Changes in Side Chain Reactivity Accompanying the Binding of Heme to Sperm Whale Apomyoglobin

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Considerable attention has been devoted recently to the study of conformational changes occurring during enzyme-substrate interaction (1, 2). Presumably the nature of the interaction between apoproteins and their prosthetic groups is essentially similar to that between enzymes and substrates, with the exception that no catalytic step occurs subsequent to the initial binding. Studies of the physicochemical changes accompanying the binding of heme to sperm whale apomyoglobin would seem to be of particular interest in that the three-dimensional conformation of sperm whale metmyoglobin is now known (3), and conformational changes occurring on heme-globin interaction may therefore be more amenable to interpretation.

Kinetic studies of heme-globin interaction suggest that the initial product of the reaction must rearrange to form the native heme-protein (4). Conceivably this rearrangement involves a change in protein conformation. Optical rotatory dispersion studies of metmyoglobin give values for the $\alpha$-helix content of approximately 75% (5, 6), whereas similar studies of globin suggest a helix content of 50% (1). In view of the differing Cotton effects in the two proteins, however, it is difficult to assess the significance of this disparity. In a preliminary communication (7), this laboratory cited discrepancies between the $H^+$ ion titration curves of globin and metmyoglobin which could possibly be interpreted as indications of subtle conformational differences. The primary aim of the present study is to examine in greater detail the significance of the relative reactivities of globin and myoglobin derivatives to $H^+$ ion. Secondarily the observed differences in reactivity of imidazoles to $H^+$ ion in the two proteins will be related to changes in reactivity to bromoacetic acid, a reagent for which the reaction with metmyoglobin imidazoles has been recently documented (8).

EXPERIMENTAL PROCEDURE

Reagents—Sperm whale myoglobin was generously supplied by Professor F. R. N. Gurd. All reagents, unless otherwise specified, were analytical grade, and water was glass-distilled.

Preparation of Globin—Globin was prepared only from metmyoglobin. To insure that myoglobin was entirely in the Fe$^{3+}$ state before removal of heme, all myoglobin preparations were examined spectrophotometrically, and any reduced protein present was oxidized by either CuCl$_2$ (9) or K$_3$Fe(CN)$_6$ followed by exhaustive dialysis finally against distilled H$_2$O.

Initially globin was prepared from metMb$^2$ by acetone precipitation according to the method of Theorell and Åkesson (10). Subsequently, a modification of Teale's 2-butanol extraction procedure (11) was employed. An approximately 1% denized solution of metMb was lowered to pH 1.5 at 0°F and then extracted at 4°C, first with an equal volume of 2-butanol (Merck reagent) and twice more with half-volumes of 2-butanol, although the last extraction was generally found to be superfusible. The resulting, slightly straw-colored solution was then immediately dialyzed at 4°C in 18/32 Visking tubing, usually first against several changes of dilute NaHCO$_3$ (50 mg per liter) and then centrifuged to remove some colored precipitate, the protein solution itself being completely colorless in good preparations. This solution was then adjusted to be 0.16 M in KCl, and any additional precipitate was removed by centrifugation. (It was found, however, that only when the original NaHCO$_3$ dialysis was omitted was any additional protein precipitated by KCl, so that this step is unnecessary if NaHCO$_3$ dialysis is introduced.) Many properties of the globin so obtained have already been described (7). Two additional observations are also of interest. First, removal of the heme is completely insured only if the pH of the initial metMb solution is near 1.5. Second, the stability of the resultant globin is markedly lowered in the presence of heavy metal ions. In the cold, in 0.16 M KCl, the presence of trace metal ions will result in the gradual precipitation of denatured globin. In recent preparations, inclusion of a dialysis step with $1 \times 10^{-4}$ M disodium EDTA before dialysis against redistilled H$_2$O resulted in globin preparations which were perfectly stable for over 1 month when stored in 0.16 M KCl at 4°C. Apart from stability on standing, however, no differences among all the globin preparations in properties such as $[\alpha]_20^\circ$, sedimentation constant, or titration were apparent, irrespective of the method of preparation.

Globin solutions were standardized either directly by dry weight determinations or indirectly by the Folin-Lowry (12) assay, with a standard curve obtained with known weights of globin.

Combination of Globin with Heme—The heme preparations used for recombination studies were either hematin, C grade (Calbiochem), or analyzed hemin, 8.61% iron (British Drug

* The abbreviations used are: metMb, sperm whale metmyoglobin; Mb, myoglobin; metHb, methemoglobin; Hb, hemoglobin.
Hemoglobin, Ltd., London). The combining capacity of globin for heme was determined as follows. A small quantity of heme was dissolved in 0.05 ml of NaOH and immediately diluted in borate buffer, pH 9.2, to a final concentration of 1 mM. Increasing aliquots of the heme solution were then immediately added to globin solutions in borate buffer, pH 9.2, ionic strength 0.16. At varying time intervals, aliquots were taken and diluted in FIDTA-acetate buffer, pH 5.6 (13), and the optical density at 409 μm was compared with a similar series of solutions containing heme but no globin. With this method, combination was found to be almost complete within 5 minutes and completed within 2½ hours. The combining capacity so determined was 1 mole of heme per mole of globin, although spurious results were obtained if the heme solution was allowed to remain in alkali too long before addition to globin.

