Electrostatic Effects in Myoglobin. Hydrogen Ion Equilibria in Sperm Whale Ferrimyoglobin†

S. J. Shire, G. I. H. Hanania,‡ and F. R. N. Gurd*

ABSTRACT: The Tanford-Kirkwood theory of intramolecular electrostatic interactions in proteins has been applied to sperm whale ferrimyoglobin. A modification of the method was used employing static solvent accessibility factors which reflect the exposure to and usually the extension into solvent of the charged amino acid residues in the protein. The computations for the major component (component IV) were compared with the experimental titration curves at different ionic strengths. The crystallographic coordinates and the amino acid sequence of component IV, with the replacements of asparagine-122 and -132 by aspartic acid-122 and -132 in the minor component (component II), were then used to obtain the theoretical titration curve of the latter. The results show reasonable agreement with experimental titration curves.

It has long been recognized that the acid–base titration curves of proteins cannot be accounted for solely on the assumption of independent sites, and it is in fact reasonable to expect the various binding sites to interact. Since protein titration curves vary with ionic strength, it is reasonable to assume that the major intramolecular interactions between titratable sites are electrostatic, and therefore can be interpreted with an electrostatic theory (Tanford, 1961).

The traditional approach to this problem has been to consider the charged residues of the protein molecule to be grouped into classes of intrinsically identical sites, and to regard the charge of the sites to be smeared over the surface of the molecule. The equation relating α, the fraction of dissociated sites of a given kind, and W_{el} the electrostatic contribution to the free energy of the molecule is (Tanford, 1961)

$$\mathrm{pH} - \log [\alpha/(1-\alpha)] = \mathrm{p}K_{int} - (1/2.303kT)(\partial W_{el}/\partial \bar{Z}) \quad (1)$$

where $\bar{Z}$ is the average net charge on the protein molecule, and $\mathrm{p}K_{int}$ is the intrinsic ionization pK (*i.e.*, the pK of the site in the protein when $\bar{Z} = 0$). If the protein is assumed to be an impenetrable sphere of radius b, and a larger ion exclusion radius a, immersed in a solvent with dielectric constant D, then eq 1 becomes (Linderstrøm-Lang, 1924)

$$\mathrm{pH} - \log [\alpha/(1-\alpha)] = \mathrm{p}K_{int} - 0.868wZ \quad (2)$$

where

$$w = e^2/2DkT\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right)$$

and e is the electronic charge and κ is the Debye–Hückel parameter.

In the Kirkwood treatment (Kirkwood, 1934) the Debye–Hückel theory was used to determine the electrostatic free energy for a set of discrete point charges on a sphere of radius b and ion-exclusion radius a immersed in a solvent (usually water) with an external dielectric constant D. The charges were placed either on the surface or buried a distance, d, below the surface in the interior of the sphere which was assumed to be a continuous medium with a low internal dielectric constant D_i. This model was first applied to dissociation constants of dicarboxylic acids (Kirkwood and Westheimer, 1938). In these cases, electrostatic interaction between the two acidic groups is reflected in the ratio of the two dissociation constants. Using this information and assuming a suitable structure, the distance R separating the dissociating groups is calculated and found to be a reasonable value. Tanford (1957a) showed that in order to obtain these reasonable values the sites must be buried approximately 1 Å below the surface of the sphere. When the Kirkwood formulation was extended to the treatment of protein titrations (Tanford and Kirkwood, 1957) and applied to simple models of proteins (Tanford, 1957b), it was found that again reasonable results could be obtained only if the sites were buried 1 Å below the surface.

Orttung (1969, 1970) later computed the variation of the average net charge with pH for hemoglobin. Although the computations were in good agreement with experiment, it was found necessary to place all the charges on the surface of the sphere, that is, at $d = 0$. Recently, Tanford and Roxby (1972) developed an efficient iterative algorithm and applied the theory to the titration of lysozyme. Again, although the computations were in good agreement with experimental results the sites had to be placed only 0.4 Å below the surface rather than the 1-Å distance inferred from Tanford's earlier work.

