Kinetic and Equilibrium Studies of the Ligand Binding Reactions of Eight Electrophoretic Components of Sperm Whale Ferrimyoglobin†

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ABSTRACT: The reactions of eight electrophoretic components of sperm whale ferrimyoglobin with fluoride, azide, and cyanide have been studied. There do not appear to be significant differences in rate constants or equilibrium constants among the various components. We conclude that at pH 7.0 in 0.05 *M* potassium phosphate these ligand binding kinetics and equilibria are insensitive to the net charge on the protein. The variation of the azide equilibrium constant with ionic strength from μ 0.01 *M* to 0.11 *M* is not in accord with the predictions of the Debye-Hückel theory. On the other hand, azide association kinetic and equilibrium constants are respectively six- and threefold greater for beef ferrimyoglobin than for the isoelectric whale ferrimyoglobin (band V). An examination of the data for whale, horse, and beef myoglobins reveals that large differences in azide (but not CO) association rate constants are associated with amino acid substitutions at residues 45 and 99 in the heme cavity.

Kinetic studies of the reactions of myoglobins and hemoglobins in the ferric state have been reported for a variety of ligands, such as azide (Blanck et al., 1961; Goldsack et al., 1965; Duffey et al., 1965; Gibson et al., 1969), thiocyanate (Gibson et al., 1969), cyanate (Goldsack et al., 1965; Blanck et al., 1961), cyanide, fluoride, and nitrite (Blanck et al., 1961; Gibson et al., 1969). In the work of Gibson et al. (1969), the sensitivity of some of the reactions to the amino acid sequence, appearing as an intrinsic difference in reactivity between the hemes in the α and β chains, was clearly shown.

To what may such differences be attributed? One possibility is that small distortions of the heme give rise to the variations in reactivity at the iron site. For instance, if the force constant for out-of-plane distortion of the heme were 4.7×10^5 dyn/cm, a displacement at the pyrrole nitrogen of only 0.07 Å would lead to a potential energy change of 1.4 kcal/mol. Such a change in the *free energy* of activation would span the tenfold variation in ligand-binding rate constants found in diverse myoglobins. A full three-dimensional X-ray analysis on the azide derivative of sperm whale myoglobin at 2 Å did not reveal any significant differences at that resolution in the heme geometry or of the position of the iron when compared with the parent compound, ferrimyoglobin (Stryer et al., 1964). No changes in the planarity of the heme (2.8-Å resolution) were observed in crystallographic studies of hydroxide and cyanide binding. In the cyanide form, there were some small shifts in the positions of two pyrrole nitrogens and a distortion of the E helix with respect to the ferrimyoglobin structure (Bretscher, 1968). Similar small changes in the positions of several histidines and a twist in the E helix were also observed in the hydroxide derivative. Other perturbations, ascribed to changes in the ionic environment, were suggested to be evidence of the onset of alkaline denaturation, the hydroxide studies having been carried out at pH 9.1 (Schoenborn, 1969). There appear as yet, however, to be no detailed crystallographic studies on two or more functionally distinct myoglobins which might permit structure-function correlations to be established.

It is well known that myoglobins from different species often differ in net charge in the pH range 6-9. In recent years, a number of equilibrium studies of azide binding to various ferric hemoglobins and sperm whale ferrimyoglobin have been interpreted in terms of a simple electrostatic theory (Beetlestone and Irvine, 1968) that proposes that the azide equilibrium constant variation with ionic strength is such that the ligand affinity decreases as the net negative charge on the protein increases and that net charges on the protein can be obtained from such experiments. Recently we reported the isolation by means of isoelectric focusing (IEF)[1] of the eight most abundant components of crystalline sperm whale myoglobin, all differing in net charge as reflected by their various p*I*'s (LaGow and Parkhurst, 1972). This paper reports: (a) the rates of binding of the ligands azide, cyanide, and fluoride, (b) the rate of dissociation of fluoride, and (c) the azide association equilibrium constant for all eight whale components at pH 7.0 and 50 m*M* potassium phosphate, (d) the azide association equilibrium and rate constants for bands I and IV at pH 7.0 as a function of ionic strength, (e) azide equilibrium constants for bands I and IV at ionic strength 0.05 *M*, pH 7.6, (f) the fluoride association equilibrium constants for bands I and IV at pH 7.0, 50 m*M* potassium phosphate, (g) the association rate constant for azide as a function of ionic strength for the major component of beef heart myoglobin (beef I), and (h) the azide equilibrium association constant for the two isoelectric proteins, beef I and whale band V, at pH 7.0 and 50 m*M* phosphate.