Isolation of regenerated metMb apparently free of either excess globin or heme was accomplished on a large scale by mixing a 1% solution of globin with a slight excess of hemin in borate buffer, pH 8.9, ionic strength 0.16. The resultant solution was exhaustively dialyzed against H₂O, and a slight precipitate (presumably containing excess heme and some protein) was removed by centrifugation. Dry weight, spectral, and titration studies of the resulting supernatant revealed the product to be essentially indistinguishable from native metMb, as will be discussed subsequently. Recovery of metMb by this method was 82%.

Ultracentrifugal Studies—Sedimentation rates of globin and metmyoglobin were determined in a Spinco model E ultracentrifuge equipped with phase plate schlieren optics and automatic temperature control. With Spectroscopic II-G plates (Kodak) for metMb studies, usable patterns could be obtained without the use of special filters at concentrations near 1% by increasing the exposure time to 7 seconds.

Hydrogen Ion Equilibria—The isoionic pH was determined after passage through a Dintzis deionizing column (14). Continuous potentiometric H⁺ ion titrations were performed as previously described (13). For globin and metMb, continuous titration curves were obtained between pH 11 and 3 at 25° and 41° and between pH 12 and 3 at 9°. Several additional points at more alkaline pH were obtained by discontinuous titration.

Spectrophotometric studies of phenolic equilibria were made with globin, metMb, CN⁻-metMb, and CO-myoglobin. CO-Mb for this purpose was prepared as described by Hermans (15), stored under CO, and standardized by dry weight determination. CN⁻-metMb studies were conducted with metMb in the presence of 0.01 M NaCN; the cyanide derivative was spectrophotometrically demonstrated to be stable at pH 12.9 at 25°.

In globin, studies of tyrosine ionization were conducted at both 245 and 295 μm with essentially identical titration curves calculable at both wave lengths. As previously cited (7), globin tyrosine ionization studies at 0.16 ionic strength gave identical results whether conducted at 0.05% protein concentration in glycine-KCl buffer or at 0.4% protein concentration in KCl alone. All studies reported here, therefore, were conducted at approximately 0.05% protein concentration in 0.01 M glycine-KCl buffer, 0.16 ionic strength. The optical densities of all protein solutions were obtained versus H₂O with the appropriate buffer blanks subsequently subtracted.

To insure adequate temperature regulation, water from a central thermostatted bath was pumped in parallel through the thermospacers of the Beckman DU spectrophotometer and the water-jacketed, covered titration vessel in which the optical density and pH, respectively, were simultaneously recorded. The Radiometer TTT1a pH meter was used for all studies at 25° and 41°, and the Radiometer model 4 was used for studies at 9°. pH standards used were 0.05 M potassium hydrogen phthalate, Beckman pH 7 and pH 10 standards, and 0.01 M NaOH in 0.14 M KCl with Harned activity coefficients (16). For studies at 25° and 41°, protein solutions were prepared at room temperature before optical density and pH recording. For studies at 9°, solutions were prepared at 0° and then equilibrated at 9° to minimize irreversible temperature effects (although this has subsequently been found unnecessary). At 41°, a faint turbidity was present in globin solutions near neutrality at 0.16 ionic strength, but not at 0.02 ionic strength. Values of percentage of ionization at this temperature were therefore calculated by using the molar extinction at pH 7 found for the lower ionic strength.

All optical densities were determined in 1 cm covered cuvettes. Temperature regulation was within ±0.1° at 25° and 41°, and within ±0.5° at 9°.

Optical Rotation Studies—Optical rotation studies were performed at the sodium D-line with a Schmidt and Haensch polarimeter, model 88, in a room regulated at 19°. Studies of the effect of pH on rotation of globin at different temperatures were performed as follows: An approximately 1% solution of globin in 0.16 M KCl was adjusted with NaOH to the desired pH at the appropriate temperature. The solution was then transferred to the covered polarimeter tube which was immersed in a water bath at the same temperature. The tube was maintained in the water bath until immediately before the readings, at which time it was quickly dried and readings were taken over a period of about 5 minutes and extrapolated to zero time. The temperature of each solution can then probably be regarded as accurate to only within ±2°.

Reaction of Globin with Bromoacetic Acid—Bromoacetic acid (Fisher, “highest purity”) was recrystallized from tolulene and petroleum ether.

To native globin in 0.16 M KCl, pH 7.4, enough of a self-buffered solution (pH 7.9) of K₂HPO₄ and bromoacetic acid was added so that the final conditions were 1% globin, 1 M phosphate, and 0.2 M bromoacetic acid, pH 7.4. The mixture was allowed to stand at room temperature with occasional shaking for 7 days. Moderate precipitation was generally observed during the course of the reaction. In one reaction, therefore, precipitate and supernatant fractions were separated and then individually exhaustively dialyzed against H₂O and lyophilized. Amino acid analyses of the two fractions indicated no significant differences. In the subsequent reaction, then, precipitate and supernatant were not separated but dialyzed, lyophilized, and analyzed together. Amino acid analyses of the two runs gave identical results. Tryptic hydrolysis and peptide mapping of the carboxymethylated globin preparations were performed by Dr. L. J. Banaszak and Professor F. R. N. Gurd as previously described (8).