In the present work an attempt is made to apply a modified Tanford–Kirkwood formulation to the computation of hydrogen ion titration curves of sperm whale ferrimyoglobin and of related ionization equilibria for specific sites on the molecule. This species of myoglobin was chosen because its detailed tertiary structure and spatial coordinates are known (Watson, 1969). Moreover, myoglobin is a globular protein for which spherical symmetry may be assumed as a first approximation and, in contrast to hemoglobin, it is monomeric. Groups with ionization pK in the range of pH 5–9 (histidines, terminal valine, iron-bound H_2O) are particularly interesting, as are the masked histidine groups which unfold as a result of conformational changes on denaturation of the protein (Breslow and Gurd, 1962).

A number of myoglobins from different species have been titrated, in particular the major components of native and acid-denatured sperm whale, harbor seal, and common porpoise myoglobins by Hartzell *et al.* (1968). Janssen (1970) has ana-

† From the Department of Chemistry, Indiana University, Bloomington, Indiana 47401. *Received March 11, 1974.* This is the 59th paper in a series dealing with coordination complexes and catalytic properties of proteins and related substances. For the preceding paper, see Gurd *et al.* (1974). This work was supported by Public Health Service Research Grant HL-05556.

‡ Permanent address: Department of Chemistry, American University of Beirut, Beirut, Lebanon.

lyzed titration curves of equine myoglobin by the differential titration method. In this paper we report a comparison of the theoretical with experimental titration curves for the major component and one of the minor components of sperm whale ferrimyoglobin.

Materials and Methods

Detailed potentiometric studies of sperm whale ferrimyoglobin have already been made on purified lyophilized protein (Hartzell *et al.*, 1968; Nakhleh, 1971). We have repeated these titrations using unlyophilized freshly prepared and purified myoglobin with the purpose of obtaining data at ionic strength $I = 0.01$ and 0.10 M.

Materials. Myoglobin was prepared as described previously (Hapner *et al.*, 1968), and the major component IV and minor component II (nomenclature of Edmundson and Hirs, 1962) were freshly isolated and stored in water at 4°. Distilled deionized water was boiled and kept under a CO_2 trap, and this water was used throughout. All chemicals were of reagent grade. Stock 0.1 N NaOH and 0.1 N HCl were standardized against primary standard potassium hydrogen phthalate.

Apparatus. The titration apparatus consisted of a water-jacketed titration vessel with a glass electrode (Radiometer, G222B semi-micro) and a water-jacketed calomel reference (Radiometer K497) connected by a bridge of agar-saturated KCl in a thin polyethylene tube. A magnetic stirrer was positioned under the vessel. The entire titration assembly with stirrer was enclosed within a Faraday box, grounded, and connected to a Radiometer pHM4 meter. A calibrated microburet (Micrometric Instrument Co., Model SB2) fitted with a glass syringe and a thin polyethylene tube was used to deliver acid or base as required.

Procedure. Nitrogen gas (Matheson Co. prepurified) was scrubbed and allowed to pass continuously over the surface of the liquid in the titration vessel. Immediately prior to titration the pH scale was linearized using primary standard phosphate, phthalate, and borate buffers (National Bureau of Standards). Approximately 6 ml of water was then passed through an ion-exchange column (Bio-Rad AG 501-X8(D)) directly into the vessel containing the requisite amount of KCl for adjustment of ionic strength, and was titrated against 0.1 N HCl or NaOH delivered from the microburet covering the pH range up to 10 and down to 3.5.

In a separate run, an aliquot of stock myoglobin solution, to make about 6 ml of final concentration near 0.1 mM, was passed through the ion-exchange column directly into the vessel containing the necessary amount of KCl, and was titrated with acid or with base. At the end of every titration the pH scale was rechecked, the volume of the myoglobin was measured, and the protein concentration was determined spectrophotometrically as ferrimyoglobin-cyanide complex with a molar absorptivity of 112×10^3 M^{-1} cm^{-1} at 423 nm. The titrations were repeated several times and concordant results obtained. All measurements were made at 25° and at total molar ionic strength of 0.01 or 0.10 M.

The experimental results obtained are to be compared with the theoretically computed titration curves, as discussed below.