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[1] Abbreviations used are: IEF, isoelectric focusing electrophoresis using carrier ampholytes; Mb^+, ferrimyoglobin; MbN_3, azide complex with ferrimyoglobin.

Experimental Section

Compounds. All inorganic chemicals were reagent grade. Crystalline sperm whale myoglobin was purchased from Calbiochem and from Miles Laboratories. Beef heart myoglobin was isolated from fresh beef heart obtained from a local slaughterhouse.

Preparation of Beef Heart Myoglobin. The myoglobin was isolated according to a modification of the method of Yamazaki et al. (1964). Cubed beef heart was homogenized for 5 min with two volumes of cold (4°) deionized water and filtered through a single layer of cheesecloth. During homogenization, the pH of the acidic homogenate was adjusted to approximately 7.5 with 2 *N* NH_4OH. The filtrate was brought to 70% saturation by stepwise addition of solid $(NH_4)_2SO_4$; after each addition, the pH was readjusted to pH 7.5 as described above. The precipitate, which contained most of the hemoglobin, was removed by centrifugation at 4000*g* for 15 min using a Sorvall RC2-B centrifuge equipped with the GSA preparative head. To the supernatant was added enough solid $(NH_4)_2SO_4$ to achieve 100% saturation and precipitation of the myoglobin. The pH was readjusted to 7.5. A small amount of Celite, approximately 0.1 g, was added and the resulting suspension was stirred for 0.5 hr. The myoglobin-containing Celite suspension was then vacuum filtered through a Büchner funnel. The myoglobin was redissolved in a minimum of cold deionized water and gravity filtered to remove the Celite. The protein solution was dialyzed vs. 50 m*M* potassium phosphate buffer (pH 7.0) for 2–3 hr. Gel filtration of 75 ml on an 85 × 2.2 cm G-100 Sephadex column equilibrated with the dialysis buffer separated the myoglobin from the hemoglobin, the latter constituting about 10% of the total heme protein. Dialysis vs. saturated $(NH_4)_2SO_4$ (pH 7.0) for 24–48 hr resulted in crystallization of the myoglobin, which was approximately 90% oxymyoglobin as determined spectrophotometrically. All operations were carried out at 4°. The yield of myoglobin was typically 300 mg from a 2.5-lb heart.

Isolation of Components. The eight most abundant electrophoretic components of whale myoglobin and beef I were isolated by IEF according to a procedure described elsewhere (LaGow and Parkhurst, 1972).

Kinetic Measurements. All measurements were made by stopped-flow. These experiments were carried out on an apparatus designed by one of us (L.J.P.) and described elsewhere (Boelts and Parkhurst, 1971). The data were acquired and analyzed by an on-line computer interfaced with the stopped-flow as previously described (LaGow and Parkhurst, 1972).

The rate constant for the combination of cyanide with myoglobin was determined by flowing a fresh solution of 10 m*M* (before mixing) KCN against an equal volume of the protein. The time course of the reaction was followed at 419 nm. Azide combination measurements utilized 1 m*M* (before mixing) solutions of KN_3 in 50 m*M* potassium phosphate buffer (pH 7.0). The wavelength employed for observing this reaction was 417 nm. Fluoride combination experiments employed 0.25 *M* NaF (before mixing) solutions; the observation wavelength was 409 nm. Fluoride dissociation was measured by the replacement by cyanide of the fluoride from the fluoroferrimyoglobin. The protein solution, which was 0.25 *M* in fluoride before mixing, was flowed against a 10 m*M* KCN solution, and the reaction was observed at 415 nm. The equation assumed for the replacement reaction rate in terms of the microsopic rate constants was identical with that derived by Lowry and John (1910) and by Fleck (1971) for a replacement reaction with an unliganded intermediate. All of the above reactions were carried out at 20° in 50 m*M* potassium phosphate buffer (pH 7.0). All protein concentrations were 10 μM before mixing. Each rate constant reported represents an average of a minimum of six experiments and 1200 raw data points. For the determination of some constants, 20,000 raw data points were collected and processed.

The azide association rate constants for whale bands I and IV and beef band I were measured at pH 7.0 as a function of ionic strength. Potassium phosphate buffers were made up at ionic strengths of 0.015, 0.04, 0.08, and 0.11 *M*. At least ten stopped-flow experiments were performed at each ionic strength, in which each experiment entailed the collection of 200 data points.