One reaction with acid-denatured globin was also investigated. Here, globin in 0.16 M KCl was first allowed to stand at pH 2.94 Amino acid analysis and peptide mapping of carboxymethylated globin were performed by Professor F. R. N. Gurd and Dr. L. Banaszak.
Properties of Regenerated Metmyoglobin—The ease of regenerating native metMb from globin prepared by acid-acetone precipitation has been well documented (10). On the other hand, globin prepared by 2-butanone extraction has not been as thoroughly studied. As previously stated (see “Experimental Procedure”), we have found no demonstrable differences in the globin prepared by these two procedures provided that all material insoluble in 0.16 M KCl at pH 7 to 8 is removed. In Table 1, the positions and intensities of the major absorption bands in native metMb and in regenerated metMb prepared from 2-butanone-extracted globin are compared. It is readily apparent that no significant difference exists between the two proteins. In Fig. 1, the titration curve of the same regenerated metMb is compared with that obtained for native metMb. No significant differences between the two proteins are apparent upon titration from pH 9 to 3 with HCl. The back-titration curve, obtained by rapid titration from pH 3 with NaOH, is similar to that obtained with some but not all metMb preparations. The reason for this behavioral difference among various metMb preparations is not apparent, but as the continuous rapid back-titration represents a nonequilibrium situation during which some native protein appears to be regenerated, it may be simply a reflection of differences in protein concentration or in the length of time for which the protein was allowed to remain at pH 3. In any event, the globin studied appears to have a conformation capable of reacting with heme to give metMb. Presumably the preparation of globin by the 2-butanone extraction procedure, which involves exposure to pH 1.5, is not deleterious despite the fact that globin is denatured at this pH (7), because this denaturation, like that of metMb itself, is reversible.

Ultracentrifugal Studies of Globin and Metmyoglobin—A somewhat crude indication of the relative conformations of globin and metMb may be obtained from the ratio of their frictional coefficients ($f_{\text{globin}}/f_{\text{metMb}}$). From the known molecular weight of the two proteins and their sedimentation rates at infinite dilution under comparable conditions of pH and ionic strength, this ratio may be obtained from the equation

$$
\frac{f_{\text{globin}}}{f_{\text{metMb}}} = \frac{\rho_{20,w}(\text{metMb})}{\rho_{20,w}(\text{globin})} \times \frac{\text{mol. wt. globin}(=17,197)}{\text{mol. wt. metMb}(=17,830)}
$$

(1)

The sedimentation rates of globin and metMb at several concentrations in phosphate-KCl buffer, ionic strength 0.16, were determined at 25°C and, although unnecessary for comparative purposes, were corrected to $s_{20,w}$ by using values of 0.74 for the partial specific volume and density and viscosity data interpolated from the International Critical Tables. The results are shown in Fig. 2. For metMb, $s_{20,w}$ at infinite dilution is 1.98. For globin, the best value of $s_{20,w}$ at infinite dilution is 1.87. The measured ratio of frictional coefficients, 1.02, would seem to suggest that no major conformational difference exists between the two proteins. Theorell and Åkeson (10) have reported values of $s_{20,w}$ at infinite dilution of 1.84 and 1.96 for horse apomyoglobin and CO-myoglobin, respectively. On the assumption that the horse and whale myoglobins have the same molecular weights, these sedimentation data similarly suggest that no definitive conformational differences between globin and myoglobin are observable by such gross measurements. Viscosity studies by Eylar¹ of sperm whale globin and metMb lead to similar conclusions.

Equilibria of Imidazoles with $H^+$ Ions in Globin and in Metmyoglobin—Analysis of the titration curve of metMb has shown that only 6 of the 12 metMb imidazoles are in $H^+$ equilibrium in the native protein and that the remainder were masked in the unprotonated form and released upon acid denaturation (13). In a preliminary account (7), differences in $H^+$ ion titration between globin and metMb in the neutral pH range were interpreted to indicate the release of 2 to 3 additional imidazoles into $H^+$ ion equilibrium upon removal of heme from metMb. In

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¹ E. Eylar, personal communication.

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Table 1

<table>
<thead>
<tr>
<th>$\lambda_{max}$ (nm)</th>
<th>$\epsilon_{max}$ (X10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>Regenerated</td>
</tr>
<tr>
<td>488</td>
<td>488</td>
</tr>
<tr>
<td>498</td>
<td>498</td>
</tr>
<tr>
<td>505</td>
<td>505</td>
</tr>
<tr>
<td>630-635</td>
<td>630-635</td>
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</table>

Fig. 1. Experimental titration curves of native metMb and metMb regenerated from globin and heme. Temperature, 25°C; ionic strength, 0.16. Filled circles: titration from pH 9 to pH 3; open circles: back-titration from pH 3. For native metMb: ---, titration from pH 9 to pH 3; ----, back-titration of two different preparations from pH 3.

for 30 minutes at room temperature and then rapidly adjusted to pH 7 with NaOH before addition of K$_2$HPO$_4$ and bromoacetic acid.
some small part, this analysis was based on a tentatively revised (and now abandoned) assumption of a total of 11 histidine residues in metMb. The following more detailed accounting of globin imidazole H+ ion equilibria will rest on the now more definite total of 12 metMb imidazoles. It should be noted, however, that there is a discrepancy between the observed isionic pH values of metMb and globin and the most recent amino acid analyses (17). Some small part of which may reside in the imidazole analysis.