Method of Computation

General Formalism. The titration curve is obtained by calculating the net charge of the protein as a function of pH. To do this, we start with eq 1. However, since the equation refers to a class of sites, the relation must be rewritten as

$$\mathrm{pH} - \log\left[\alpha_i/(1-\alpha_i)\right] = (\mathrm{p}K_{\mathrm{int}})_i - (1/2.303kT)(\partial W/\partial z_i) \quad (3)$$

where at a given pH, α_i is the fraction dissociated of one particular site i with charge z_i; $(\mathrm{p}K_{\mathrm{int}})_i$ is the chemical (pH independent) pK of the site, that is, the pK value for a site in a hypothetically discharged protein molecule rather than the Linderstrøm-Lang value at $\bar{Z} = 0$, which was defined previously in connection with eq 1. W is the total electrostatic work done in placing all the other charged sites on the molecule, and is given by

$$W = \sum_{j \neq i} W_{ij} \quad (4)$$

where W_{ij} is the free energy of interaction of a pair of point sites with charges z_i and z_j placed on a sphere of radius b and ion exclusion radius a ($a - b$ being a measure of the average radius of counterions). W_{ij} is given by

$$W_{ij} = e^2 z_i z_j \{(A_{ij} - B_{ij})/b - C_{ij}/a\} \quad (5)$$

where e is the electronic charge; A_{ij}, B_{ij}, and C_{ij} are mathematical functions which depend on b, a, D_i, D, r_{ij} (distance between sites), as well as the depth parameter d; $(A_{ij} - B_{ij})$ represents the interaction at zero ionic strength and C_{ij} represents the effect of finite ionic strength on this interaction (Tanford and Kirkwood, 1957).

Since W_{ij} is linear in z_i (eq 5), it follows that the derivative $\partial W/\partial z_i$ is independent of the z_i value. Consequently, the changes in pK for site i as a result of its interactions with all other sites, j, is not determined by z_i but only by z_j. Hence the effective pK for site i, pK_i, will be given by

$$\mathrm{p}K_i = (\mathrm{p}K_{\mathrm{int}})_i - \sum_{j \neq i} \frac{W_{ij}}{2.303 z_i kT} \quad (6)$$

It is to be noted that the effect of a positive site j is always to decrease the pK_i of site i and that of a negative site is to increase pK_i.

For a given set of model parameters at temperature T: b, a, D_i, and D, and a given d (all charges being placed at the same depth within the sphere), it is seen that the functions A_{ij}, B_{ij}, and C_{ij} will depend only on the variable r_{ij}.

Calculation of W_{ij}. The first stage of the calculation for a given set of structural parameters (b, a, D_i, D, d, T) is to compute a table of W_{ij} values as a function of r_{ij} for a pair of sites placed anywhere on or within a sphere, and at distances varying by 0.1 Å to the maximum value possible, $2b$.

Calculation of pK_i. The interative method of Tanford and Roxby (1972) was used to perform the calculations. In this method, an intrinsic pK (based on model compound studies) is assigned to every site j, and hence the charge z_j of every site is calculated at any particular pH. For a given set of sites with distances r_{ij}, the tabulated W_{ij} values are used (eq 5) to calculate a set of effective pK_i values (eq 6). These new pK_i values are then used to calculate a new set of z_i values; and the calculations are repeated until the set of z_j values and hence pK_i values no longer change by more than 0.1 (about three to six iterations). When all the pK_i values calculated no longer change the entire computation is self consistent.

The sum of all the final charges gives the average net protein charge at the given temperature, pH, and ionic strength. These calculations can then be repeated for different pH values and ionic strengths.

Application to Myoglobin

Structural Parameters of the Model. Sperm whale myoglobin is an oblate spheroid $42 \times 42 \times 25$ Å with molecular volume (Kendrew and Parrish, 1957) about 29,000 $Å^3$, and thus the average radius $b = 18$ Å. Assuming the average ionic radius in solution to be 2 Å, $a = 20$ Å. The dielectric constant of

the interior of the molecule was taken to be the same as that used in the calculation for lysozyme (Tanford and Roxby, 1972), and hemoglobin (Orttung, 1970), $D_i = 4$. The external dielectric constant is that of water at 298°K, $D = 78.5$. As discussed below, the effect of varying d between 0 and 1.0 was explored. A static solvent accessibility parameter is introduced and defined below.