Azide Equilibrium Constants. The azide equilibrium constants of all eight whale bands and of beef band I at pH 7.0, 20°, in 50 m*M* potassium phosphate buffer were measured by reacting 5 μM solutions of ferrimyglobin (Mb^+) with microliter aliquots of a 1 m*M* NaN_3 solution. The reaction was followed by monitoring the absorbance changes from 440 to 400 nm on a Cary 14 recording spectrophotometer, and the calculations based on absorbance changes at 409 nm. The association constants were followed as a function of ionic strength at pH 7.0 for whale bands I and IV; the association constant at ionic strength 0.05 *M* was determined for these bands at pH 7.6.

Fluoride Equilibrium Constants. The fluoride association constants for whale bands I and IV in 50 m*M* potassium phosphate buffer (pH 7.0), 20°, were determined by reacting 5 μM solutions of the proteins with aliquots of 0.5 *M* KF. The reaction was followed by monitoring the absorbance changes from 440 to 400 nm, and calculations were based on the absorbance changes at 409 nm, corrected for dilution effects.

Results

Isoelectric Focusing. IEF on 100 mg of beef heart myoglobin over a Sephadex gel strip of 20 × 3 × 0.4 cm produced three visible bands. The major band, band I (numbered from the cathode), which was estimated to be 90% of the total, was isoelectric with whale band V, with a p*I* of 7.36. The two minor bands had more acidic p*I*'s and were not included in the studies reported here. IEF results for whale myoglobin were as previously reported (LaGow and Parkhurst, 1972).

Kinetic Measurements. The rate constants for the azide, cyanide, and fluoride measurements at pH 7.0, 50 m*M* potassium phosphate buffer, 20°, for all eight whale myoglobin bands are summarized in Table I. For a particular ligand, a standard *F*-test analysis showed no difference, at the $F(0.01)$ confidence level, in reactivity among the eight bands. When the azide association rate constants for whale bands I and IV were measured at pH 7.0 as a function of ionic strength over the range μ = 0.015–0.11 *M*, no differences or changes were observed; the values obtained were all within ±6% of our reported value of 3.6×10^3 M^{-1} sec^{-1} in 50 m*M* phosphate. The same insensitivity of the reaction rate to ionic strength was obtained with the beef band I. The measurements as a function of ionic strength were within ±9% of the value we report at 50 m*M* phosphate, 2.3×10^4 M^{-1} sec^{-1}, a value 6–7-fold greater than for whale myoglobin. These results are also tabulated in

Table I: Ferric Rate Constants, Whale Myoglobin.[a]

IEF Band	% Abundance	pI	CN^- Association Rate Constant ($M^{-1}sec^{-1} \times 10^{-2}$)	N_3^- Association Rate Constant ($M^{-1}sec^{-1} \times 10^{-3}$)	F^- Association Rate Constant ($M^{-1}sec^{-1}$)	F^- Dissociation Rate Constant ($sec^{-1} \times 10^2$)
Whale						
1	74.0	8.11	5.0	3.6	2.4	5.8
2	1.2	7.87	5.0	3.6	2.2	5.5
3	5.6	7.71	5.9	3.6	2.6	6.0
4	5.8	7.53	5.6	3.8	2.4	5.6
5	2.6	7.36	5.4	3.6	2.1	5.9
6	0.5	7.22	5.6	3.7	2.1	5.9
7	0.5	7.05	5.7	3.4	2.3	6.2
8	0.4	6.84	4.8	3.5	2.4	6.2
Beef						
1	90.0	7.36		22.5		

[a] Protein concentrations were 10 μM (before mixing). Reactions were run in 0.05 M potassium phosphate buffer (pH 7.0) at 20°. Percent abundance refers to percent of a given band found in crude myoglobin. In whale myoglobin, approximately 10% remained unfocused or focused in other minor bands. In beef myoglobin, two other bands accounted for the other 10%.

