species are representative of their cetacean suborders. The shapes of the profiles reflect the reversible two-state transition recognized previously (Hermans & Acampora, 1967; Hartzell et al., 1968; Friend & Gurd, 1979b; Gurd et al., 1980). Similar results were obtained at μ = 0.10 M with the acid transition occurring at a higher pH in all cases.[1]

Figure 2 shows plots of log K vs. pH at μ = 0.01 and 0.10 M for all four suborders. The linearized form allows quantitation of $\Delta\bar{\nu}_{H^+}$ and extrapolation of the log K plots to a common pH at each ionic strength (Hermans & Acampora, 1967). The results from these computations are collected in Table II. Table IIA shows the number of data points for each species, n, the pH of the midpoint of the transition, pH_{mid}, the slope, $\Delta\bar{\nu}_{H^+}$, and the value of log K at pH 4.0. These data are for an ionic strength of 0.01 M at 25.0 °C. Table IIB shows corresponding data for an ionic strength of 0.10 M, the value of log K applying to pH 4.5. The values for pH_{mid} and log K follow roughly the phylogenetic suborders, with the exception of the beaked whales. The difference in log K referred to sperm whale myoglobin, $\delta\Delta G_{sw}$, is shown in the next column. According to the results of the statistical analysis performed within suborders (see Experimental Procedures), the myoglobins of the sperm, baleen, and beaked whales were found

[5] Programs ATMSORT, GENCART, GENSYM, BMFIT3, CART21, and NEWCOMB[4] from the Indiana University Structure Center XTEL interactive program library were utilized to construct an interactive structure-manipulation and graphics package for peptide and protein structures. The controlling programs were written by M.A.F. This package ran on a CYBER 172 at the Academic Computing Center of Indiana University.

[6] See paragraph at end of paper regarding supplementary material.

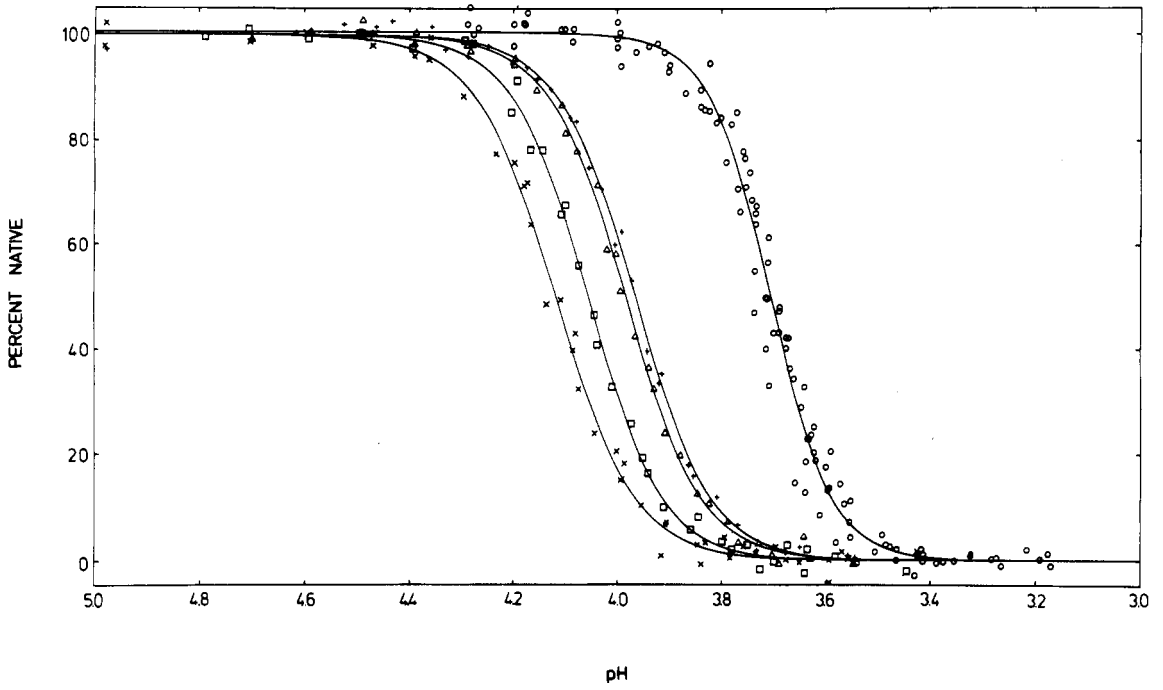

FIGURE 1: Acid-denaturation profiles of representative species of cetacean myoglobins at μ = 0.01 M, 25.0 °C. The percent native structure is plotted as a function of pH for the myoglobins of sperm whale (○), goose-beaked whale (+), killer whale (△), minke whale (□), and Hubb's beaked whale (×). Increasing hydrogen ion activity is to the right.