Site Positions. Assuming that the crystal structure of sperm whale myoglobin is little altered in aqueous solution (Hugli, 1968; Hugli and Gurd, 1970), we have examined the detailed space-filling model based on the atomic X-ray coordinates of 1.6-Å resolution (Watson, 1969) to categorize the proton binding sites on the molecule. Table I lists three classes of sites to be taken into account, of which the first two enter into hydrogen ion equilibrium. For these sites the charges i and j were placed at a distance d below the surface in the computation model and separated by the distance r_{ij} as determined from the crystallographic coordinates.

Site Assignments. The first class of sites in Table I comprises all available groups with normal intrinsic pK values, *i.e.*, those groups for which changes in the pK value are determined mainly by electrostatic interactions. The second consists of other sites available to the hydrogen ion equilibrium with abnormal intrinsic pK values, *i.e.*, those groups under the influence of nonelectrostatic forces such as hydrogen bonding.

The third class represents the masked sites. In these cases the decision to exclude the groups from the calculation rests on a combination of lines of evidence indicating that the potential sites will not bear charges over the titration region under consideration. The choices made are partly heuristic, and will be discussed further in conjunction with the results of the computations.

The pair of oppositely charged groups, glutamic acid residue 148 and lysine residue 145, have a separation distance of less than 2 Å. Because of the small separation distance of this pair and its limited exposure to the solvent (Lee and Richards, 1971), it has been treated as internally neutralized and taken out of the calculation. The effect of including these sites in the calculation is very small. A number of other pairs of residues may be involved in salt bridges (Schoenborn, 1971). Whether or not these residues are included in the calculation has little effect on the overall results.

The histidine-24, -36, -82, -93, and -97 were assumed to be masked (Hugli and Gurd, 1970; Gurd, 1970; Nigen and Gurd, 1973) and to fall in the uncharged class in the native protein (Breslow and Gurd, 1962). They are not included in the calculation. Histidine-64 shows limited reactivity toward organic reagents (Hugli and Gurd, 1970; Nigen and Gurd, 1973), but structural considerations suggest that it is as accessible to solvent to a similar degree as the iron-bound water molecule.

As will be discussed further below, arginine-45 was excluded from the calculation. This residue is probably involved in anion binding (Watson, 1969). The results of Cameron *et al.* (1966) indicate that at least one chloride ion will be bound under the conditions of the experiments. The exclusion of arginine-45 from the charged-site roster is tantamount to identifying it as the primary ion-pairing group for the bound chloride ion. The guanidinium group of arginine-45 is involved in multiple interactions, and is believed to bind divalent anions in conjunction with neighboring residues (Stryer *et al.*, 1964; Gillespie *et al.*, 1966).

Intrinsic pK Values. For the purpose of calculation an intrinsic pK value must be assigned to every available site. As defined above for the discrete charge model, an intrinsic pK value refers to the group in a hypothetically discharged protein molecule, that is, with the exclusion of all electrostatic interactions. Intrinsic pK values have been obtained from several types of studies of model compounds (Cohn and Edsall, 1943; Tanford, 1962; Nozaki and Tanford, 1967; Gurd *et al.*, 1971). It is noteworthy, however, that these values are operational estimates and as such are subject to slight variations. Table I gives the intrinsic pK values assigned to the "normal" groups in our calculations. It was also assumed that in hydrogen bonding the carboxylic groups, being electron donating, would have lower pK_{int} than normal. On this basis the pK_{int} values for hydrogen-bonded glutamic and aspartic acids were assigned as in Table I. Conversely, hydrogen bonding in a donor group such as the hydroxyl of tyrosine-146 should raise the pK value.