Table II: Fluoride and Azide Association Equilibrium Constants

IEF Band	% Abundance	$K_{assn}^{F^-}$ Calcd (M^{-1}) [d]	$K_{assn}^{F^-}$ (measured), pH 7.0 (M^{-1}) [a,b]	$K_{assn}^{N_3^-}$ pH 7.0 ($M^{-1} \times 10^{-4}$) [a,b]	$K_{assn}^{N_3^-}$ pH 7.6 ($M^{-1} \times 10^{-4}$) [a,c]
Whale					
1	74.0	41	40	3.4	3.3
2	1.2	40		3.3	
3	5.6	43		3.2	
4	5.8	43	38	3.2	3.3
5	2.6	36		3.3	
6	0.5	36		3.2	
7	0.5	37		3.5	
8	0.4	39		3.5	
Beef					
1	90.0			11.2	

[a] Protein concentrations were 7–8 μM. [b] Buffer composition was 0.05 M in potassium phosphate, pH 7.0, 20°. [c] Buffer composition was potassium phosphate, initial ionic strength of 0.05 M, pH 7.6, 20°. [d] Equilibrium constants calculated from association rate constant/dissociation rate constant. Values for these rate constants are in Table I.

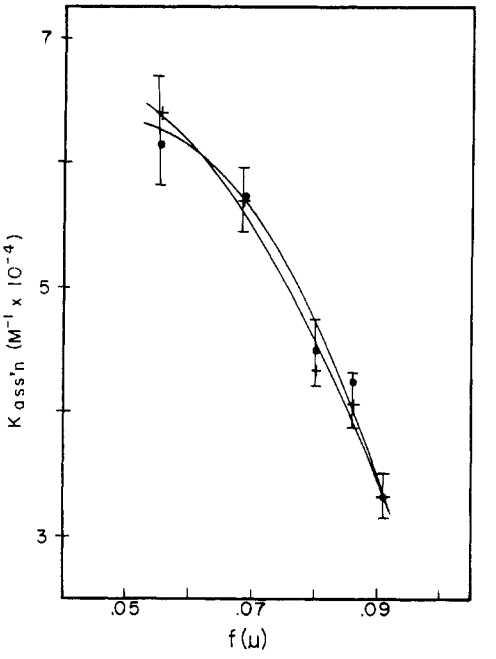

FIGURE 1: Azide association equilibrium constants, K(obsd), vs. ionic strength, bands I and IV, sperm whale myoglobin, pH 7.0, 20°. Buffer composition was potassium phosphate at appropriate ionic strengths. Myoglobin concentrations were 7–8 μM; (+), whale myoglobin, band I; (●) whale myoglobin, band IV.

Table I.

Azide Equilibrium Constants. The azide association constants for all eight whale bands, determined at pH 7.0, 20°, and 50 mM phosphate buffer, are summarized in Table II. No significant differences could be seen among the individual bands; all were within ±11% of the average value of 3.7×10^4 M^{-1}, and were calculated to be not significantly different at the $F(0.01)$ confidence level. Figure 1 illustrates the results of equilibrium measurements on the myoglobin band I and band IV at pH 7.0, 20°, and five ionic strengths. Whereas all of the association constants decrease as a function of increasing ionic strength, at any particular ionic strength, there are no differences, within experimental error, between the proteins. When the equilibrium constants were measured at pH 7.6 at $\mu = 0.05$ M, there was no difference between bands I (pI = 8.11) and IV (pI = 7.53), even though the charges on the two proteins at this intermediate pH are opposite in sign. Values for these pH 7.6 experiments are listed in Table II.

Also tabulated in Table II is the value for the association constant for beef band I at pH 7.0, 20°, 50 mM phosphate buffer. The value of 1.1×10^5 M^{-1} is three times larger than the average value for the whale bands under the same conditions of pH, temperature, and ionic strength.

Fluoride Equilibrium Constants. In Table II, the values for the association constant calculated from the ratio: association rate constant/dissociation rate constant, 20°, pH 7.0, 50 mM phosphate, are tabulated for each whale band; the measured association constants for bands I and IV are listed also.

Discussion

For the three ferric ligands in this study, only the azide rate constant has been measured previously for whale myoglobin, using the unfractionated protein, and under the fol-