In Table II, the current estimate of titratable groups in metMb is given. The isionic pH of metMb is 7.86 and is unchanged by essentially complete guanidination (18). No lysine residues therefore titrate below pH 7.86, and even if complete deprotonation of imidazoles is assumed at this pH (an assumption not completely warranted by the estimated imidazole log $k'$), it is apparent that at pH 7.86 the calculated charge should be at least 1.5. In globin this discrepancy is again seen. The isionic pH of 8.63 is compatible with that of metMb when the effect of heme removal is allowed for (7). Here, even generously allowing for complete deprotonation of the carboxyls, $\alpha-NH_2$, 12 imidazoles, and 0 to 1 $e-NH_2$ group at this pH (a number in accord with the maximal acid-binding capacity of 34 to 35 (7)), the calculated charge is 1 to 2 at pH 8.63. At present, however, there is no recourse other than to assume the correctness of the amino acid analysis as given in Table II.

The difference in titration behavior of the native and acid-denatured forms of metMb, i.e. the titration difference curve, is primarily a reflection of the difference in $w$, the electrostatic work factor; and the number of imidazoles in H+ ion equilibria in the two species (13). As a first approximation, the value of $w$ for a given class of groups in both native metMb and native globin may be considered the same, as the sedimentation data indicate no major conformational differences between the two native proteins. Moreover, $w$ for the two acid-denatured proteins appears to be the same. Analysis of carboxyl ionization in denatured globin at 0.16 ionic strength and $25^\circ$ can be shown to give average values of 0.033 and 4.49 for the 21 globin carboxyls, in good agreement with the values of 0.034 and 4.49 obtained for the 29 carboxyls of denatured metMb (13). A rough approximation of the relative number of imidazoles released into H+ ion equilibrium in globin and in metMb can then be obtained from the relative magnitudes of the difference curves alkaline to the pH of denaturation. At $25^\circ$ and 0.16 ionic strength, estimation of the difference curve is complicated by precipitation of the denatured protein near pH 6. The pH of precipitation, however, is increased to above 7 if the temperature is lowered to $9^\circ$, and more reproducible difference curves are obtained. In Fig. 3, the titration curves of globin and metMb at $9^\circ$ and 0.16 ionic strength are shown. At this temperature, globin denaturation appears to occur gradually between pH 5.5 and 5.2 as compared to a more abrupt transition near pH 4.6 for metMb. In Table III, the relative heights of the difference curves ($\Delta \Delta \eta$) in the two proteins are shown. If a release of 6 imidazoles upon acid denaturation in metMb is assumed, the number of imidazoles released in globin upon denaturation most closely approximates 3. The number of imidazoles estimated by this method to be in H+ ion equilibrium in native globin therefore would be 9, as compared to 6 in native metMb.

A more accurate measure of the number of imidazoles in H+ ion equilibrium would depend only upon analysis of the titration curve of the native protein. A preliminary titration curve analysis based upon the increased number of groups titrating in globin relative to metMb in the neutral pH range suggested that 8 or 9 imidazoles were in H+ ion equilibrium in native globin (7). This analysis rested on the assumption that the intrinsic pK of the titratable globin histidines was identical with that found for the same in metMb. A more precise analysis may be obtained from the equation:

$$p\mathrm{H} = \log k' - wZ(0.808) - \log[\bar{\eta}_\text{in}/(n_1 - \bar{\eta}_\text{in})]$$

where

- $w$ is the intrinsic H+ ion association constant;
- $Z$ is the net protein charge;
- $n_1$ is the number of titratable groups in a given class;
- $\bar{\eta}_\text{in}$ is the number of protonated groups in that class.

This assumption is based on the more approximate treatment of titration curves with a distributed charge model (19). It is recognized, however, that with the fixed charge model of Tanford and Kirkwood (21), $w$ might be expected to vary for different classes of groups within the same protein.
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METMb TITRATION. 9°C.

\( \text{pH} 11 \rightarrow 3 \)

\( \text{pH} 3 \rightarrow 8 \)

GLOBIN TITRATION. 9°C.

\( \bullet \) \( \text{pH} 11 \rightarrow 3 \)

\( \circ \) \( \text{pH} 3 \rightarrow 8 \)

GLOBIN TITRATION, 41°C.

\( \Delta \Delta \) \( \text{pH} 8 \rightarrow 11 \)

The data obtained suggest that the titration curves are most readily fit with a value of \( n_i = 9 \) if a single class of histidines is assumed. The titration curves shown in Fig. 5 can be shown to be completely reversible under the existing titration conditions to a value of \( Z = +9 \). However, significant deviations from the alkaline branch of the globin titration curve, but does not significantly affect the results. The other log \( k' \) values are those shown in Table II.

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**FIG. 3.** Titration curves of globin and metMb at 9°C, ionic strength 0.16, and partial titration curve of globin at 41°C, ionic strength 0.16. For metMb: —, titration from pH 11.3 to pH 3; ---, back-titration from pH 3. For globin at 9°C: ●, titration from pH 11.4 to pH 3; ○, back-titration from pH 3. For globin at 41°C: ▲, titration from pH 8 to pH 11.

**FIG. 4.** Continuous titration of globin at 25°C in KCl. At ionic strength 0.16: ●, pH 9 to pH 3; ○, back-titration from pH 3. At ionic strength 0.06: ■, pH 9 to pH 3; □, back-titration from pH 3.

**FIG. 5.** Plots of pH + log \( k'/(n_i \cdot v_{im}) \) against \( Z \) for globin imidazole groups; \( n_i = 8 \). ●, ionic strength 0.16; ○, ionic strength 0.06. Temperature, 25°C.