The 12 histidine residues form a group of special interest. The titration behavior of the histidine residues has already been studied by the Linderstrøm-Lang treatment (Hartzell *et al.*, 1968; Breslow and Gurd, 1962). As pointed out by Gurd (1970) the simple Linderstrøm-Lang treatment is not fully adequate, and moreover the histidine residues would be expected

TABLE I: Intrinsic pK Values and Static Solvent Accessibility Factors Used in the Computations.[a]

Residues	pK_{int}
Normal Sites	
Asp-20 (0.56), Asp-44 (0.85), Asp-126 (0.93)	4.0
Glu-4 (0.76), Glu-18 (0.44), Glu-41 (0.82), Glu-52 (0.54), Glu-54 (0.54), Glu-59 (0.69), Glu-83 (0.74), Glu-85 (0.43), Glu-105 (0.59), Glu-109 (0.58), Glu-136 (0.59)	4.5
Gly-153 (0.95)	3.6
Propionic-1 (0.26), propionic-2 (0.46)	4.0
Arg-31 (0.49), Arg-118 (0.32), Arg-139 (0.16)	12.0
His-12 (0.54), His-81 (0.85), His-113 (0.50), His-116 (0.58), His-119 (0.14)	6.3
Lys-16 (0.29), Lys-34 (0.44), Lys-47 (0.48), Lys-50 (0.63), Lys-56 (0.68), Lys-62 (0.40), Lys-63 (0.78), Lys-77 (0.50), Lys-78 (0.39), Lys-79 (0.61), Lys-87 (0.55), Lys-96 (0.84), Lys-98 (0.84), Lys-102 (0.62), Lys-133 (0.52), Lys-140 (0.82), Lys-147 (0.85)	10.6
Val-1 (0.89)	7.7
Tyr-103 (0.19), Tyr-151 (0.47)	10.2
Abnormal Sites	
Asp-17 (0.50), Asp-60 (0.48), Asp-141 (0.43)	3.50
Glu-6 (0.30), Glu-38 (0.42)	4.00
His-48 (0.68)	6.80
His-64 (0.10)	7.80
Lys-42 (0.28)	8.4
Tyr-146 (0.05)	11.50
$Fe \cdot H_2O$ (0.01)	8.32

Masked Sites[b]	Basis for Omission
Glu-148, Lys-145	Salt bridge
Arg-45	Ion binding site
His-24, -36, -82, -93, -97	Lack of chemical reactivity, masked in deprotonated form

[a] In each case, static accessibility fractions are given in parentheses, computed as described in the text from data of Lee and Richards (1971). [b] These sites are omitted from the calculation.

to have a wide spread of individual intrinsic pK values in the Linderstrøm-Lang sense, *i.e.*, when the average net charge on the protein equals zero. The recent work of Cohen *et al.* (1972) using proton nuclear magnetic resonance (nmr) measurements on sperm whale myoglobin has shown that there are seven available histidine side chains with pK values ranging from 5.3 to 8.0. Specifically, two of the groups appear to have abnormally high pK values most likely due to hydrogen bonding. We have therefore assigned abnormal intrinsic pK values (from chemical and structural considerations) to residues 48 and 64, which lead to calculated values similar to the values determined from the nmr data.[1] The nmr measurements in question were performed in D_2O without correction of the pH meter readings. They may be taken to underestimate pD by approximately 0.4 unit, a correction that is not necessarily offset by the isotope effect proper. Therefore, the correlation between the nmr and titration values in H_2O may be no more than approximate (Bradbury and Brown, 1973).

The pK_{int} of the iron-bound water was chosen so that the final calculated value agreed with that of Hanania and coworkers (Hanania and Irvine, 1970; Nakhleh, 1971) at zero ionic strength.

Various observations on reactions with myoglobin maybe explained by assuming the presence of certain lysine amino groups with relatively low characteristic pK values (Hugli and Gurd, 1970, Nigen and Gurd, 1973).[2] The crystalline structure (Watson, 1969) moreover presents a wide variety of environments for these groups. Detailed investigation of the model shows that one lysine in particular, residue 42, is hydrogen bonded to the backbone carbonyl oxygen of lysine-98 under circumstances of reduced static solvent accessibility (Lee and Richards, 1971). This residue was chosen for the assignment of a lower intrinsic pK value. Other less obvious possibilities may well exist. All groups were assigned integral intrinsic charges depending on their chemical nature.

A constant pK_{int} is assumed in the computations for all ionic strengths. It can be shown that the correction is no larger than 0.04 pK unit at I = 0.01 M and 0.1 pK unit at I = 0.1 M.