Table II: Acid-Denaturation Behavior of Cetacean Myoglobins at 25 °C

(A) μ = 0.01 M

	n	pH_{mid}	$\Delta\bar{\nu}_{H^+}$	pH 4.0 log K	$\delta\Delta G_{sw}$ (kcal/mol)	ΔG_{el} (kcal/mol)	$\delta\Delta G_{el,sw}$ (kcal/mol)
sperm whale	52	3.70	6.7	1.99		−10.14	
dwarf sperm whale	12	3.72	6.7	1.82	0.231	−8.95	1.19
pygmy sperm whale	41	3.66	6.7	2.27	−0.381	−8.97	1.17
pilot whale	11	3.99	6.4	0.090	2.58	−12.11	−1.97
killer whale	12	3.99	6.4	0.090	2.58	−12.10	−1.96
bottlenosed dolphin	11	3.96	6.4	0.260	2.35	−12.10	−1.96
minke whale	12	4.06	5.7	−0.340	3.17	−13.02	−2.88
sei whale	10	4.11	5.4	−0.570	3.48	−12.77	−2.63
humpback whale	13	4.11	4.4	−0.500	3.37	−12.77	−2.63
goose-beaked whale	12	3.97	6.1	0.180	2.46	−11.94	−1.80
Hubb's beaked whale	15	4.12	5.4	−0.640	3.58	−11.33	−1.19

(B) μ = 0.10 M

	n	pH_{mid}	$\Delta\bar{\nu}_{H^+}$	pH 4.5 log K	$\delta\Delta G_{sw}$ (kcal/mol)	ΔG_{el} (kcal/mol)	$\delta\Delta G_{el,sw}$ (kcal/mol)
sperm whale	34	4.19	5.4	1.65		−10.99	
dwarf sperm whale	17	4.17	5.8	1.94	−0.394	−10.13	0.87
pygmy sperm whale	19	4.13	6.4	2.35	−0.952	−10.14	0.86
pilot whale	13	4.48	5.8	0.135	2.06	−12.52	−1.53
killer whale	12	4.48	5.7	0.140	2.05	−12.50	−1.51
bottlenosed dolphin	12	4.44	6.5	0.420	1.67	−12.50	−1.51
minke whale	35	4.51	5.4	−0.065	2.33	−13.32	−2.32
sei whale	27	4.55	5.4	−0.255	2.59	−12.97	−1.97
humpback whale	32	4.54	5.4	−0.205	2.52	−12.97	−1.97
goose-beaked whale	12	4.44	5.6	0.315	1.82	−12.50	−1.51
Hubb's beaked whale	12	4.59	5.9	−0.515	2.94	−12.05	−1.06

to be distinguishable.[7] In the dolphin suborder, killer and pilot whales were found to be distinguishable from bottlenosed dolphin but not from each other (see Figure 2B). Values of $\Delta\bar{\nu}_{H^+}$ were found to be distinguishable within the beaked whale suborders at μ = 0.01 M. At μ = 0.10 M, values of $\Delta\bar{\nu}_{H^+}$ were distinguishable within all suborders but the baleen whales (see Table II). The best fit lines drawn through the data in Figure 2 were calculated from the statistical analysis. Note that the ionic strength effects are substantial and indicate the importance of electrostatic interactions in promoting the stabilization of the native form of the myoglobin molecules. The continuum of ionic strength effects observed for sperm whale myoglobin (Friend & Gurd, 1979a) may be taken to apply to the myo-

[7] Myoglobins within the suborders were considered distinguishable when the data showed differences significant at the 95% confidence level.[4]

