# Proton Nuclear Magnetic Resonance Study of Histidine Ionizations in Myoglobins of Various Species. Specific Assignment of Individual Resonances†

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ABSTRACT: Individual residues in a series of myoglobins from 16 species, comprising 12 cetaceans, 2 pinnipeds, horse, and man, were examined and assigned in terms of C-2 proton resonances observed at 220 MHz. The cetacean myoglobins examined were isolated from muscles of sperm whale, dwarf sperm whale, sei whale, minke whale, humpback whale, California grey whale, Pacific common dolphin, pilot whale, Amazon River dolphin, bottlenosed dolphin, common or harbor porpoise, and Dall porpoise. The pinnipeds were harbor seal and California sea lion. The various myoglobins contained between 9 and 13 histidine residues in their known sequences. Clearly resolvable, titrating histidine $C^{\epsilon}$ proton resonances represented four to eight residues in the species studied. Those corresponding to histidine residues 8, 12, 35, 113, 116, 128, and 152 were fully identified by comparisons between the myoglobin species. Those corresponding to histidine residues 36, 48, 81, and 119 are found in all the myoglobins studied; their assignment required additional evidence from chemical modification. The modifications used were obtained by carboxymethylation with bromoacetate under a variety of conditions and by preparing a specific adduct to the $NH_2$-terminal $\alpha$-amino group. Five histidine residues, at positions 24, 64, 82, 93, and 97, are common to the species studied but were not observable by this method.

Proton NMR is capable of yielding direct and detailed information about the smaller, well-characterized proteins (Meadows et al., 1967; Wüthrich et al., 1970; Migchelson and Beintema, 1973; Cohen and Hayes, 1974; Markley, 1975). The present paper and the following one in this issue (Botelho et al., 1978) deal with the determination of histidine p$K$ values and with some conformational changes that can be recognized by observing histidine C-2 proton resonances in myoglobins. A group of myoglobins from 16 animal species was studied in this way (Botelho, 1975). These myoglobins offer a considerable range of variation in homologous polypeptide sequences. Within this range three cetacean families, the sperm whales, the baleen whales, and the dolphins and porpoises, are emphasized to provide some close structural comparisons (Jones et al., 1977). The majority of the sequences of these myoglobins have been determined since the NMR data were collected and first analyzed (Botelho, 1975). Every amino acid substitution encountered so far has been found to fit qualitatively into the three-dimensional structure of sperm whale ferrimyoglobin (Watson, 1969; Takano, 1977). Between the species studied, numerous spectral and other physical similarities have been noted in the present work and in related studies (Wüthrich et al., 1970; Hartzell et al., 1968a,b; Marks et al., 1971; Friend et al., 1977). The group of myoglobin species, therefore, provides a set of structurally homologous proteins that can be used for the purpose of NMR resonance assignments (Botelho, 1975), determination and interpretation of p$K$ values (Shire et al., 1974a,b, 1975; Botelho et al., 1978), and recognition of effects on conformation and reactivity with changes in pH or heme ligand (Hartzell et al., 1968a; Botelho, 1975; Friend et al., 1977). The three-dimensional structure of the sperm whale myoglobin (Takano, 1977) provides a reference, and this myoglobin was chosen for most experiments involving chemical modification.

The myoglobin species compared here contain between 9 and 13 histidine residues. Five of these residues that are conserved throughout all the species, those at positions 24, 64, 82, 93, and 97, are not readily observed by proton NMR (Hayes et al., 1975). Four more, at positions 36, 48, 81, and 119, are both conserved and observable by high-resolution proton NMR. These conserved residues require assignment through chemical-modification procedures and other evidence. The remaining histidine residues in the present set of myoglobins, at positions 8, 12, 35, 113, 116, 128, and 152, are uniquely identified by their presence or absence in one or another sequence. The evidence is redundant; only two of these histidine residues represent unique substitutions in the myoglobins studied. Another form of redundancy in this work was not anticipated at the time the NMR measurements were completed. The sequences of the myoglobins of two of the baleen whales, sei whale and grey whale, are now known to be identical (Jones et al., 1979), and the sequence of the humpback whale myoglobin differs from these two only by the substitution of alanine for glycine at position 121 (Lehman et al., 1978). The sequences of the common dolphin[1] myoglobin and the bottlenosed dolphin myoglobins are identical (Jones et al., 1976; Wang et al., 1977) and so are those of the Dall porpoise myoglobin and common porpoise myoglobin (Meuth et al., 1978; Bradshaw and Gurd, 1969).[2] These identities are helpful

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[1] The sequence of the myoglobin studied here of the Pacific Common Dolphin, *Delphinus delphis* (Wang et al., 1977), differs from that of the Black Sea Dolphin for which the same species designation was used (Kluh and Bakardjieva, 1971).

[2] Meuth et al. (1978) found no difference between the sequence of the Dall porpoise myoglobin and that of a preparation of the same common porpoise myoglobin muscle source as was used for the present work. They revised the identification of residues 83 and 85 of the common porpoise myoglobin (Bradshaw and Gurd, 1969) to be aspartic acid and glutamic acid, respectively.

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TABLE I: Observable Histidine Residues in Myoglobins.[q]

| species | histidine residue | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 8 | 12 | 35 | 36 | 48 | 81 | 113 | 116 | 119 | 128 | 152 |
| **sperm whale and dwarf sperm whale** | | | | | | | | | | | |
| sperm whale[a] | | x | | x | x | x | x | x | x | | |
| dwarf sperm whale[b] | | x | x | x | x | x | x | x | x | | |
| **baleen whales** | | | | | | | | | | | |
| sei whale[c] | | | | x | x | x | x | x | x | | |
| Calif. gray whale[d] | | | | x | x | x | x | x | x | | |
| humpback whale[e] | | | | x | x | x | x | x | x | | |
| minke whale[f] | x | | | x | x | x | x | x | x | | |
| **dolphins and porpoises** | | | | | | | | | | | |
| common dolphin[g] | | | | x | x | x | x | x | x | | x |
| bottlenosed dolphin[h] | | | | x | x | x | x | x | x | | |
| pilot whale[i] | | | | x | x | x | x | x | x | | x |
| Amazon River dolphin[j] | | | | x | x | x | x | x | x | | x |
| common porpoise[k] | | | | x | x | x | x | x | x | | x |
| dall porpoise[l] | | | | x | x | x | x | x | x | | x |
| **miscellaneous** | | | | | | | | | | | |
| harbor seal[m] | x | | | x | x | x | x | x | x | | x |
| Calif. sea lion[n] | | | | x | x | x | x | | x | x | x |
| horse[o] | | | | x | x | x | x | x | x | | |
| man[p] | | | | x | x | x | | | x | | |