**TABLE III**

Relative magnitudes of difference curves in globin and in metmyoglobin at 9°C and 0.16 ionic strength

<table>
<thead>
<tr>
<th>pH</th>
<th>( \Delta pH )</th>
<th>Globin</th>
<th>MetMb</th>
<th>Globin imidazoles released by denaturation*</th>
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<tbody>
<tr>
<td>5.8</td>
<td>1.8</td>
<td>4.0</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>1.8</td>
<td>3.6</td>
<td>3.0</td>
<td></td>
</tr>
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<td>6.2</td>
<td>1.7</td>
<td>3.2</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>1.4</td>
<td>2.6</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>6.8</td>
<td>1.0</td>
<td>2.0</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>0.5</td>
<td>1.6</td>
<td>1.9</td>
<td></td>
</tr>
</tbody>
</table>

* These values are based on the assumption that the number of imidazoles released by acid denaturation in metMb is 6.

by the usual plot of log \( v_{im}/(n_i \cdot v_{im}) \) against \( Z \), assuming varying values of \( n_i \), to determine log \( k' \) and \( w \) for the globin histidines. The average of a large number of globin titration curves at 0.16 and 0.06 ionic strength and 25°C are shown in Fig. 4. In Figs. 5, 6, and 7, the aforementioned plots are calculated from the data assuming values for \( n_i \) of 8, 9, and 10, respectively, at the two ionic strengths. (Values of \( n_i = 7 \) can readily be shown to give unreasonable results.) \( v_{im} \) was determined from the number of groups titrating below the isoionic pH, with corrections for \( \epsilon \)-NH, \( \alpha \)-NH, and carboxyl titration made by using respective log \( k' \) values of 10.30, 7.80, and 4.48. (The \( \epsilon \)-NH log \( k' \) was determined by independent analysis of

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linearity in Fig. 5, calculated for $n_i = 8$, begin to occur at $Z = +7$. Moreover, assuming no major change in molecular dimensions between globin and metMb, the theoretical value of $w$ calculated from approximate $H^+$ ion titration theory (19) has been calculated as 0.065 to 0.069 at 0.16 ionic strength and as 0.106 to 0.113 at 0.06 ionic strength (13). For metMb, the experimentally obtained values of $w$ at these ionic strengths were 0.050 and 0.055, respectively. Tanford and Kirkwood (21) have pointed out that although significant deviations from the theoretically derived value of $w$ may be expected if the immediate charge environment of each ionizing group and the number of groups in each class are considered, the change of $w$ with ionic strength should remain relatively constant. The value of 0.060 for the change in $w$ with ionic strength ($\Delta w$) found for $n_i = 8$ seems somewhat high in view of the theoretical value of 0.042 and the experimental value of 0.035 found for metMb (13). On the other hand, the data shown in Fig. 6 for $n = 9$ form a linear plot exactly to the pH where denaturation begins, and then show the expected deviations. Moreover, the calculated values of $w$ at both ionic strengths studied are in essentially perfect agreement with theory. Similar plots for values of $n = 10$ (Fig. 7) also show the expected deviations from linearity in the known region of denaturation, and the effect of ionic strength on $w$ is intermediate between that expected from theory and that found for metMb. However, values of $w$ so obtained for $n = 10$ seem very high, although they might be reconcilable with those of metMb with the use of more rigorous titration curve treatment (21).

**Reaction of Globin Imidazoles with Bromoacetic Acid**—The interpretation of the $H^+$ ion titration curves of metMb by approximate titration theory, indicating the presence of 6 reactive and 6 unreactive histidine residues, was seemingly substantiated by the reactivity of the protein to p-nitrophenyl acetate (13). Recent carboxymethylation studies of metMb (8) suggest that 8 histidines are available for reaction with bromoacetic acid. Conceivably this apparent discrepancy is due to the irreversibility of carboxymethylation, or it might be that more rigorous treatment of the metMb titration curve would disclose the presence of 1 or more additional reactive histidines with slightly abnormal log $k'$ values. Regardless of these difficulties, however, the difference in relative reactivity of globin and metMb imidazoles to bromoacetic acid offers another convenient parameter by which subtle changes in the environment of histidine side chains may be assessed.

Reaction of native globin with bromoacetic acid was carried out in a manner analogous to that for metMb (8). In Table IV, the amino acid analyses of carboxymethylated globin are compared with the theoretical amino acid composition. Only 1 histidine residue appears to be unreactive in globin as compared with 4 unreactive histidines in metMb. The identity of this unreactive residue was established by peptide mapping of trypsin-digested carboxymethylated globin as previously described (8). Only the FG3 histidine could be detected as a nonreactive residue.

One possible cause of the greater reactivity of globin than of metMb imidazoles to bromoacetic acid could be the increased ease of globin denaturation. The possibility therefore exists that the carboxymethylated globin analyzed was no longer "native" in conformation. However, as shown in Table IV, the methionine content of native globin (as with native metMb) was unchanged by carboxymethylation; i.e. the methionine residues were unreactive to bromoacetic acid. Upon acid, alkali, or heat denaturation of metMb (8), and also upon Cu(II) denaturation, at least 1 methionine residue becomes reactive to bromoacetate. Similarly, carboxymethylation of acid-denatured globin definitively indicates an increased reactivity of methionyl residues. It would seem, therefore, that the conformation of carboxymethylated native globin differs significantly from that of the acid- or heat-denatured protein, in which the previously internal methionyl residues apparently become exposed.