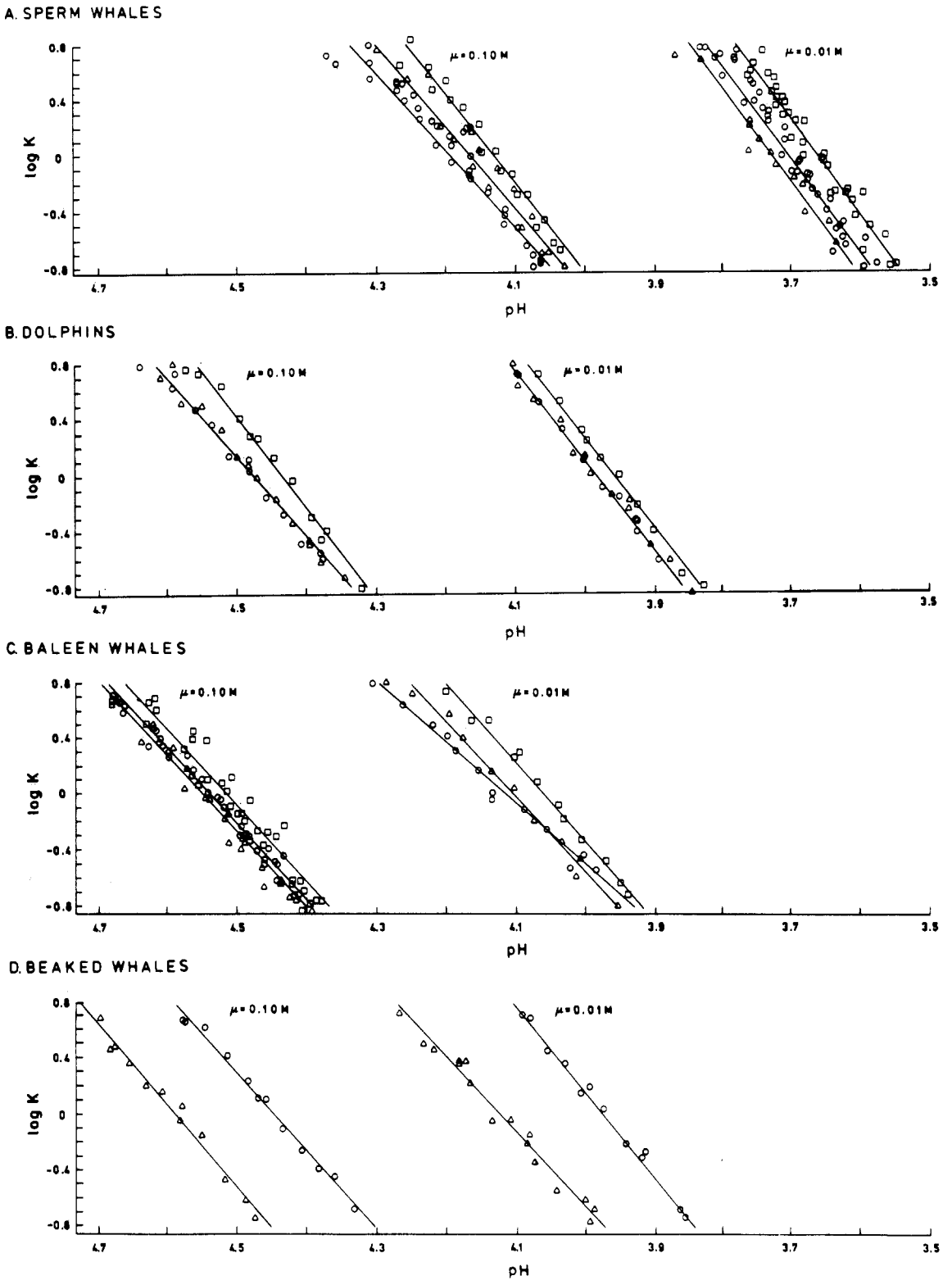

FIGURE 2: Dependence of log K on pH at two ionic strengths, μ = 0.01 and 0.10 M. Increasing hydrogen ion activity is to the right. (A) Sperm whales. At μ = 0.01 M, the least stable myoglobin is from dwarf sperm whale (△), followed by sperm whale (○), and pygmy sperm whale (□) myoglobins. At μ = 0.10 M, the order is sperm whale (○), dwarf sperm whale (△), and pygmy sperm whale (□). (B) Dolphins. At μ = 0.01 and 0.10 M, pilot whale (○) and killer whale (△) myoglobins are indistinguishable, and bottlenosed dolphin myoglobin (□) is more stable. (C) Baleen whales. At μ = 0.01 M, the least stable myoglobin is from humpback whale (○), followed by sei whale (△) and minke whale (□) myoglobins. At μ = 0.10 M, the order is sei whale, humpback whale, and minke whale myoglobins. (D) Beaked whales. At μ = 0.01 and 0.10 M, Hubb's beaked whale myoglobin (△) is less stable than goose-beaked whale myoglobin (○).

globins of the other species as well.