[a] *Physeter catoden,* Edmundson (1965); Romero-Herrera and Lehman (1974). [b] *Kogia simus,* Dwulet et al. (1977). [c] *Baleanoptera borealis,* Jones et al. (1978b). [d] *Eschrichtius gibbosus,* Bogardt et al. (1976). [e] *Megaptera novaeangliae,* Lehman et al. (1978). [f] *Baleanoptera acutorostrata,* Lehman et al. (1977). [g] *Delphinus delphis,* Wang et al. (1977). [h] *Tursiops truncatus,* Jones et al. (1976). [i] *Globicaphala melaena,* Jones et al. (1978a). [j] *Inia geoffrensis,* Dwulet et al. (1975). [k] *Phocoena phocoena,* Bradshaw and Gurd (1969); Meuth et al. (1978). [l] *Phocoenoides dalli dalli,* Meuth et al. (1978). [m] *Phoca vitulina,* Bradshaw and Gurd (1969). [n] *Zalophus californianus,* Vigna et al. (1974). [o] *Equus caballus,* Dautrevaux et al. (1969). [p] *Homo sapiens,* Romero-Herrera and Lehmann (1971). [q] The symbol x designates an observable histidine C-2 proton resonance referable to the position in the cited sequence.

in testing the reproducibility of the results under practical conditions.

The titration results for histidine p$K$ values offer a test of the application of the Tanford–Kirkwood electrostatic theory (Tanford and Kirkwood, 1957; Tanford and Roxby, 1972), as extended by Shire et al. (1974a,b). The extended treatment takes into account the surface topology to express the degree of solvent accessibility (Lee and Richards, 1971) of each charged group. The solvent accessibility factor provides for the relative degree of electrostatic communication through the aqueous medium of high dielectric constant containing moving ions and the internal medium of low dielectric constant (Shire et al., 1974a; Tanford and Roxby, 1972). For these and all other computations, the recently redetermined structural coordinates for sperm whale ferrimyoglobin have been used (Takano, 1977). Matthew and co-workers (1978) have shown that the new coordinates of Takano (1977) often lead to distinctly different solvent-accessibility results for sperm whale myoglobin from those derived from the earlier coordinates published by Watson (1969). The corresponding estimates of solvent accessibility for the other species considered here may be estimated by cautious extrapolation from the sperm whale myoglobin structure (Botelho et al., 1978). The solvent-accessibility results also guide the assignment of the histidine ring nitrogen position to be taken as the dominant hydrogen-ion dissociation locus for a given residue (Reynolds et al., 1973; Botelho, 1975). This assignment, in turn, is used in the following paper in this issue (Botelho et al., 1978) to control the choice of the intrinsic p$K$ value for a given residue.

The present paper reports the resonance assignments for the $^1$H NMR spectra of the various myoglobins. These assignments are established either by direct comparisons between species or, where necessary, by evidence from various chemical-modification procedures. The following paper in this issue (Botelho et al., 1978) presents the individual p$K$ values and their interpretation in terms of the structural substitutions and electrostatic theory.

## Experimental Section

### *Materials*

The major myoglobin component from each species was prepared by the method of Hapner et al. (1968). Phosphate buffer, ionic strength 0.1, was used at room temperature to fractionate the crude extract and purify the principal component on C-50 CM-Sephadex,[3] the pH being adjusted between 6.4 and 6.8 depending on the isoelectric point of the particular protein. The homogeneity of the purified myoglobin was verified by cellulose-acetate electrophoresis at pH 9.2 and 5.2. The purified, deionized myoglobin samples were lyophilized and stored at −20 °C.

Table I lists the species studied, showing the sequence positions for those histidine resonances observed to undergo changes of chemical shift with pH. In addition to the residues given in Table I, all species studied contained histidine residues at positions 24, 64, 82, 93, and 97 that were not observable. The species in Table I are grouped according to similarities in the details of the complete sequences which also reflect biological affinities (Jones, 1977; Bogardt, 1978).

### *Methods*

*Preparation of Carboxymethyl Derivatives of Sperm Whale Myoglobin.* The standard carboxymethylation procedure by treatment with 0.2 M bromoacetate at pH 6.8 was that used by Hugli and Gurd (1970b) and Nigen and Gurd (1973).

[3] Abbreviations used: CM, carboxymethyl; SPTC, sulfophenylthiocarbamyl; AT-Lys, $N^{\epsilon}$-acetimidyl-L-lysine.

Certain experiments were run at pH 9.0 after initial adjustment of all solutions to that pH value. The reaction pH was maintained by the pH-stat for 10 days, after which the solution was exhaustively dialyzed against water and lyophilized. For carboxymethylation of the protein in the crystalline state, the type A monoclinic crystals (Kendrew and Parrish, 1956) were treated as described by Hugli and Gurd (1970a), followed by solution in $^2H_2O$ (Oak Ridge) and deionization. The analytical procedures to gauge the extent of formation of $N^{\pi}$-, $N^{\tau}$-, and $N^{\pi},N^{\tau}$-carboxymethyl products have been described (Hugli and Gurd, 1970a,b; Gurd, 1972; Nigen and Gurd, 1973). The preparation and isolation of cyanogen bromide fragments followed the procedure of Dwulet et al. (1975).

*Preparation of $N^{\alpha}$ Adduct of Sperm Whale Myoglobin.* Sperm whale myoglobin was treated at pH 10.5 with a 50-fold excess of methyl acetimidate per lysine residue until the free lysine content was less than 5% (Garner and Gurd, 1975). The derivative was then treated at pH 7.0 in the dark for 24 h with a tenfold molar proportion of 4-sulfophenyl isothiocyanate (Birr et al., 1970). The $N^{\epsilon}$ protecting groups were then selectively removed (Garner and Gurd, 1975; Gurd et al., 1977) by treatment for 24 h at pH 10.5 in a buffer of ammonium hydroxide-acetic acid (15:1, v/v). The product was shown by amino acid analysis and sequential degradation analysis (Edman and Begg, 1967) to correspond to 80% $N^{\alpha}$-sulfophenyl thiocarbamate, as described under Results.