Preliminary Calculations. Using the above set of physical parameters, charged sites, and spatial coordinates, and a variety of sets of intrinsic pK values varying somewhat from those in Table I, a number of trials were made at computation of the titration curve for sperm whale ferrimyoglobin. To obtain good agreement with experiment it was found necessary to set the burial parameter d = 0 as was done for hemoglobin (Orttung, 1970). Even with this restriction it was found that the variation of the pK with ionic strength for the ionization of iron-bound water was not in agreement with experiment (Hanania and Irvine 1970; Nakhleh, 1971; Shire *et al.*, 1974). A modified computation as discussed below gave results in agreement with these various types of experiment, for which the set of intrinsic pK values given in Table I was adopted.

Inclusion of Static Solvent Accessibility Factors. Tanford and Roxby (1972) have pointed out that in order to obtain good agreement with experiment the charged sites in a protein must be placed closer to the surface of the model than the minimum 1-Å depth required in the study of small molecules. They attributed this result to the fact that the titratable groups of a protein really protrude into the surrounding solution and thus do not feel the effects of the internal, low dielectric medium as much as is implied by the formalism of the model. Since the groups protrude or are buried to different degrees it is clear that, strictly, every individual residue should have its own burial parameter, d. Moreover, since myoglobin deviates from the spherical, the physical significance of the burial parameter is not clear. To reflect the degree of exposure of each point charge to the solvent with high dielectric constant, we propose the inclusion of a new parameter which is characteristic for each group.

We make use of Lee and Richards' static solvent accessibilities for sperm whale myoglobin (Lee and Richards, 1971). These accessibilities are a measure of the degree to which a residue in a protein is exposedto solvent in the crystallographic model. Since these accessibilities may differ slightly when the protein is in solution they have been termed static solvent accessibilities to denote the average position of the atoms which are really in motion. By dividing the accessibility for the entire side chain of a residue, X, in a protein by that of the residue in the model tripeptide Ala-X-Ala a fractional exposure can be computed. If we then assume that the electrostatic interaction of two groups is reduced in direct proportion to the amount of exposure to solvent, we can rewrite eq 6 as

$$pK_i = (pK_{int})_i - \frac{1}{2.303kT} \sum_{j \neq i} \frac{1}{z_i}(W_{ij} - SA_j W_{ij}) \quad (7)$$

where SA_j is the fractional accessibility of site j. Now by treating all the sites as point charges on the surface of the model (d = 0) we can use the above equation to attempt to take into account the effect Tanford and Roxby (1972) describe. It is clear that this approach is not strictly correct since eq 7 would give no electrostatic correction as SA_j tends to the value of 1 (*i.e.*, 100% exposed). However, since one cannot be sure of the correct functional form of eq 7 the simple linear form was used. This choice is not unreasonable since the SA_j are an indirect measure of the local variation of dielectric constant and the electrostatic free energy is inversely proportional to the dielectric constant. Since no residue in the molecule is 100% exposed the problem of the electrostatic correction vanishing was not encountered. On the other hand, a few residues of interest in the protein are almost totally buried and in these cases an arbitrarily small value of 0.01 was assigned. The static solvent accessibility values taken for the various sites are included in Table I.

It was possible to fit the titration curve for the main component IV at ionic strength 0.01 M with d set equal to 1.0. However, in combination with the static solvent accessibility terms, this choice of d introduced such large electrostatic correction terms (eq 7) that in the low pH region, the effective pK became much too small, and even negative in many cases. This effect extended to the more acidic histidine groups. Hence, the introduction of the static solvent accessibility factors does not accommodate the d = 1.0 case, provided that unreasonable adjustments of parameters are not accepted.

Results and Discussion

Using the above procedure and set of parameters, including the intrinsic pK values in Table I, the calculations were applied to sperm whale ferrimyoglobin (pure component IV) at zero ionic strength and at a series of finite salt concentrations. Figure 1 shows a comparison of the calculated and the experimental titration curves at 25° and I = 0.01 M. It is seen that the calculated net charge of the protein is zero at pH 8.19 which agrees with the experimental isoionic pH of 8.20 for 0.1 mM protein. The agreement is nearly as close throughout the titration curve.

Without changing any of the parameters, the calculations

[1] The nmr observations of Cohen *et al.* (1972) have been substantially confirmed in this laboratory by L. H. Botelho, M. H. Garner, and G. I. H. Hanania.