lowing conditions: Duffey et al. (1965) measured the azide association and dissociation rate constants as a function of pH by both stopped-flow and temperature jump, but their investigations did not proceed above pH 6.5 and thus are not directly comparable with ours. Goldsack et al. (1965) also measured both rate constants as a function of pH using the temperature-jump method. The difference between their value, at 25°, in phosphate buffer and 0.1 M KNO_3, of 4.4×10^3 M^{-1} sec^{-1}, and ours at 20° (3.7×10^3 M^{-1} sec^{-1}) could well be due to the temperature difference, since, under these conditions, the Arrhenius activation energy is about 7 kcal/mol for this reaction, which would convert their value to 3.8×10^3 M^{-1} sec^{-1} at 20°. The association rate constant for horse myoglobin at pH 7.05 and 21–23° has been reported (Blanck et al., 1961) to be 7.0×10^3 M^{-1} sec^{-1}, or roughly double the rate constant determined in this work for whale myoglobin. Ver Ploeg and Alberty (1968) studied, by stopped-flow, the binding of cyanide to whale myoglobin at 25° from pH 4.66 to 10.60 as a function of buffer concentration and ionic strength. Their discovery that the apparent association rate constant at many pH values was strongly dependent on the nature and concentration of the buffer even though the ionic strength was held constant by addition of KNO_3 (to a total of 0.11 M) led to their reporting rate constants extrapolated to zero buffer concentration. Their results are thus not directly comparable with ours. Awad and Badro (1967) measured the cyanide association rate constant as a function of pH at 25° in Tris-maleate–potassium chloride buffers of constant ionic strength of 0.15 M; thus, their results, too, are not comparable with ours. To our knowledge, no kinetic data exist for the reactions of whale ferrimyoglobin with fluoride.

The spectrophotometrically measured fluoride equilibrium constants for bands I and IV at pH 7.0, 20°, 50 mM phosphate buffer, are in excellent agreement with those calculated from the association and dissociation rate constants, differing by 2 and 7%, respectively, from the means of the calculated values for each of the two bands. Our value (average) of 39 M^{-1} is significantly different from that of $55M^{-1}$ reported by Beetlestone and Irvine (1969) at pH 6.95, 20°, and ionic strength of 0.05 M (exclusive of KF) in phosphate buffer. The discrepancy may derive from the method employed for determining the end point of the ligand titration. In our work, saturation was achieved by adding excess solid KF to the myoglobin solution and recording the end point spectrophotometrically. Beetlestone and Irvine obtained their end point from an extrapolation.

The binding of azide to ferrihemoglobin (Beetlestone and Irvine, 1968) and ferrimyoglobin (Bailey et al., 1969) has been treated in terms of the Debye–Hückel theory. In terms of ferrimyoglobin, one obtains (Beetlestone and Irvine, 1968, eq 6)

$$\log K_{N'} = \log K_N^0 - A(q_{Mb^+}^2 - q_{MbN_3}^2)\sqrt{\mu}/(1 + Ba\sqrt{\mu}) \quad (1)$$

where A and B are the usual Debye–Hückel parameters, μ, the ionic strength, q is the net charge on the protein, a is taken as 24 Å, the mean radius of myoglobin (from crystallographic data) plus the radius of two water molecules (Bailey et al., 1969). Three apparent equilibrium constants are defined (Anusiem et al., 1966)

$$K_a = (MbOH)(H^+)/(Mb^+OH_2) \quad (2)$$

$$K_{obsd} = (MbN_3)/((Mb^+OH_2) + (MbOH))(N_3^-) \quad (3)$$

$$K_N = (MbN_3)/(Mb^+OH_2)(N_3^-) = K_{obsd}(K_a + (H^+))/(H^+) \quad (4)$$

where MbOH and Mb^+OH_2 are respectively alkaline and acid forms of ferrimyoglobin. $K_{N'}$ is related to K_N through eq 5

$$\log K_{N'} = \log K_N + A\sqrt{\mu}/(1 + \sqrt{\mu}) \quad (5)$$

(Beetlestone and Irvine, 1968) in which the second term on the right corrects for the activity coefficient of azide, with Ba for azide taken = 1. For whale ferrimyoglobin, pH 7.0, 20°, μ = 0.05 M, the addition of azide is accompanied by the uptake of approximately 0.1 mol of H^+/mol of ferrimyoglobin. Thus

$$q_{MbN_3} = q_{Mb^+} - 0.90 \quad (6)$$

We may therefore rewrite eq 1 as

$$\log K_{N'} = \log K_N^0 - A(1.8q_{Mb^+} - 0.81)f(\mu) \quad (7)$$

where

$$f(\mu) = \sqrt{\mu}/(1 + Ba\sqrt{\mu}) \quad (8)$$

Thus, if this theory were correct, the charge, q, on various myoglobins could be obtained from the slope of a plot of log $K_{N'}$ vs. $f(\mu)$ throughout the ionic strength range for which eq 7 was valid (taken as μ = 0.005–0.05 M by Beetlestone and Irvine (1968)).