*Sample Preparation and NMR Measurements.* Ferrimyoglobin samples for $^1H$ NMR experiments were prepared by dissolving appoximately 200 mg of lyophilized protein in 2 mL of $^2H_2O$, 0.1 M NaCl. The solution was filtered through glass wool, centrifuged, and then was allowed to equilibrate for 24 h at room temperature to facilitate exchange of the amide protons with the $^2H$ isotope from the solvent. Adjustments of pH were made with 0.1 M solutions of $^2HCl$ (Diaprep) and $NaO^2H$ (Merck). Measurements of pH were made with a Radiometer pH M4 meter equipped with a combination glass electrode. Primary standard NBS buffer solutions in $^2H_2O$ were used for standardization without correcting the pH reading to a p$^2H$ scale (Bradbury and Brown, 1973). In every case, pH was measured at the probe temperature before and after NMR data accumulation, and agreement within ±0.02 pH unit was generally obtained.

Transients were averaged on a Nicolet 1080 computer connected to a Varian high-resolution 220-MHz spectrometer. The 5-mm probe was operated at a temperature of 16 ± 1 °C. Chemical shifts are expressed as parts per million downfield of $Me_4Si$ with acetone as a pH-independent internal standard. The chemical-shift range usually examined for pH dependence of the histidine C-2 protons was 6- to 10-ppm downfield of $Me_4Si$.

Each myoglobin species was titrated identically from approximately pH 4.7 to 10.5 in 0.1 M NaCl. To avoid sample dilution and to maintain a constant ionic strength, two stock solutions of 5 mM protein were prepared, one near pH 10.5 and the other near pH 5.0; intermediate pH values were obtained by mixing in appropriate proportions. Adjustments of pH were made with 0.1 M solutions of $^2HCl$ and $NaO^2H$. Spectra were recorded at intervals of 0.1 to 0.2 pH unit. Duplicate conditions were studied frequently, and many experimental points used for computation are omitted from the illustrations for clarity. The data were analyzed according to the Henderson-Hasselbalch relation or to two linear versions of it. In cases where two distinct processes contributed to a chemical-shift titration curve, the following expressions (Botelho, 1975) were used:

$$\delta = \delta_a - K(\delta - \delta_b)/[H^+] \quad (1)$$

$$\delta = \delta_b + (\delta_a - \delta)[H^+]/K \quad (2)$$

where $\delta$ is the observed chemical shift at a given pH value, $\delta_a$ and $\delta_b$ are the limiting chemical shifts observed at low and high pH, respectively, $[H^+]$ is taken directly as the negative antilogarithm of the pH value, and $K$ is the dissociation constant.

The resonance positions in the spectrum for the imidazolium ion are weighted averages of the dissociated and undissociated species such that the ionization can be described by the Henderson-Hasselbalch equation in terms of chemical-shift positions as a function of pH. Computer programs were used to analyze the data to obtain p$K$ values to an estimated uncertainty of ±0.06.

A nonlinear least-squares fitting program for the Henderson-Hasselbalch equation was used to fit the single proton dissociations and respective acid and base chemical-shift limits to ±0.05 ppm. If perturbations in the titration curves indicated another group titrating with a p$K$ 2 or more pH units removed from the group being observed, the dissociation curve was treated as a sum of two single proton dissociation curves and calculated independently (Marshall and Hanania, 1975). The errors on the acid and base chemical-shift limits are again ±0.05 ppm. If titration curves exhibited an interacting group with a p$K$ within 2 pH units of the group being observed or if the curves showed any further perturbations, the chemical-shift data were instead analyzed by a linear version of the Henderson-Hasselbalch equation (see above). For example, given the base-titration limit $\delta_b$, the slope of the linear portion of a plot of $\delta_{obsd}$ vs. $(\delta - \delta_b)/[H^+]$ determines the p$K$ value for the group of interest, and the intercept determines the chemical-shift acid-titration limit.

## Results and Discussion

*General Characteristics of Titrations.* The assignment of each histidine C-2 proton resonance in the full set of myoglobins from 16 animal species can be simplified if it is found that the chemical shift and titration characteristics of the resonance of any given residue in one myoglobin are similar to those of the corresponding residue in all other myoglobins in the set. Such evidence of overall similarity would, in turn, provide a rationale for interpreting the effects on function or stability of the differences in protein composition introduced by amino acid substitutions between species or by chemical manipulation (DiMarchi et al., 1978; Friend et al., 1977). The results to be presented below show that such similarities do indeed exist and that each histidine C-2 proton resonance observed in one species of myoglobin has a counterpart in all other species that share the particular histidine residue.

The C-2 proton resonances that have been observed to titrate according to the one- or two-stage Henderson-Hasselbalch relation do not correspond to all the histidine residues in the myoglobins. The remaining residues presumably correspond to those that are not titratable in the native protein (Breslow and Gurd, 1962; Gurd, 1970) and that are not accessible to carboxymethylation under conditions in which the native protein structure is retained (Gurd, 1970). The carboxymethylation results define the masked histidine residues as 24, 64, 82, 93, and 97 (Hugli and Gurd, 1970a,b; Nigen and Gurd, 1973). Presumably, these residues are not sufficiently mobile within the protein structure to provide $^1H$ NMR resonances that are narrow enough to be observed in this work.

Four histidine residues that are common to all the myoglobins studied here are the residues 36, 48, 81, and 119. In one way or another these residues can be distinguished on the basis of patterns of carboxymethylation, and this approach has been

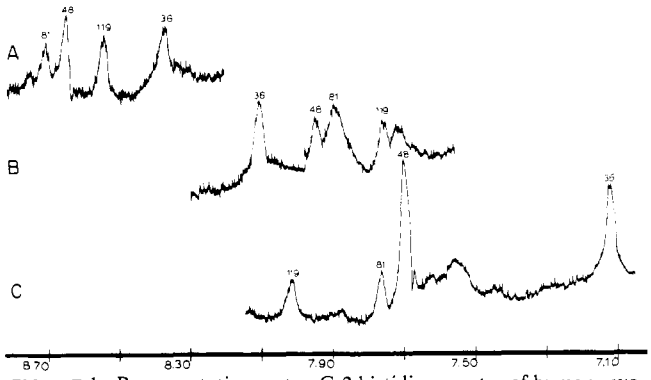


FIGURE 1: Representative proton C-2 histidine spectra of human myoglobin obtained at pH values of (A) 5.1, (B) 7.0, and (C) 9.8.

followed here for residues 36, 48, and 119. Residue 81 has been identified by a separate modification of the amino terminus in such a way as to perturb the C-2 resonance of this nearby histidine residue. Residues 36 and 48 are distinguishable by comparison of carboxymethylation products obtained in solution and in the crystalline state (Hugli and Gurd, 1970a,b). Residue 119 forms the $N^{\pi}$-carboxymethyl derivative slowly in the crystalline state. The resonance positions and titration behavior of unmodified histidine residues 36, 48, and 119 are very little affected by the alkylation of other residues, so that positive correlations can be safely made with the protein derivative preparations.