A major stabilizing factor for the denatured state comes from the protonation of newly exposed histidine residues that are contained within the native structure in the unprotonated state (Breslow & Gurd, 1962; Acampora & Hermans, 1967; Friend & Gurd, 1979b). Significantly, the value of $\Delta\bar{\nu}_{H^+}$ always exceeds 4 (Table II), which is the number of buried histidines that become protonated on denaturation (Takano, 1977; Botelho et al., 1978). The physical basis of variations in $\Delta\bar{\nu}_{H^+}$ among the species has been suggested to be solely a reflection of the pH region in which denaturation occurs (Hermans & Acampora, 1967). To test this hypothesis, $\Delta\bar{\nu}_{H^+}$

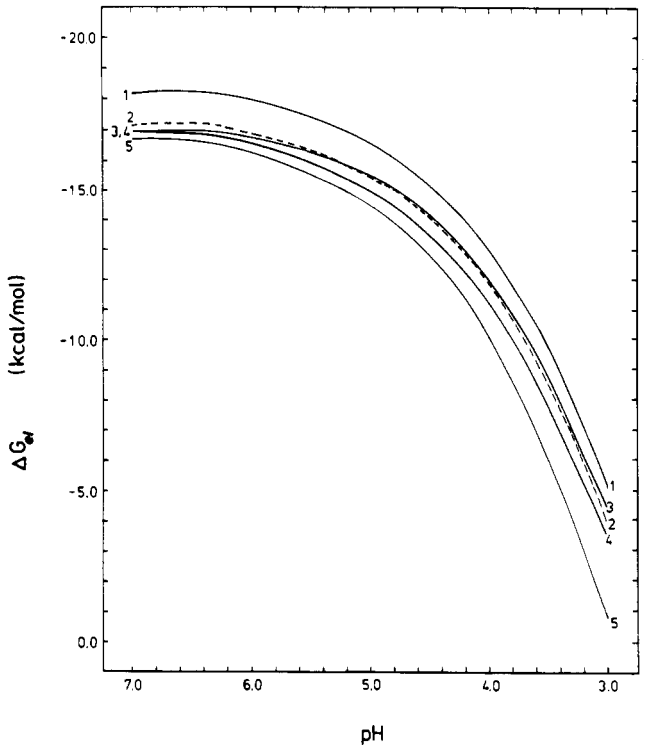

FIGURE 3: Electrostatic free energy, ΔG_{el}, in kilocalories per mole, at μ = 0.01 M and 25.0 °C of the native structure of (1) minke whale, (2) killer whale, (3) goose-beaked whale, (4) Hubb's beaked whale, and (5) sperm whale myoglobins. Increasing hydrogen ion activity is to the right.

for a particular species was plotted against the corresponding pH_{mid} value (Table II). At μ = 0.01 M, $\Delta\bar{\nu}_{H^+}$ and pH_{mid} are found to have a linear dependence significant at the 99% confidence level. As will be discussed in the later section, the humpback whale myoglobin is the only apparent deviate from this correlation.

The sequences given in Table I show that the differences in denaturation behavior between the major groups of myoglobins in Figure 2, parts A–D, do not correlate well with the numbers of substitutions between them. For example, although the bottlenosed dolphin differs from sperm whale myoglobin in 15 sequence positions, its stability relative to sperm whale is similar to that of harbor seal myoglobin (Friend & Gurd, 1979a), which differs from the sperm whale protein at 26 sequence positions (Hartzell et al., 1968; Gurd et al., 1980). Likewise, between the dolphins and baleen whales, the number of sequence differences ranges between 13 and 16, yet the observed stability difference is on the order of 1 kcal/mol. Most significantly, the two closely related beaked whales show differences in stability comparable to those seen between the three other main suborders, even though the beaked whales themselves differ at only two sequence positions. Possible energetic sources of differences in stability are considered first by comparing suborders differing in several concurrent substitutions and then in terms of the closely related species within these suborders.

Electrostatic Contributions to Stability. Figure 3 shows the summed electrostatic free energy, ΔG_{el}, as a function of pH between 3 and 7 computed for the native structure of several representative myoglobins at μ = 0.01 M and 25.0 °C.[8]

[8] These calculations are different from those reported previously (Friend, 1979) and reflect improvements in the method for placement of side-chain residues.

Table III: Change in Buried Surface Area among Representative Cetacean Myoglobins

	native accessible surface area (Å²)	denatured accessible surface area (Å²)	buried surface area (Å²)[b]
sperm whale	7870	25 997	18 127
minke whale	7946	25 950	18 004 (−123)
killer whale	8004	25 788	17 784 (−343)
goose-beaked whale	8038	25 935	17 896 (−230)

[a] Denatured accessible surface area was calculated by the method of Chothia (1976). [b] Numbers in parentheses indicate the change in buried surface area relative to sperm whale myoglobin.