A plot of log $K_{N'}$ vs. $f(\mu)$ at pH 7.0 gives, from least-squares fitting of the data, q's of 6.0 and 5.7, for bands I and IV, respectively, of whale ferrimyoglobin. Hartzell et al. (1968a) have published titration curves for sperm whale, harbor seal, and porpoise myoglobins. These three curves need only slight translations along the pH axis to become superimposable over the range pH 4–9.5, suggesting that the minor differences in amino acid sequences occurring among these three myoglobins do not significantly change the titration behavior ±1.5 pH units from the isoelectric points. From the sperm whale myoglobin curve, a net charge of 2.4 at pH 7.0 can be obtained. This net charge can be assigned to band I since this component comprises about 75% of the unfractionated sperm whale myoglobin. This net charge is also what one would calculate from the pI of liganded myoglobin with respect to ferrimyoglobin, assuming $(\partial pI/\partial q) \sim (\partial q/\partial pH)^{-1}$, the latter obtained from titration data. By plotting the net charge (zero at the pI, 8.11, and 2.4 at pH 7.0, 1.1 pH units away from the pI) vs. the displacement from the pI, one can estimate a net charge of 1.2 for band IV, which, at pH 7.0, is 0.53 unit from its pI (7.53). Figure 2 shows a plot of log $K_{N'}$ vs. $f(\mu)$ for whale band IV. Although the data are not obviously linear, a least-squares line is shown. The dashed line shows the expected theoretical line from eq 7, with q = 1.2, and displaced vertically to coincide with the experimental data at $f(\mu)$ = 0.08. We conclude that reliable charges cannot be obtained from eq 1 or 7. Somewhat similar discrepancies between the predictions of classical titration theory and the observed dependence of K_a (eq 2) on net charge for chemically modified myoglobins have been reported by Hartzell et al. (1968b).

Even after conversion into $K_{N'}$, the equilibrium constants reported in this work at pH 7.0 bands I and IV agree at the $F(0.01)$ confidence level, using a standard F-test analysis at all ionic strengths. At pH 7.7, the net charges on bands I and IV should differ in sign, and yet at μ = 0.05 M the $K_{N'}$ association constants for the two bands differ by less than 5% of their mean value. Examination of Tables I and II

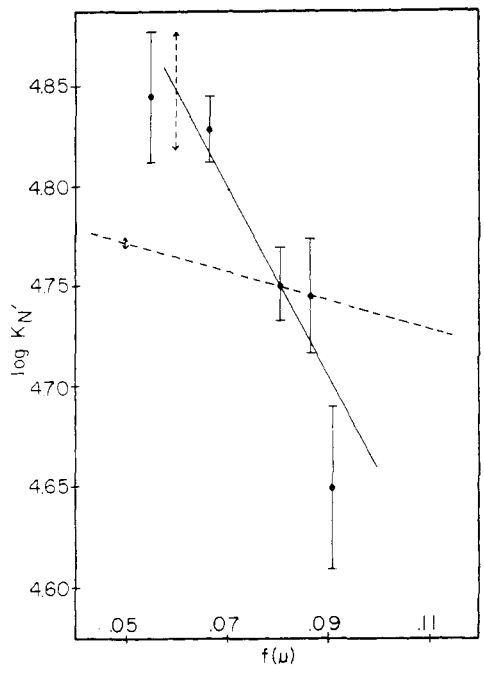

FIGURE 2: Log $K_{N'}$ vs. ionic strength function, $f(\mu)$, band IV, sperm whale ferrimyoglobin, pH 7.0, 20°. The dashed line is the theoretical line, eq 8, with charges derived from titration data. The solid line is parallel to the least-squares fit through the data. The two lines are translated to coincide at the mean value of log $K_{N'}$ for $f(\mu) = 0.08$. The vertical arrows depict standard deviations in the slopes.

shows that there are no significant differences among the various whale myoglobin bands for any of the kinetic or equilibrium constants. This total insensitivity to net charge is striking in view of the recent results of Garner et al. (1974) which show that the charge differences among the various sperm whale myoglobin bands do not arise solely from deamidations, but rather, the electrophoretic bands arise from genetic multiplicity, and show compositional differences comparable to those seen among distinct animal species.

It was of interest to compare azide equilibrium and rate constants for two different mammalian myoglobins which had the same isoelectric point. The azide association constant of beef heart myoglobin I (isoelectric with whale band V) is three times greater than that of whale, measured in 50 m*M* phosphate buffer, 20°, pH 7.0, and the association rate constant is more than six times greater. The net charge on the two proteins should be very nearly the same at pH 7.0 owing to the small displacement in pH from their common isoelectric point and the amino acid homologies for whale vs. beef.