The carboxymethylation conditions used here appear to preserve the native structure of the protein without consecutive processes that might lead to the exposure of normally masked histidine residues (Banaszak et al., 1963; Banaszak and Gurd, 1964; Gurd, 1970; Nigen et al., 1973). The titration experiments have been done under conditions in which structural alterations by acid or base have been minimized (Breslow and Gurd, 1962; Clark and Gurd, 1967; Hartzell et al., 1968a; Friend et al., 1977). There is evidence from other studies to suggest that the distal histidine residue 64 will become protonated under slightly more acid conditions than are used here (Wilbur and Allerhand, 1977).

The chemical-shift ranges and titration behavior of the common histidine residues 36, 48, 81, and 119 are similar throughout the whole set of myoglobins. The similarities are well illustrated by comparing the titrations of human and common porpoise myoglobins. The spectra of the human myoglobin reflect only the common histidine residues (Figure 1), whereas the porpoise myoglobin shows the additional resonances of histidine residues 113, 116, and 152 (Table I). The two myoglobin sequences differ at 15 other positions out of a total of 153 residues (Romero Herrera and Lehmann, 1971; Meuth et al., 1978). The full titrations shown for human myoglobin in Figure 2 and for porpoise myoglobin in Figure 3 show strong similarities between the corresponding common residues 36, 48, 81, and 119. The parallels between the conserved histidine residue titrations extend without exception to all other myoglobins in the set under study. As shown below, general correspondences among other resonances are also found.

Nine of the 11 histidine C-2 proton resonances encountered in this study showed titration ranges, $\delta_a - \delta_b$, between the respective acid ($\delta_a$) and base ($\delta_b$) titration limits of $1.00 \pm 0.04$ ppm almost without exception. This range is characteristic of a titration process unperturbed by secondary changes affecting the chemical shift (Bradbury and Brown, 1973; Markley, 1975). Of particular interest are the remaining two cases in which deviations from this norm are quite marked, those of the

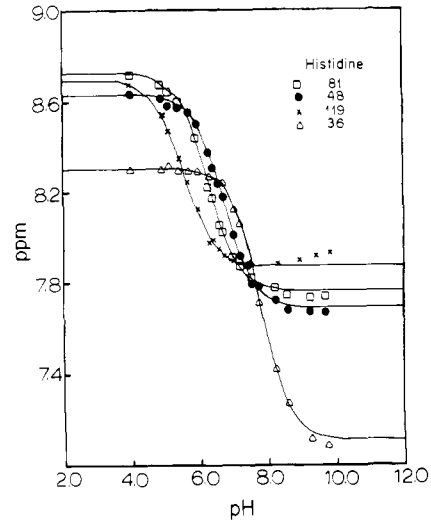


FIGURE 2: Titration curves for human myoglobin with histidine residues identified on the figure.

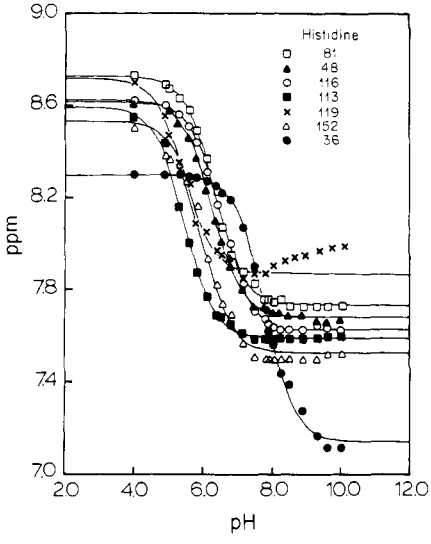


FIGURE 3: Titration curves for common porpoise myoglobin with histidine residues identified on the figure.

resonances shown below to correspond to histidine residues 36 and 119. Figure 1 shows that in the spectra of human myoglobin the normal, upfield trend for the resonance of residue 119 is reversed between spectrum B at pH 7.0 and spectrum C at pH 9.8. The progress of this resonance is seen in detail in Figure 2 where it reaches an upfield end point and then progressively veers back in the pH range above 8. This effect is also shown for the common porpoise myoglobin in Figure 3 and is found without exception in the whole set of myoglobins. Here $\delta_a - \delta_b$ for the primary (lower pH) process is approximately 0.80 ppm.

The influence of a secondary change supervening at the alkaline end of the titration range for the resonance of residue 36 is also observed in all cases. As Figures 2 and 3 show, this process exaggerates the upfield trend of the chemical shift of the primary titration. It is most easily evaluated by the plot shown in Figure 4 (eq 1) in which two p$K$ values of 7.9 and 8.6 are found to fit the major (lower pH) and minor (higher pH) chemical-shift changes with pH. The combination of these two processes corresponds to observed overall values of $\delta_a - \delta_b$ of approximately 1.18 ppm for residue 36 in the whole set of myoglobins. The minor changes reflect the structural conse-

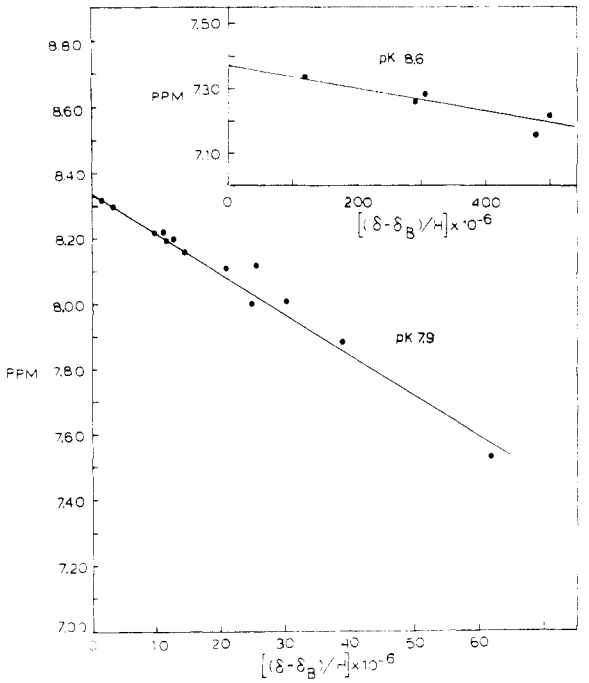


FIGURE 4: Linearized plots as explained under Methods to determine pH dependence of the chemical shift of histidine-36 C-2 proton of sperm whale myoglobin in terms of dominant processes in the lower and higher (inset) pH ranges.

quences of the hemic acid dissocation (Botelho et al., 1978).