The greatest stabilization is shown in the curves for the myoglobins of the minke whale, followed by the killer and beaked whales, and lastly by the sperm whale. A similar set of curves is obtained at μ = 0.10 M with somewhat reduced differences and generally lessened stabilization due to increased ionic screening.

The last two columns of Table II list ΔG_{el}, the free energy of stabilization of the native structure, and $\Delta G_{el,sw}$, the additional stabilization of each of the species relative to sperm whale myoglobin. It is apparent from Figure 3 and Table II that on the basis of the putative three-dimensional structures, the myoglobins of sperm whales are stabilized less by electrostatic interactions than are the other myoglobins. This conclusion runs counter to observed behavior (Figures 1 and 2, Table II) and indicates that some compensating nonelectrostatic characteristics may contribute specific stabilization to the myoglobins of the sperm whale suborder. Otherwise, the free energy associated with electrostatic interactions generally reflects the experimentally observed order of stability among the remaining species. This agreement is contingent on the homology of electrostatic interactions with sperm whale myoglobin, which has been assumed in the modeling process.

The observed values of $\Delta\bar{\nu}_{H^+}$ were compared with computed values at the midpoint of the transition. For this purpose, it was assumed that the denatured state underwent titration as a sum of groups all titrating with their individual pK_{int} values. The differences in the numbers of bound protons between native and denatured states for each myoglobin species were then calculated at the experimentally observed pH_{mid} of denaturation. The linear correlation between the calculated and observed values is the same as that found between pH_{mid} and $\Delta\bar{\nu}_{H^+}$. Therefore, it can be assumed that the correlation observed here is due primarily to variations in the pH at which $\Delta\bar{\nu}_{H^+}$ is computed from the electrostatic interaction model.

Hydrophobic Contributions to Stability. The buried surface area of a globular protein has been postulated to be proportional to the hydrophobic free-energy contribution to stability of its native conformation (Chothia, 1974, 1976; Chothia & Janin, 1975). The accessible surface area of the native structure, accessible surface area of the denatured structure [cf. Chothia (1976)], and, by difference, the buried surface area of representative myoglobin species were calculated (Lee & Richards, 1971; Matthew et al., 1978a) and are shown in Table III. Sperm whale myoglobin has the greatest buried surface area and therefore could be considered to have the greatest hydrophobic free energy favoring stability. However, the significance of this result is limited by the small differences between species, which amount to 1 or 2% of the total buried surface area. Uncertainty in the placement of substituted residues can account for as much as 40 Å² per residue (Friend, 1979; M. A. Flanagan, results not shown), which amounts to

Table IV: Stability Differences between Closely Related Species of Myoglobin[a]

	μ = 0.01 M (pH 4.0)		μ = 0.10 M (pH 4.5)		
	$\delta\Delta G_{obsd}$ (kcal/mol)	$\delta\Delta G_{el}$ (kcal/mol)	$\delta\Delta G_{obsd}$ (kcal/mol)	$\delta\Delta G_{el}$ (kcal/mol)	
Ile-28 → Val	−0.2	*b*	−0.4	*b*	killer whale → bottlenosed dolphin
Glu-83 → Asp	0.0	0.0	0.0	0.0	pilot whale → killer whale
Gly-121 → Ala	−0.1	*b*	−0.1	*b*	sei whale → humpback whale
Arg-118 → Lys Ala-129 → Gly	1.1	0.6	1.1	0.5	goose-beaked whale → Hubb's beaked whale
Gln-8 → His Asp-122 → Glu	−0.2	−0.2	−0.2	−0.4	humpback whale → minke whale
Ser-51 → Thr Ser-132 → Thr	−0.6	0.0	−0.6	0.0	dwarf sperm whale → pygmy sperm whale

[a] 25.0 °C. [b] The input for the electrostatic interaction calculation did not change for these pairs of species.

approximately 1 kcal/mol at 25 cal/(mol·Å^2) (Clothia, 1975). Although the calculated surface-area differences correctly predict the sperm whales to be more stable than the other suborders, the relative importance of hydrophobic interactions is not clearly established.

Other Contributions to Stability. Other types of noncovalent interactions have been shown to contribute significantly to protein stability (Kauzmann, 1959; Privalov, 1979; Finney et al., 1980). The observed differences in stability may contain components due to changes in hydrogen-bonding capability, configurational entropy, packing, or state of ionization, as well as those already discussed. Unfortunately, at this time it is not possible to estimate a contribution from each of these components that will give the resolution necessary for the present study.