Han et al. (1970) have published the amino acid sequence of beef heart myoglobin. Assuming that beef myoglobin has the same three-dimensional structure as the whale protein, an estimate can be made, using computer-generated stereodrawings of sperm whale myoglobin (Dickerson and Geis, 1969; Johnson, 1970), of the positions of the amino acid substitutions in which charge differences occur between the two proteins. There are six such substitutions. Inspection of the stereodrawings leads to the conclusion that the side chains of the residues in question are either far removed from the heme site or are on the surface of the molecule directed toward the solvent. Perutz et al. (1968) have determined 15 residues with side chains in contact with the heme or its ligands. A comparison of these residues in beef and whale myoglobins shows that all but two of the 15 are identical. At position 99 (all numbering is according to Kendrew and Watson [Watson, 1968]), in beef a valine has been substituted for the isoleucine in whale. This residue (FG 5) has at least two contacts (interatomic distances less than 4 Å) on the proximal side with the vinyl group on pyrrole II in hemoglobin, and is similarly located in myoglobin (Perutz et al., 1968). The other substitution is a lysine in beef myoglobin which replaces the arginine in whale at residue 45. In whale myoglobin, this arginine forms a salt bridge to the propionate side chain on pyrrole III. Both of these substitutions are conservative; there is no change in charge. Thus, differences between the two proteins in reactivity at the heme cannot be attributed to differences in local electrostatic charges. The structural comparison of myoglobins can be carried further to include horse heart myoglobin, the primary sequence of which is known (Dautreveaux et al., 1969). Again, assuming the same three-dimensional structure, an examination of the primary sequence shows that in those substitutions where charge differences occur between horse and whale, the residues are either far removed from the heme site or else have their side chains directed into the solvent. Of the 15 important contact residues, there is only one difference between horse and whale, at residue 45 where, as in beef, a lysine has been substituted for the arginine.

With these differences in mind, a comparison of the reactivities among the three proteins toward a few selected ligands is interesting. If the association rate constants for CO in the ferrous state are examined, the values for whale, beef, and horse myoglobins are all quite similar: $5.4 \times 10^5\ M^{-1}\ sec^{-1}$ for whale band I and beef I (LaGow and Parkhurst, 1972; LaGow and Parkhurst, unpublished results) and $5.0 \times 10^5\ M^{-1}\ sec^{-1}$ (Antonini et al., 1965) for horse. In the ferric state, however, the azide association rate constants are quite different: $3.5 \times 10^3\ M^{-1}\ sec^{-1}$ for whale, $7.0 \times 10^3\ M^{-1}\ sec^{-1}$ for horse (Blanck et al., 1961), and $20 \times 10^3\ M^{-1}\ sec^{-1}$ for beef, all at pH 7.0, 20°. While enough data certainly do not exist to make a definitive statement, it is tempting to try to correlate the number and location of substitutions at the heme site with reaction rates. The single replacement in horse, that of an arginine with lysine at residue 45, can be associated with a doubling in the azide association rate constant when referenced to whale; in beef, the further substitution of a valine for the isoleucine of whale at position 99 seems to be accompanied by a further threefold increase in the azide association rate constant. This is perhaps too simplified a view, since there are other substitutions in the molecules which, although conservative in nature, may affect the conformation of the protein in some subtle fashion and thus alter the heme geometry so as to generate the differences in reactivity toward azide. The apparent effects of these substitutions in the heme cavity, however, are striking enough to warrant comment.

References

Antonini, E., Gibson, Q. H., and Wyman, J. (1965), in Hemoglobin and Myoglobin in their Reaction with Ligands, Antonini, E., and Brunori, M., Ed., Amsterdam, North-Holland Publishing Co., pp 88, 226.

Anusiem, A. C., Beetlestone, J. G., and Irvine, D. H. (1968), *J. Chem. Soc. A*, 960.

Awad, E. S., and Badro, R. G. (1967), *Biochemistry 6*, 1785.

Bailey, J. E., Beetlestone, J. G., and Irvine, D. H. (1969), *J. Chem. Soc. A*, 241.