*Assignment of Resonances by Comparisons between Species.* Figure 5 compares spectra of the myoglobins of (A) sperm whale, pH 4.7; (B) common porpoise, pH 5.0; (C) California sea lion, pH 4.7; (D) minke whale, pH 5.1; and (E) dwarf sperm whale, pH 5.6. The resonances for sperm whale myoglobin were first numbered 1 through 7, starting at the downfield peak (Figure 5A). As new resonances were encountered during the titrations of the myoglobins of the various species, the numbers 8 through 11 were added (Figure 5B–E). After each resonance had been assigned a recognition number, its chemical-shift position was followed throughout the titration range for the determination of acid and base chemical-shift limits, the fitting of titration curves, and the estimation of p$K$ values. This procedure was repeated with the ferrimyoglobins of all 16 animal species.

Assignments by difference were made under comparable conditions of pH, as exemplified by the comparison between the myoglobins of (A) sperm whale and (B) gray whale at pH 5.0 in Figure 6. This comparison establishes the identity of the resonance of residue 12 that is found only in the myoglobins of the sperm whale and the dwarf sperm whale (Table I). Comparisons between the latter two myoglobin species, in turn, established the identity of the resonance of residue 35 that is found only in the dwarf sperm whale in this set (Table I). The complete set of assignments of resonances shown in Figure 5 is collected in Table II, in which all but the common resonances 36, 48, 81, and 119 are established by comparisons between species. The table includes values of $\delta_a$ and $\delta_b$ as well as p$K$ values determined from least-squares fitting to the Henderson–Hasselbalch relation.

*Carboxymethylation in the Dissolved State.* Histidine residue 36, represented by the resonance of p$K$ 7.97 in sperm whale myoglobin (Table II), was recognized by characteristic differences in its carboxymethylation pattern under various conditions. The resonance was unchanged after the alkylation treatment in solution at pH 6.8 (Figure 7) but was no longer

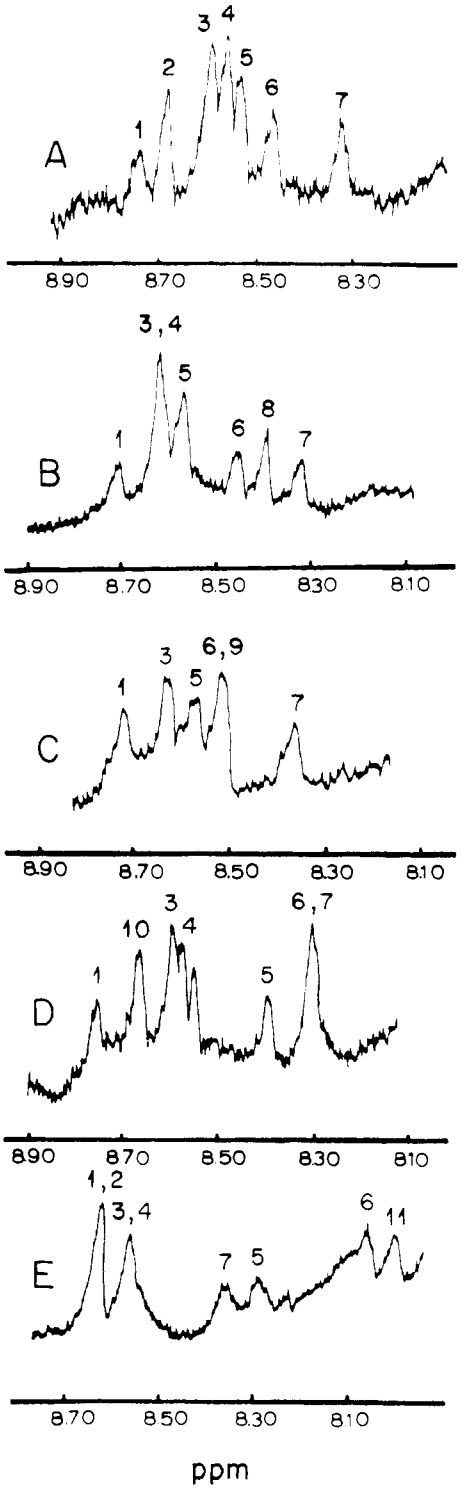


FIGURE 5: Representative proton C-2 histidine resonances in aquoferrimyoglobins of various species. Resonances are numbered for reference as explained in the text and in Table II: (A) sperm whale, pH 4.7, illustrating resonances 1 through 7 (residues 81, 12, 48, 116, 119, 113, and 36, respectively); (B) common porpoise, pH 5.0, illustrating the additional resonance 8 (residue 152); (C) sea lion, pH 4.7, illustrating resonance 9 (residue 128); (D) minke whale, pH 5.1, illustrating resonance 10 (residue 8); (E) dwarf sperm whale, pH 5.6, illustrating resonance 11 (residue 35).

observed after alkylation at pH 9.0 (Figure 8), in keeping both with its unique lack of reactivity among the observable histidines at the lower pH (Hugli and Gurd, 1970a,b; Nigen and Gurd, 1973) and with its relatively high p$K$. Furthermore, this resonance was distinct from the other histidine C-2 protons in

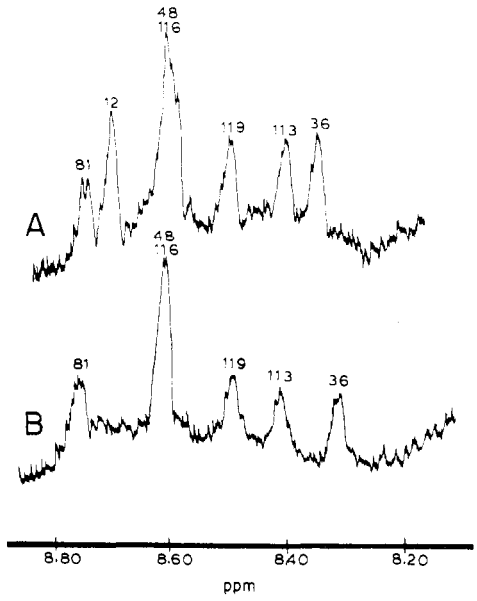


FIGURE 6: Comparison at pH 5.0 of spectra of (A) sperm whale myoglobin and (B) gray whale myoglobin to illustrate the assignment of resonance for histidine residue 12.