A major focus of this paper is to gain additional information from the observed stability differences on the kinds of interactions that are perturbed by conservative substitutions. It has been demonstrated in the analysis of electrostatic and hydrophobic contributions to the observed stability differences that the differences in computed contributions are for the most part very small. Uncertainty in the placement of substituted side-chain residues introduces an error into the calculation of these computed differences that can be as large as the computed differences among the species. A comparison between very closely related species of myoglobin, i.e., those differing in only one or two sequence positions, eliminates the accumulation of these uncertainties. The differences in electrostatic interactions are limited and well defined in that they can be understood in terms of interactions between individual pairs of charged residues. Therefore, they can be an asset in interpreting the structural basis of the observed stability differences.

Comparisons of Closely Related Myoglobins. Table I contains certain residues marked in italics to draw attention to distinctions between closely related species. In three cases, pairs of species differ at only one residue position and in three others at two. The six pairs for comparison are listed in Table IV. The species and residue substitutions are listed first, followed by free-energy differences, $\delta\Delta G$ in kilocalories per mole under the two reference conditions of pH 4.00 and μ = 0.01 M and pH 4.5 and μ = 0.10 M. For each of these reference conditions, there are two entries, $\delta\Delta G_{obsd}$ and $\delta\Delta G_{el}$ (from Table II). The results shown in Table IV illustrate a wide range of possible effects. In three cases, charged groups are substituted, and in three cases, they are not. In two of the cases where charged groups are substituted, differences in electrostatic stabilization are computed; in the third, the charge array is found to be substantially equivalent in the species compared. Individual electrostatic interaction energies were computed for the experimental conditions and are shown in Table V. See Theoretical Procedures for definitions of $\delta\Delta G_{el,ij}$ and $\delta\Delta G_{el,i}$.

Table V: Change in Individual Electrostatic Interaction Energies between Closely Related Species of Myoglobin[a]

	$\delta\Delta G_{el,ij}$ (kcal/mol)	$\delta\Delta G_{el,i}$ (kcal/mol)
Pilot Whale → Killer Whale		
Glu-83 → Asp		−0.03
Asp-141	0.10	
Lys-145	−0.18	
Humpback Whale → Minke Whale		
Gln-8 → His		0.25
Asp-4	−0.20	
Lys-16	0.24	
Lys-79	0.19	
His-119	0.10	
Glu-122	−0.11	
Asp-122 → Glu		−0.58
Lys-16	−0.42	
Goose-Beaked Whale → Hubb's Beaked Whale		
Arg-118 → Lys		0.56
Asp-20	0.32	
His-119	0.13	

[a] The values shown were calculated at μ = 0.01 M, pH 4.0, and 25.0 °C.

All the substitutions in Table IV can be considered conservative, representing pairs with low indices of difference by multivariate analysis with none of them falling among the 11 positions at which nonconservative changes have been recognized in mammalian myoglobins (Bogardt et al., 1980). The conservative nature of the substitutions was confirmed by an examination of their helix-forming probabilities with the method of Chou & Fasman (1978). In all cases but one (Gly-121 → Ala), the substitutions were found to leave unaltered the prediction of stabilization of secondary structure.

Residue Ile-28 → Val. The substitution Val-28 → Ile results in stabilization of the bottlenosed dolphin myoglobin relative to that of the killer whale. Rotation around the C^{β}–C^{γ_1} bond axis of Ile-28 in sperm whale myoglobin has been found by carbon-13 nuclear magnetic resonance (^{13}C NMR) analysis to be particularly restricted both in absolute terms and by comparison with other isoleucine residues (Gurd et al., 1980). Removal of C^{δ_1} with the substitution of a valine may relieve packing constraints.

Residue Glu-83 → Asp. The substitution of Asp-83 → Glu produces no significant stabilization of the killer whale myoglobin relative to that of the pilot whale. The experimental data were found to be indistinguishable at both ionic strengths (Table II and Figure 2B). The electrostatic calculation pre-

dicts no detectable differences in stability of an Asp or Glu at this position due to the opposing effects on Asp-141 and Lys-145 (Table V).

Residue Gly-121 → Ala. This substitution produces a statistically significant difference in slope (Figure 2C) between the denaturation profiles of the sei and humpback whale myoglobins. These myoglobins have identical midpoints of denaturation, yet the values of $\Delta\bar{\nu}_{H^+}$ are 5.4 and 4.4, respectively, at $\mu = 0.01$ M (Table IIA). The substitution at position 121 produces no computed change in buried surface area, packing constraints, or static accessibility of neighboring charged groups. Therefore, the electrostatic array parameters for interaction calculations were not changed in consequence of this substitution.