On the other hand, urea denaturation of metMb does not seem to cause a similar increase in reactivity of methionyl residues (8), so that absence of methionine reaction may not be a necessary indication of native conformation. A preliminary investigation...
of the relative conformations of native globin and carboxymethylated native globin by other criteria has been initiated by ultracentrifugal studies. A 1% solution of carboxymethylated native globin at pH 7.5 in 0.16 m KCl migrated at 25° as a single symmetrical peak with $\eta_{20, w} = 1.73$ compared with an average value of $\eta_{20, w}$ for globin under similar conditions of 1.78. From the molecular weights of the two proteins, the frictional ratio, $f_{\text{carboxymethylated globin}}/f_{\text{globin}}$, may be calculated as 1.09. These results suggest some conformational change or reaction of globin with bromoacetic acid, but one apparently insufficient to expose methionyl residues or to cause the polymerization noted for acid-denatured globin (22). Moreover, the similarity in sedimentation of metMb and carboxymethyl-metMb has also been noted (8) and consideration of the difference in molecular weight of these two proteins suggests that a similar minor conformational change may occur on reaction of metMb with bromoacetic acid.

It would appear, then, that removal of heme from metMb results in the exposure of 3 additional histidines to bromoacetic acid and presumably involves no major change in heme-protein attachment. That CN–metHb Zurich is electrophoretically inseparable from normal CN–metHb at pH 6.5 while migrating more slowly towards the anode at pH 8.5 (24) would seem only to be explicable in terms of an essentially normal log $k''$ for that histidine in normal hemoglobin. To the extent that metHb and metMb structures are similar, then, the unexpected reactivity of $\varepsilon$-arginine to bromoacetic acid in metMb may be correlated with its reactivity to $H^+$ ion in metHb. Moreover, this correlation suggests that on removal of heme from globin, only 1 or 2

These conclusions are based upon study of a large scale model of metMb, built from the Kendrew structure, in the laboratory of Professor F. R. N. Gurd.
remotely possibly 2 additional histidine, the F_3 and possibly the FG_3, should be released into H^+ ion equilibrium in the absence of conformational changes. From the titration data alone, then, the increased reactivity of 3 histidines to H^+ ion is indicative of a conformational difference between globin and metMb.

It is interesting to note here that the shapes of the titration curves of globin and metMb also suggest another means by which their relative helix contents might be assessed. The greater stability of myoglobin derivatives than of globin to acid (and also to alkali) is undoubtedly due in part to stabilization of the native structure by heme. It appears in Fig. 3, moreover, that metMb denaturation occurs over an appreciably narrower pH range than does that of globin. If the denaturation may be viewed rather simply as a polypeptide helix → coil transition, then the treatment of such transitions, as for example by Schellman (25), may be cited to indicate that the broadening of the transition in globin could be due to a decreased number of residues involved in the unfolding process as well as to the absence of heme stabilization. It would seem, therefore, that a detailed study of the denaturation of both globin and metMb could lead to an estimate of their relative helical contents.

Phenolic Equilibria—In a preliminary communication (7), the ionization of the 3 globin tyrosines was compared with the data of Hermans (15) for phenolic equilibria in CO-Mb. The marked difference observed between the two proteins was most simply attributable to the exposure in globin of the tyrosyl residue found buried in CO-Mb, although it was noted that a possible conformational change occurred in globin within the pH range of tyrosine titration. The relative tyrosine titration curves of globin and several myoglobin derivatives have therefore been re-examined under identical conditions. In Fig. 8, the change in molar extinction at 245 μm in glycine-KCl buffer, ionic strength 0.16, is shown for globin, CO-Mb, and CN^-metMb at 25° as well as for CN-metMb at 9°. The not unexpected similarity of the CO-Mb and CN^-metMb titrations is readily apparent, and the discrepancy between globin and Mb phenolic equilibria is similar, although not quite as striking, as that previously deduced from comparison of globin titrations from this laboratory with CO-Mb titrations from Hermans.

As previously cited, the degree of globin ionization is independent of time within the entire pH range studied, and the titration curve is essentially reversible from pH 13, although a slight shift to approximately 0.2 lower pH unit between pH 11 and 12 on back-titration is discernible (7). In the Mb derivatives, however, time-dependent increases in optical density can be observed above pH 12, becoming more rapid as the pH is raised. Above this pH, the extent of optical density increase at 245 μm can be generally correlated with the extent of denaturation (specifically observed here by optical density changes at 350 μm), an observation compatible with Hermans’ thesis that 1 tyrosyl residue in solution is raised above 11, although even after exposure to pH 13, only a fraction of the total globin is precipitated at pH 7. More dramatic evidence of a major globin conformational change occurring at 25° above pH 11 is seen from a study of the optical rotation at the sodium D-line with change of pH. At 25°, a small decrease in [α]_D from -18° at pH 7 to -21° at pH 11 occurs, followed by a sharp decrease to -45° at pH 12 and to -50° at pH 12.5. No further decrease above this pH is demonstrated.

Actually, preliminary calculations suggest that log k' for lysine may be slightly higher in metMb than in globin. Consideration of this would shift the theoretical globin curve to still more alkaline pH.
The average value of 0.16 at 41° (O), 25° (C), and 9° (A). All values of percentage which no significant optical density increased seemed to occur below pH 7 and 100% ionization at the pH values shown, above which no significant optical density increases seemed to occur. The average value of ΔE265 was 11,500 per tyrosine.