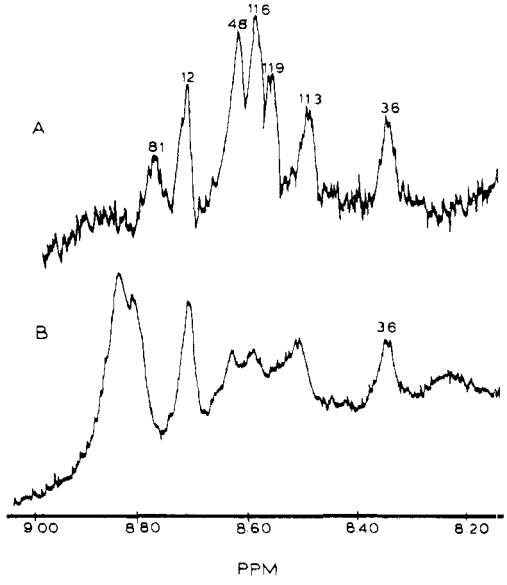


FIGURE 7: Comparison at pH 4.7 of spectra of (A) ummodified ferrimyoglobin and the (B) preparation previously treated with 0.2 M bromoacetate, 0.5 M phosphate (pH 6.8) for 10 days.

showing relatively slow attenuation by exchange with the $^2H_2O$ solvent at pH 7.0 over a period of 8 days at 38 °C (Markley, 1973). The histidine reaction products in residues per myoglobin molecule following treatment with 0.2 M bromoacetate at pH 6.8, 0.5 M phosphate, for 10 days were: diCM, 4.6; $N^{\tau}$-CM, 1.2; $N^{\pi}$-CM, 0.9; unmodified histidine, 5.3; for a total of 12.0 residues as expected from the composition (Edmundson, 1965). Following the treatment at pH 9.0 for 10 days, the corresponding values were 3.2, 2.7, 0.8, and 5.0 for a total of 11.7 residues accounted for. Several comparable preparations were studied. Previous analyses of the course of the carboxymethylation of sperm whale myoglobin have shown similar results and have established that the monocarboxymethyl derivatives are formed as precursors of the dicarboxymethyl form and, in addition, occur preferentially at certain residues

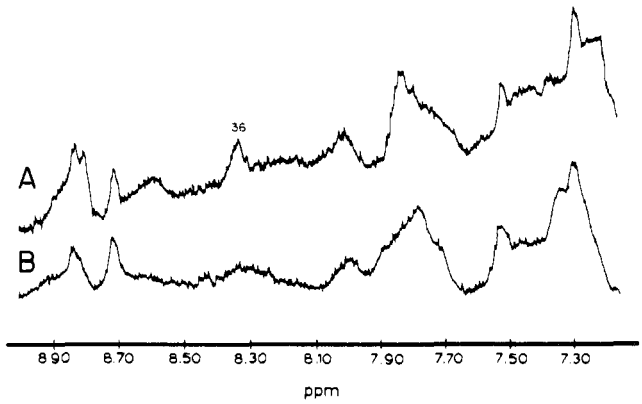


FIGURE 8: Comparison at pH 7.0 of sperm whale myoglobin following carboxymethylation for 10 days with 0.2 M bromoacetate (A) at pH 6.8 (cf. Figure 7B) and (B) at pH 9.0.

TABLE II: Representative Histidine C-2 Proton Titration Data.[a]

| resonance no. | representative species | $\delta_a$ | $\delta_b$ | p$K$ | His res |
|---|---|---|---|---|---|
| 1 | sperm whale | 8.76 | 7.74 | 6.17 | 81 |
| 2 | sperm whale | 8.71 | 7.71 | 6.28 | 12 |
| 3 | sperm whale | 8.62 | 7.63 | 6.73 | 48 |
| 4 | sperm whale | 8.60 | 7.63 | 6.55 | 116 |
| 5 | sperm whale | 8.70 | 7.89 | 5.46 | 119 |
| 6 | sperm whale | 8.58 | 7.58 | 5.49 | 113 |
| 7 | sperm whale | 8.34 | 7.16 | 7.97 | 36 |
| 8 | bottlenosed dolphin | 8.53 | 7.51 | 6.12 | 152 |
| 9 | sea lion | 8.65 | 7.74 | 5.53 | 128 |
| 10 | minke whale | 8.72 | 7.66 | 6.10 | 8 |
| 11 | dwarf sperm whale | 8.49 | 7.46 | 5.52 | 35 |

[a] The resonance peaks are identified as shown in Figure 5. $\delta_a$ and $\delta_b$ are the acid- and base-titration limits of the resonance chemical shifts, respectively, defined as parts per million downfield of $Me_4Si$.

(Banaszak et al., 1963; Hugli and Gurd, 1970b; Nigen and Gurd, 1973; Ray and Gurd, 1967).

All carboxymethylated myoglobin samples studied by NMR were subjected to cyanogen bromide cleavage and isolation of the three pure fragments to confirm the distributions of carboxymethylated histidine forms (Dwulet et al., 1975). The isolation of smaller individual peptide fragments to confirm the previous studies was done on separate samples before the preparations for NMR (Nigen and Gurd, 1973; Dwulet et al., 1975). The identification of the resonance assigned to residue 36 in Figure 7B was confirmed by its titration with a p$K$ of 7.9 in the same NMR sample. Apart from residue 36, the histidine residues that do not undergo carboxymethylation under these conditions at pH 6.8 occur at positions 24, 64, 82, 93, and 97 (Nigen and Gurd, 1973), none of which is observable by $^1H$ NMR under the experimental conditions.

The products of carboxymethylation in solution lead directly and unequivocally to the identification of the resonance of residue 36 alone. However, an approximate correlation between the carboxymethylation pattern of individual histidine residues and the NMR spectra of the modified preparations can be drawn. Conversion of a histidine residue to the dicarboxymethyl form produces a shift of the C-2 proton resonance approximately 0.3 ppm to low field, and conversion to a monocarboxymethyl form produces a corresponding shift of 0.1 or 0.2 ppm (Bradbury and Norton, 1975). Figure 7B shows distinct resonances in the appropriate regions, 8.90 to 8.70 ppm for the dicarboxymethyl derivatives and 8.70 to 8.50 ppm for

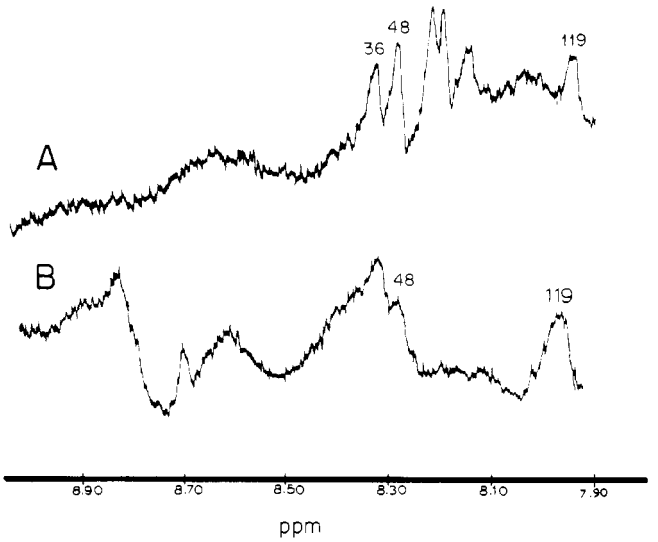


FIGURE 9: Comparison at pH 6.4 of (A) unmodified sperm whale ferrimyoglobin and (B) a preparation previously treated for 7 days in the crystalline state in 70% saturated ammonium sulfate containing 0.5 M phosphate (pH 6.8) with 0.2 M bromoacetate.