A possible consequence of the substitution Ala-121 → Gly is to decrease the stability of the β-turn between the G helix and the H helix. The turn is initiated with the invariant Pro-120. According to the secondary structure prediction method of Chou & Fasman (1978), Gly-121 is well suited to stabilization of the β-turn whereas Ala-121 is not.

Residues Gln-8 → His and Asp-122 → Glu. The two substitutions produce a marginal stabilization of the minke whale myoglobin relative to that of the humpback whale. The result is predicted on electrostatic grounds (Table IV), in which a net destabilization from the Gln → His substitution at position 8 is overridden by the stabilizing effect of the Asp-122 → Glu substitution (Table V). The positive charge of His-8 is introduced into a net positive field contributed primarily by Lys-16, Lys-79, and His-119. The extension of the side chain of residue 122 brings the carboxyl group into closer proximity of the charge borne by Lys-16.[6] The minke whale myoglobin shares the Ala-121 substitution with the humpback whale myoglobin. As discussed above, a relative destabilization of the β-turn between the G helix and H helix may occur.

Residues Arg-118 → Lys and Ala-129 → Gly. The two substitutions produce a strong destabilization of the Hubb's beaked whale myoglobin relative to that of the goose-beaked whale. The substantial effects computed on electrostatic grounds, reflecting differences in charge placement and static accessibility of residue 118, are exceeded by the observed effects (Table IV). The replacement of Arg-118 by Lys-118 disturbs the hydrogen bonding for which the guanidinium group is well suited, notably with residues Asp-20 and Glu-27 (Puett, 1973; Takano, 1977). The structural modeling used here, which is based in part on the seal myoglobin structure[6] (Scouloudi, 1978; H. Scouloudi, unpublished results), indicates that the interaction with Asp-20 would be particularly diminished. The introduction of Lys-118 in the model coordinates of Hubb's beaked whale lessens the computed stabilizing electrostatic interaction with Asp-20 and increases the destabilizing one with His-119 (Table V). Additionally, the loss of the hydrogen-bonding capability with Asp-20 would further destabilize the lysine in this position (Finney et al., 1980). No structural basis for a stability difference could be found for the substitution Ala-129 → Gly.

Residues Ser-51 → Thr and Ser-132 → Thr. The two identical substitutions produce a considerable stabilization of the pygmy sperm whale myoglobin relative to that of dwarf sperm whale. The side-chain hydroxyl group of residue 51 in the sperm whale myoglobin structure is hydrogen bonded with the carbonyl O of Glu-54. There is no solution evidence available to explain the stability differences seen between Ser and Thr in this case.

Possible Basis of Extreme Stability of the Sperm Whale Myoglobins. Figures 1 and 2 show the extreme stability of the myoglobins of the sperm whales relative to those of other species. However, Figure 3 suggests that the sperm whales are the least stable with respect to electrostatic interactions. This may be partially offset by hydrophobic free-energy differences (Table III). Additional compensating stabilization may arise from the hydrogen-bonding interactions of Arg-45 (Puett, 1973), which is replaced by Lys-45 in all other cetacean myoglobins. The particularly rich hydrogen-bonding network around Arg-45 allows the foundation of three hydrogen bonds, two more than when a lysine occupies this position (Takano, 1977; Scouloudi, 1978). A parallel case was considered above for the Arg-118 → Lys substitution between goose-beaked and Hubb's beaked whales. Arg-45 has also been implicated in the increased resistance of sperm whale myoglobin over other species to denaturation by copper(II) ions (Hartzell et al., 1968).

Functional Roles and Interdependence of Classes of Stabilizing Interactions. The correlations drawn here between sequence substitutions and stability differences exploit small, circumscribed structural and functional changes to analyze the small net differences no matter how large the total values for the various kinds of interactions may be. The different interactions invoked to explain the present observations play different roles in the structural stability of the native state (Kauzmann, 1959; Tanford, 1968, 1970; Pain, 1978; Finney et al., 1980). Their potentially compensatory nature (Bogardt et al., 1980) is demonstrated by the examples in the present study.

The stability of the myoglobin structure depends on the interplay of these and other interdependent energy forms. The contribution of each type of stabilizing interaction cannot be optimized independently of the others (Finney et al., 1980), but some general functional distinctions between them have been proposed. The interconnected, mobile hydrophobic pools surrounding the heme play a functional role that cannot be substituted by other types of components. Their role depends in turn on the stabilizing polar interactions represented by the electrostatic lattice and hydrogen-bond interactions. The polar interactions retain their stabilizing action throughout the repertoire of motions of the molecules by retaining attractive restoring forces. The approach followed here recognizes the partitioning of the free-energy differences among the different categories of noncovalent interactions and provides a method for probing the structural compensation necessary to converve functional features among evolutionarily related proteins.