Beetlestone, J. G., and Irvine, D. H. (1968), *J. Chem. Soc. A*, 951.
Beetlestone, J. G., and Irvine, D. H. (1969), *J. Chem. Soc. A*, 735.
Blanck, J., Graf, W., and Scheler, W. (1961), *Acta Biol. Med. Ger. 7*, 323.
Boelts, K., and Parkhurst, L. J. (1971), *Biochem. Biophys. Res. Commun. 43*, 637.
Bretscher, P. A. (1968), *Nature (London) 219*, 606.
Dautreveaux, M., Boulanger, Y., Han, K.-K., and Biserte, G. (1969), *Eur. J. Biochem. 11*, 267.
Dickerson, R. F., and Geis, I. (1969), The Structure and Action of Proteins Stereo Supplement, New York, N.Y., Harper and Row.
Duffey, D., Chance, B., and Czerlinski, G. H. (1965), *Biochem. Biophys. Res. Commun. 19*, 423.
Fleck, G. M. (1971), Chemical Reaction Mechanisms, New York, N.Y., Holt, Rinehart and Winston, eq 10.14, 10.24, pp 159–160.
Garner, M. H., Garner, W. H., and Gurd, F. R. N. (1974), *J. Biol. Chem. 249*, 1513.
Gibson, Q. H., Parkhurst, L. J., and Geraci, G. (1969), *J. Biol. Chem. 244*, 4668.
Goldsack, D. E., Eberlein, W. S., and Alberty, R. A. (1965), *J. Biol. Chem. 240*, 4312.
Han, K.-K., Dautreveaux, M., Chaila, X., and Biserte, G. (1970), *Eur. J. Biochem. 16*, 465.
Hartzell, C., Bradshaw, R., Harper, K., and Gurd, F. R. N. (1968a), *J. Biol. Chem. 243*, 690.
Hartzell, C. R., Clark, J. F., and Gurd, F. R. N. (1968b), *J. Biol. Chem. 243*, 697.
Johnson, C. (1970), ORTEP: A Fortran Thermal-Ellipsoid Plot Program for Crystal Structure Illustrations, Oak Ridge, Tenn., Oak Ridge National Laboratory.
LaGow, J., and Parkhurst, L. J. (1972), *Biochemistry 11*, 4520.
Lowry, T. M., and John, W. T. (1910), *J. Chem. Soc. 97*, 2634.
Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968), *Nature (London) 219*, 131.
Schoenborn, B. (1969), *J. Mol. Biol. 45*, 297.
Ver Ploeg, D. A., and Alberty, R. A. (1968), *J. Biol. Chem. 243*, 435.
Watson, H. C. (1968), *Prog. Stereochem. 4*, 229.
Yamazaki, I., Yokota, K., and Shikima, K. (1964), *J. Biol. Chem. 239*, 4151.

The Buoyant Titration of Native and Carbamylated Bovine Serum Mercaptalbumin†

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ABSTRACT: The buoyant density titration curves of native and carbamylated bovine serum mercaptalbumin were measured throughout the pH range 5.3–12.7. Large increments in the buoyant density were observed above pH 10, with inflection pH values of 11.2 and 11.4 for native and carbamylated bovine serum mercaptalbumin, respectively. For the modified protein in which 25 out of 58 lysine residues were carbamylated, the buoyant densities were 0.048 g/ml higher at neutral pH and 0.024 g/ml higher at the extrapolated pH 13. The carbamyl groups apparently produce a larger residual density at pH 13 than they did in the case of ovalbumin. Homopolymer buoyant density titration data were demonstrated to be of value in calculating the contributions of titratable residues to the buoyant density of both proteins. The buoyant density increment at high pH was due largely to the deprotonation of the lysines as indicated by the diminished change in buoyant density between pH 10 and 12.7 for the modified protein. These density changes were attributable primarily to a gain of cesium ions. The limited modification of the lysine residues under mild reaction conditions and the rather high intrinsic dissociation constant of tyrosine residues in mercaptalbumin may indicate a preferential modification of easily accessible lysine residues. Phenolic deprotonation is facilitated by the neutralization of normally charged lysine residues and demonstrates ionic interactions between internal lysines and certain carboxyl and tyrosine residues thereby stabilizing the native state of the protein.

The use of well-known amino acid blocking groups has found widespread employment as a means of studying various biophysical properties of proteins when specific ionizable residues are chemically modified. The technique of the carbamylation of lysine residues (Stark and Smyth, 1963) (Svendsen, 1967) was adopted for the modification of native bovine serum mercaptalbumin. The buoyant behavior of native and carbamylated BMA[1] was studied in the pH range 5.3–12.7. This work follows the recent study of the buoyant density titration of native and carbamylated ovalbumin (Ifft, 1971), the only other protein for which such data have been obtained.

BMA is a widely studied protein, characterized by its exceptional ability to bind anions and water. Although BMA

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[1] Abbreviation used is: BMA, bovine serum mercaptalbumin.