the monocarboxymethyl derivatives. The former group between 8.90 and 8.70 ppm has an integrated area corresponding approximately to the five protons implied from the amino acid analysis given above and can be accounted for with residues 12, 81, 113, and 116 as exclusively dicarboxymethyl products and the two residues 48 and 119 as partially in that form. As expected, these resonances are pH independent, and they broaden beyond detection at pH values greater than about 8.0 (Bradbury et al., 1975; Bradbury and Norton, 1975).

Three resonances appear in Figure 7B in the range of monoalkylated derivatives, 8.70 to 8.50 ppm, each corresponding to approximately 0.5 proton. Samples were withdrawn at several intermediate stages during two series of alkylation reactions to obtain indications of the assignments of these resonances by relating changes in resonance intensity with the changes in extent of modification of particular residues. That shown at 8.52 ppm in Figure 7B is tentatively assigned to the $N^{\pi}$-CM derivative (Hugli and Gurd, 1970b) of histidine residue 119 and titrated with a p$K$ of approximately 5.7. That shown at 8.64 ppm is tentatively assigned to the $N^{\tau}$-CM derivative of histidine residue 48. The resonance at 8.61 ppm in Figure 7B could not be ascribed to any predominant residue.

*Carboxymethylation in the Crystalline State.* The overall composition with respect to forms of histidine residues per molecule of the myoglobin preparation carboxymethylated in the crystalline state at pH 6.8 was: DiCM, 2.7; $N^{\tau}$-CM, 1.5; $N^{\pi}$-CM, 0.8; unmodified histidine, 7.3; for a total of 12.3 residues. Figure 9 compares the $^1$H NMR spectra at pH 6.4 of (A) unmodified sperm whale myoglobin and (B) the above preparation carboxymethylated in the crystalline state. Under these conditions, the only NMR observable histidine residues that escape modification are residues 48 and, to a large extent, 119 (Hugli and Gurd, 1970a). At the pH of the measurements the resonance for residue 48 can be distinguished from a broad downfield band representing monocarboxymethylated products and the resonance of that fraction of residue 36 that was incompletely converted to the $N^{\tau}$-carboxymethyl product. Unfortunately, under these conditions the resonance of residue 119 overlaps a nontitrating resonance band (Figure 8A) and so is exaggerated. The appropriate resonances for residue 36, 48, and 119 were found to titrate with p$K$ values characteristic

TABLE III: Amino Acid Analyses of Products Obtained in the Preparation of the $N^{\alpha}$-Sulfonylphenylthiocarbamyl ($N^{\alpha}$-SPTC) Derivative of Sperm Whale Myoglobin.[a]

| amino acid | unmodified | $N^{\alpha}$-acetimidyl | $N^{\alpha}$-acetimidyl derivative | $N^{\alpha}$-SPTC derivative |
|---|---|---|---|---|
| Asp | 8 | 7.1 | 6.9 | 7.0 |
| Thr | 5 | 3.1 | 3.2 | 3.1 |
| Ser | 6 | 6.4 | 6.2 | 6.4 |
| Glu | 19 | 18.9 | 19.0 | 18.8 |
| Pro | 4 | 3.8 | 3.9 | 4.1 |
| Gly | 11 | 11.0 | 11.0 | 11.2 |
| Ala | 17 | 17.0 | 17.0 | 17.1 |
| Val | 8 | 8.4 | 7.6 | 7.5 |
| Met | 2 | 2.2 | 2.2 | 2.0 |
| Ile | 9 | 6.5 | 6.9 | 6.5 |
| Leu | 18 | 18.4 | 18.1 | 18.0 |
| Tyr | 3 | 2.5 | 2.6 | 2.5 |
| Phe | 6 | 5.7 | 5.6 | 5.7 |
| Lys[b] | 19 | 2.3 | 2.1 | 18.9 |
| His | 12 | 11.5 | 11.8 | 12.2 |
| AT-Lys[b] | 0 | 18.3 | 18.1 | 0.0 |
| Arg | 4 | 3.0 | 3.2 | 3.1 |

[a] See Methods for preparative procedures. Samples were hydrolyzed for 24 h as described, without extrapolation to zero time for threonine and serine and without prolonged hydrolysis to cleave the Ile–Ile bond (residues 111 and 112). [b] The low recovery of $N^{\epsilon}$-acetimidyllysine (AT-Lys) is caused in part by the instability of this derivative under the standard acid hydrolysis conditions (Garner and Gurd, 1975). [c] See Table IV for extent of $N^{\alpha}$ derivatization. See also text footnote 4.

of the unmodified protein. The full series of titrations (not presented) further served to show that the intensity of the resonance assigned to histidine-48 was greater than those for residues 36 and 119 which are known under these conditions to undergo gradual modification to the $N^{\tau}$ and $N^{\pi}$ derivatives, respectively (Hugli and Gurd, 1970a).

The preparation used for the measurements in Figure 9B was treated with bromoacetate for 7 days rather than the more usual 10 days (Hugli and Gurd, 1970a). This strategy left more of the unmodified form of residues 36 and 119 to appear as markers in the spectrum. Cleavage of the preparation following NMR analysis and isolation of the appropriate peptides confirmed the presence of some unmodified residue 36 as well as of unmodified residue 48. These observations show that, in the crystalline state, residue 48 escapes carboxymethylation under conditions in which it is alkylated in solution (Hugli and Gurd, 1970a,b).

The formation of the $N^{\tau}$-monocarboxymethyl derivative of histidine residue 36 observed in the crystalline state has an interesting counterpart in the preparation carboxymethylated in solution at pH 9.0 (Figure 8). At the high pH, the yield of the $N^{\tau}$-carboxymethyl derivative in the reaction product was the highest observed in this or the preceding work (Banaszak et al., 1963; Hugli and Gurd, 1970a; Nigen and Gurd, 1973); the analysis showed 3.2 residues of di-CM derivative, 2.7 residues of $N^{\tau}$ derivative, 0.8 residue of $N^{\pi}$ derivative, and 5.0 residues of unmodified histidine for a total of 11.7 residues per myoglobin molecule. The high proportion of the $N^{\tau}$ derivative of residue 36 was confirmed by analysis of the first cyanogen bromide peptide (residues 1–55) obtained by cleavage of the protein following the completion of the NMR measurements: 1.2 residues of di-CM derivative, 1.6 residues of $N^{\tau}$ derivative, and 0.8 residue of unmodified histidine, corresponding, respectively, to residues 12, 36 + 48, and 24 (Hugli and Gurd, 1970a,b).