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Supplementary Material Available

Rationale for orientation of the side chains of Glu-4, Asp-4, Glu-27, His-36, Lys-45, Lys-118, and Glu-122, the input for the electrostatic calculations and the resultant $pK_{1/2}$ values for each of the cetacean myoglobins, the results of electrostatic calculations on sperm whale structure when each substitution is considered individually, and the results of the statistical analysis of the experimental data (19 pages). Ordering in-

formation is given on any current masthead page.

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Incorporation of Dansylated Phospholipids and Dehydroergosterol into Membranes Using a Phospholipid Exchange Protein†

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ABSTRACT: A nonspecific phospholipid exchange protein (PLEP) preparation was used to transfer dansyl[^{3}H]phosphatidylethanolamine (DNS-PE), dansyl[^{3}H]phosphatidylserine (DNS-PS), and dehydroergosterol (DHE) from sonicated lipid vesicles to electroplax plasma membrane fragments enriched in Na^+,K^+-ATPase with retention of 80–90% of Na^+,K^+-ATPase activity. The transfer of individual fluorescent lipid molecules was distinguished from a nonspecific association of lipid vesicles and membranes by including [^{14}C]triolein, a lipid that is not transferred by PLEPs, in the vesicles. Dansyl ^{3}H-labeled phospholipids (DNS-[^{3}H]PLs) or DHE was considered "incorporated" into the Na^+,K^+-ATPase membranes when fluorophores pelleted with the Na^+,K^+-ATPase preparation without the nonexchangeable [^{14}C]triolein. The locations of incorporated DHE and DNS-PLs were also described by iodide quenching experiments. DHE was not accessible to iodide for quenching, while 75% of the DNS-PLs incorporated into Na^+,K^+-ATPase membrane fragments were accessible to iodide. After a technique was developed for using PLEP to incorporate fluorescent lipids into membranes with the Na^+,K^+-ATPase preparation, DNS-PE, DNS-PS, and DHE were then analogously incorporated into electroplax plasma membranes enriched in acetylcholinesterase (AChE) and into erythrocyte ghosts in order to evaluate the fluorophores as membrane probes. In the subsequent evaluation, the fluorescent properties of membrane-incorporated DNS-PE, DNS-PS, and DHE were systematically compared to the fluorescent properties of the molecules in lipid vesicles. The fluorescence polarizations of both DNS-PLs were increased by the presence of protein in a bilayer. The fluorescence polarization of DNS-PS was greater than the polarization of DNS-PE in both membranes and vesicles. In contrast, the polarization (and the lifetime) of DHE was the same whether the fluorescent sterol was in a membrane preparation or in vesicles. Fluorescence polarization and intensity of all three fluorophores were measured in the bilayer preparations as a function of temperature. The intensities of all three probes and the polarization of DNS-PE in both membranes and vesicles decreased biphasically with a change in slope occurring at 26.0–27.5 °C. DNS-PS in lipid vesicles was depolarized biphasically with increasing temperature, but when incorporated into membranes, DNS-PS was depolarized linearly without a change in slope. The polarization of DHE in either membranous or vesicle bilayers did not change with temperature. Finally, when the Na^+,K^+-ATPase membrane preparation was used as a model system, the question was asked whether or not the fluorescent parameters of the lipid probes (i.e., intensity, polarization, and lifetime) could detect conformational changes in an intrinsic membrane enzyme. Results were negative despite localization of the fluorophores near the protein.

In the past decade, the structure and dynamics of biological membranes have been investigated with fluorescent probes. The application of such probes, however, requires scrutiny of at least two fundamental issues. First, the majority of these probes [e.g., 8-anilino-1-naphthalenesulfonate, diphenylhexatriene, (anthroyloxy)stearic acid, 2-(octadecylamino)-naphthalene-6-sulfonic acid, pyrene, and perylene] do not resemble the lipid molecules present within a membrane (Badley et al., 1973). The molecules may therefore perturb the surrounding environment to an extent where their emission reflects the environment they created rather than that of the membrane (Krishnan & Balaram, 1975; Cadenhead et al., 1975; Curtain et al., 1978). In addition, fluorescent lipid probes lacking an amphipathic structure will not be under the same restraints as membrane lipids. Their fluorescent properties will reflect events at the surface of the bilayer or within the hydrophobic core without the influence of covalent linkage to the other region. Second, the location of fluorescent probes

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