The change in rotation appears instantaneously upon increasing the pH, and is at least partially and instantaneously reversible upon lowering the pH. In two separate experiments at 25°, the rotation of a globin solution in 0.16 M KCl, exposed reversible upon lowering the pH. In one experiment in which globin was exposed to pH 12.5 for only 20 minutes, the rotation on return to pH 10.8 was also -27°. No further decrease on prolonged exposure to pH 10.8 was observed.

The approximately parallel correlation of the optical rotation change with the last third of the total tyrosine titration curve at 25° is a suggestive but not conclusive indication that 1 globin tyrosine may also be masked in the native protein and only become ionizable upon denaturation. The possibility must still be considered that the coincidence of the conformational change with tyrosine ionization is fortuitous and that all 3 tyrosines are merely equally available in native and denatured states. Evidence to the contrary, however, may be gleaned from the comparative globin tyrosine titration curves at 9°, 25°, and 41° (Fig. 9). The pH displacement between temperatures T1 and T2 of a given degree of tyrosine ionization is related to the apparent heat of ionization, Q', by the equation

\[ \Delta H = -\frac{\Delta H(T_2 - T_1)}{23R T_1 T_2} + 0.868 (w_1 Z_1 - w_2 Z_2) \]  

and to the intrinsic heat of ionization, ΔH12, by the equation

12 As defined here, the apparent heat of ionization, Q', is related to the intrinsic heat of ionization, ΔH, as the apparent association constant for H+ ion at a given pH (log k' = 0.868 wZ) is related to the intrinsic association constant (log k'). Equation 5 may therefore be derived directly from Equations 4 and 2.

The value of ΔH for tyrosine ionization, so calculated, should be insensitive to differences in charge and shape between the two temperatures. Q', however, would reflect such differences and, in the pH region of normal tyrosine ionization, should gradually decrease with pH because of the higher enthalpy of lysine ionization and the consequent larger negative charge on the protein with increasing temperature at the same degree of tyrosine ionization. It can immediately be seen from Fig. 9, however, that near 60% ionization a significant increase of titration curve pH displacement occurs, indicating an actual increase in Q' at higher pH. Between 10 and 50% ionization, an average value of Q' between 25° and 9° or between 41° and 25° may be calculated from Fig. 9 as 5.0 kcal or 4.6 kcal, respectively. Moreover, as shown in Fig. 10, no significant change in globin conformation at 25° and 9° is demonstrable by optical rotation below 60% tyrosine ionization. Assuming a value of w = 0.068 at these two temperatures within this pH range, and calculating \( \tilde{Z} \) at the two temperatures from titration data (see (7) and Fig. 3), ΔH below 60% tyrosine ionization can be calculated as 6.6 kcal, in keeping with the usually assigned value of 6.7 kcal for the ΔH of tyrosine ionization. At 41°, approximately half the globin is already denatured below pH 10.8 (see the legend for Fig. 10). By using a value of w = 0.055 (intermediate between the values for the native and acid-denatured species), an average ΔH may be calculated from the titration data (see (7) and Fig. 3) between 10 and 50% ionization as 7.1 kcal, suggesting that the tyrosines ionizing in this pH range are almost equally available in native and denatured conformations. Near 83% ionization, however, the value of Q' has become 9.8 and 11.3 kcal between 25° and 9°, respectively. Moreover, it can be shown (Fig. 10) that at both 25° and 9°, the increase in optical levorotation with pH occurs in a pH range coincident approximately with the last third of the total tyrosine titration, and therefore with a midpoint near 83% ionization. For the same degree of ionization within this temperature range, then, no large difference in w at the two temperatures should be expected, and the increase in Q' would seem to be a reflection of a real increase in ΔH of tyrosine ionization (see Equations 4 and 5). Presumably the discrepancy in Q' near 83% ionization, when calculated within the higher and lower temperature ranges, is a reflection of the fact that at 41°, at the lowest pH at which it was possible to obtain optical rotations (Fig. 10), the observed rotation indicated considerable denaturation when only 50% of the tyrosines were ionized. A difference in w for equivalent degrees of tyrosine ionization may therefore contribute to the increased value of Q'. In the main, however, the simplest conclusion invited by the above data is that part of the total tyrosine titration is conformationally dependent; or, more specifically, that 1 of the 3 globin tyrosines is ionizable only in the denatured protein.

The apparent reversibility of globin tyrosine titration upon return from pH 13 (7) remains to be considered. Admittedly, if 1 globin tyrosine is unmasked upon globin denaturation, an appreciable shift in the observed tyrosine titration to more acid pH should be apparent upon back-titration from pH 13. That this does not occur is most probably attributable to reversibility of denaturation under the titration conditions. As has already been cited, optical rotation criteria suggest that at least two-
thirds of the alkaline denaturation is instantaneously reversible upon lowering the pH. Quite possibly, under titration conditions, in which the protein concentration is more dilute than in optical rotation studies, reversibility is essentially complete. It is also possible that failure to observe complete restoration of the initial optical rotation upon back-titration is due to imperfect refolding of the molecule in areas not adjacent to the abnormal tyrosyl residue. The assumption will therefore tentatively be made that at 25° and 9°, the upper third of the globin tyrosine titration curve is accompanied by a rapid and reversible conformational change which releases a previously masked tyrosine into $H^+$ ion equilibrium.