TABLE IV: Determination of the Percentage of $N^{\alpha}$-SPTC Derivative Formed.[a]

| degradation stage | recovery | | |
|---|---|---|---|
| | amino acid | nmol | % |
| 1 | Val | 26 | 20 |
| 2 | Leu | 117 | |
| 4 | Glu | 130 | |

[a] Determined by difference as 80% from recovery of underivatized N-terminal residue in automated Edman degradation. The yield of glutamic acid (residue 4) was taken as a measure of the total apoprotein. The recovery of valine in the first round represents only cleavage from the underivatized protein chain, since the $N^{\alpha}$-SPTC-valine is not recovered by the extraction and back-hydrolysis steps.

*Incorporation of Amino-Terminal Adduct.* The proximity of the amino terminus of myoglobin to the side chain of histidine residue 81 prompted the preparation of an adduct to the amino terminus that could be expected to perturb preferentially the resonance behavior of residue 81. Table III shows the amino acid analysis of the product of coupling of 4-isothiocyanato benzenesulfonate to the derivative previously protected on the lysine residues with the acetimidyl group. The adduct on the $\alpha$-amino group, an $N^{\alpha}$-sulfonylphenylthiocarbamyl derivative, both contains an aromatic ring and, in addition, bears a negative charge on its sulfonic acid group. Characterization of intermediate products was made by the analyses reported in Table III, and the evidence for 80% coupling to the $\alpha$-amino group is provided in Table IV.[4] Figure 10 compares at pH 5.0 the spectra of (A) unmodified sperm whale myoglobin and (B) the $N^{\alpha}$-sulfonylphenylthiocarbamyl ($N^{\alpha}$-SPTC) adduct. The contribution of the characteristic resonance of the C-2 proton of residue 81 is clearly and selectively diminished by interaction with the adduct.

*Conservation of pK Values.* The representative p$K$ values in Table II show a considerable variation between the different histidine residues. Full description of the p$K$ values observed with the various myoglobin species is reserved for the following paper in this issue (Botelho et al., 1978). Figure 11 summarizes the ranges of pK values for each histidine residue in the whole set of myoglobins. The ordinate denotes the observed p$K$ value, and the vertical bars show the ranges found within each of the groups of species (Table I) for the residue denoted by the numeral above the bracket. To make some allowance for experimental error, the ranges have been extended by 0.05 p$K$ unit on each end, with a minimum bar length for clarity of 0.15 unit. As many as four bars are shown for each residue, corresponding to the four main groups of the sperm whales, the baleen whales, the porpoises and dolphins, and the miscellaneous species (Table I).

The results in Figure 11 show that the p$K$ values for a given histidine residue group rather distinctively within their ranges of 0.3 to 0.4 unit. The general similarities discussed above among the observable residues conserved among all the myoglobin species are found in terms of all p$K$ values as well. This conservation opens the way for the close comparisons drawn in the following paper in this issue (Botelho et al., 1978). It is related to the evolutionary conservation of protein charge put forward by Jukes et al., (1975).

The p$K$ assignments in this set of homologous proteins of known sequence form the basis for a more conclusive test (Botelho et al., 1978) of the electrostatic theory than was possible earlier (Shire et al., 1974a,b, 1975; Botelho, 1975).

[4] Since this work was completed, methods have been developed for the convenient isolation of the $N^{\epsilon}$-acetimidyl derivative completely free of the $N^{\alpha}$-acetimidyl form (Gurd et al., 1977; DiMarchi et al., 1978).

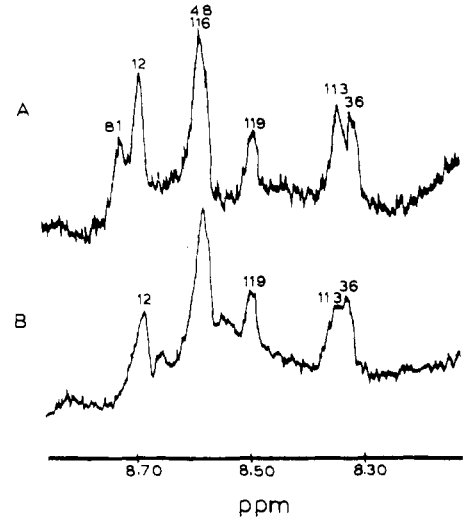


FIGURE 10: Comparison at pH 5.0 of (A) unmodified sperm whale ferrimyoglobin and (B) a preparation composed of 80% of the $N^{\alpha}$-sulfophenylthiocarbamyl derivative along with the underivatized protein.

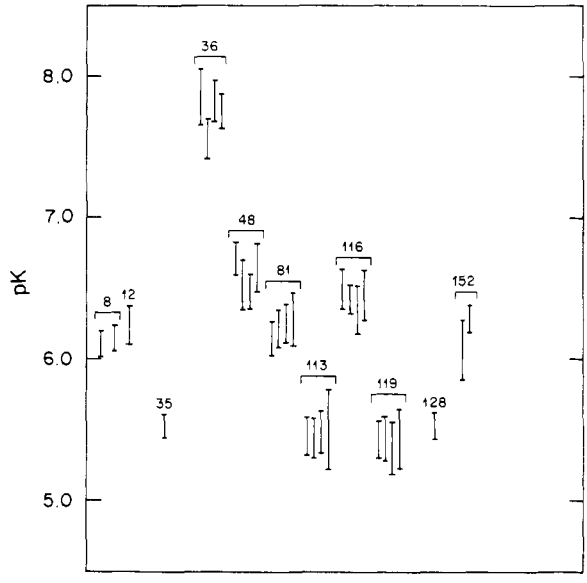


FIGURE 11: Ranges of p$K$ values for each residue observed, shown as bars extended at the limits by 0.05 unit, with a minimum range of 0.15 unit for clarity. The p$K$ values are shown as ordinate. As many as four bars are shown for each residue, bracketed beneath each residue number, corresponding in order to the main groupings (Table I) according to sperm whales, baleen whales, dolphins and porpoises, and miscellaneous; single bars apply to residues 12, 35, and 128 that find representatives in only a single group.

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