If the abnormal globin tyrosine is identical with that found buried in myoglobin derivatives, the question remains as to the underlying cause of the striking differences in globin and myoglobin tyrosine ionizations. To a certain extent, of course, this difference is a reflection of the diminished stability of globin to alkaline pH. But it is apparent from Fig. 8 that if only 2 tyrosines are in $H^+$ ion equilibrium in native globin (below pH 11) and in native myoglobin (below pH 12), a significant decrease in log $k'$ of these 2 tyrosines occurs when the heme is removed. Hermans (15) has concluded that in CO-Mb 1 of these 2 tyrosines and in native myoglobin (below pH 11), a significant decrease in log $k'$ occurs when the heme is removed. Moreover, the potentiometric titration curves of CN--metMb and CO-Mb can probably be assumed to be identical with those for metMb at 25° between pH 10 and 12 (13), and $Z$ for these proteins can therefore be calculated within this pH range. With a value of $w = 0.06$ for both globin and myoglobin derivatives, Equation 2 can be used to calculate log $k'$ for these 2 tyrosines as approximately 9.9 in globin and 10.5 in myoglobin derivatives. This latter value is in good agreement with that found by Hermans for the 2 reactive tyrosines of guanidinated metMb. Although both values of log $k'$ fall within the range often described as “normal” for protein tyrosines, the difference between them is significant.

Further evidence supporting a difference in log $k'$ between the 2 freely titratable tyrosines of globin and CN--metMb may be found in the relative values of $Q'$ for the two proteins between 25° and 9°. From the data in Fig. 8, $Q'$ for CN--metMb can be shown to rise gradually from 5.2 kcal at values of $\Delta E_{245}$ × 10$^{-3}$ near 6, to 6.4 kcal at values near 18 (a pH region in which CN--metMb is completely stable and in which the third tyrosine may be presumed buried). By using a value of 11,500 for $\Delta E_{245}$ per tyrosine in globin, the data in Fig. 9 may be recalculated in the same $\Delta E_{245}$ interval to show a decrease from 5.2 to 4.2 kcal. Although the differences in pH displacement represented by these differences in $Q'$ are only of the order of 0.05 pH unit, the reproducibility of the results, coupled with the expected decrease in tyrosine $Q'$ (in globin) as the pH is increased, lends additional support to a difference in log $k'$ between at least 1 of the 2 titratable tyrosines in the two proteins. In metMb, no tyrosines

**Fig. 10. Correlation of optical rotation changes in globin at alkaline pH with tyrosine titration curves.** The ionic strength for all studies was 0.16. The upper 40% of the spectrophotometric tyrosine ionization curves are shown for $9^\circ$, $25^\circ$, and $41^\circ$ (——). At $9^\circ$ (○) and $25^\circ$ (●), the optical rotations are plotted as the percentage of the total rotational change at the sodium D-line in alkali, assuming no change near pH 7. At $9^\circ$ (△), rotations are plotted as percentage of the total change from pH 10.85, the lowest pH at which it was possible to obtain readings, although $[\alpha]_d$ at this pH at $41^\circ$ was $-35^\circ$ as compared with $-15^\circ$ to $-18^\circ$ observed for globin samples near pH 7 at $9^\circ$ and $25^\circ$.

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13 J. Hermans, Jr., personal communication.
14 Spectrophotometric titrations of metMb itself were also conducted at 245 ma and indicated a large shift in the apparent titration curve to more acid pH relative to CN--metMb. Although some of this difference may be attributed to the ultraviolet effects of Fe(OH)$_2^+$ ionization (log $k'$ = 8.9 at 25°), further investigation is required to ascertain whether any of the apparent shift is due to an unexpected difference in tyrosine acidity between metMb and CN--metMb.
the effect of protein charge to greater differences in apparent acidity. Conceivably, large differences in apparent reactivity of tyrosines in related proteins to reagents other than H⁺ ion may also arise from smaller differences in intrinsic reactivities. Finally, it should be emphasized that although 1 tyrosine has been concluded to remain masked when the heme is removed from myoglobin, the reversibility of the globin tyrosine titration curve cannot make this conclusion unequivocal without demonstration of the complete reversibility of denaturation under titration conditions. It is apparent in any event, however, that important differences in the reactivity of at least 1 tyrosine, as well as of several non-heme-linked histidine residues, exist between globin and myoglobin derivatives. In conclusion, it should be emphasized that, as with apomyoglobin, conformational changes occurring upon specific interaction of proteins with prosthetic groups or other molecules may lead to marked changes in the reactivities of amino acid side chains not directly adjacent to the binding sites.

**SUMMARY**

1. The relative H⁺ ion titration curves of sperm whale metmyoglobin and its derivative, globin, suggest that at least 3 imidazoles are released into H⁺ ion equilibrium upon removal of heme from myoglobin.

2. Eleven imidazoles are carboxymethylated by bromoacetate in globin, as compared with 8 imidazoles in native metmyoglobin. The 1 unreactive imidazole in globin has been identified as the heme-propionate-linked imidazole in metmyoglobin. Tentative identification of the histidines released to H⁺ ion with those released to bromoacetate suggests that differences in reactivity of non-heme-linked histidine residues exist between globin and metmyoglobin.

3. Large differences in tyrosine ionization between globin and several myoglobin derivatives have been demonstrated. These differences have been tentatively attributed to a difference between the two types of proteins in the acidity of 2 freely titratable tyrosines. The release in globin of the buried tyrosine of whale myoglobin derivatives, however, cannot completely be excluded.

4. The changes in imidazole and tyrosine reactivity upon removal of heme from myoglobin suggest that binding of its prosthetic group by an apoprotein can lead to conformational changes in regions of the protein that are not necessarily proximal to the binding site.

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