[2] J. S. Morrow, P. Keim, and F. R. N. Gurd (1974), manuscript in preparation on carbamino compound formation.

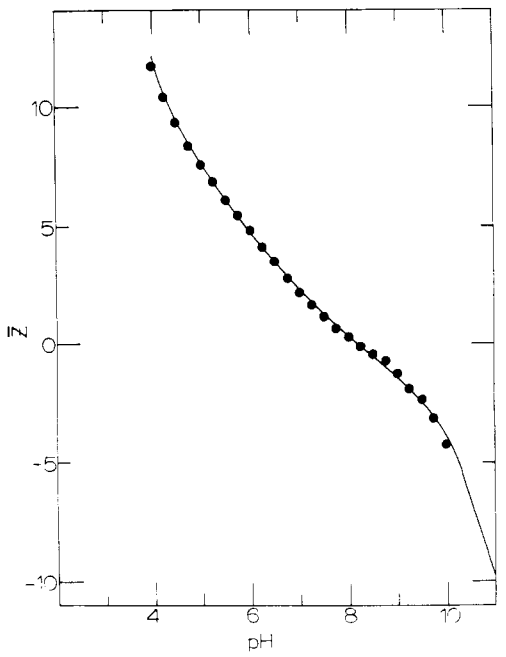

FIGURE 1: Titration curve of sperm whale ferrimyoglobin (major component IV) showing the average net protein charge $\bar{Z}$ as a function of pH. Solid curve, computed by method described in text; full circles, experimental points for 0.1 mM protein at 25° in 0.01 M KCl.

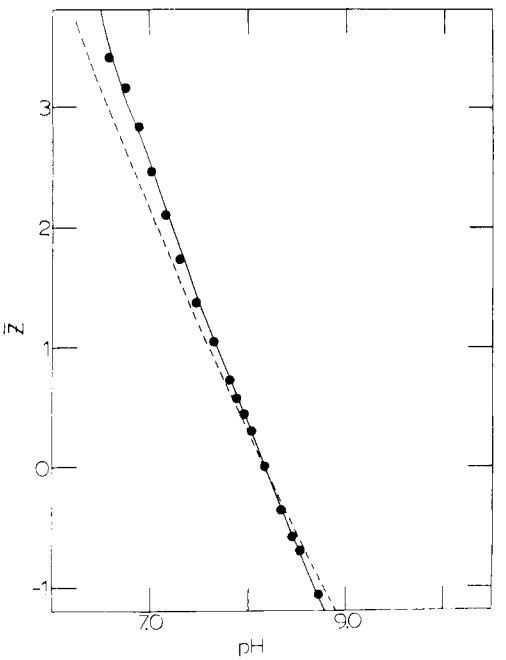

FIGURE 2: Effect of ionic strength on the titration curves of sperm whale myoglobin (major component IV). Theoretical titration curves computed for I = 0.01 M (- - - - -) and I = 0.1 M (——). The experimental points for I = 0.1 M, 0.1 mM protein, at 25° are given by full circles.

were repeated at I = 0.1 M, and compared to experimental values at I = 0.1 M. It should be noted that since the Tanford–Kirkwood theory is based on Debye–Hückel theory any calculation in the region of I = 0.1 M may not be very accurate, and that any agreement obtained must be accepted with this in mind. However, our intentions were to demonstrate that the theory can correctly predict the ionic strength variation of titration curves. The results are shown in Figure 2 in which the agreement between theory and experiment is good; the calculated pH of zero charge in this case is 8.18, in agreement with the experimental value of 8.18 at I = 0.1 M. The computed trend with increasing ionic strength is in the direction indicated by the experiments, that is for higher ionic strengths the acid side of the curve tends to more positive charge and the basic side to more negative charge, whereas the neutral region of the curve changes very little.

Semiempirical and theoretical descriptions of the ionic strength effect have already been given (Cohn and Edsall, 1943; Tanford, 1962). A clear interpretation follows from the Tanford–Kirkwood model. In the acid region, the net charge on the protein is positive, and indeed most of the carboxylic pK values are depressed indicating interactions with positive charges. A comparison of the calculations at two ionic strengths reveals that the decrease in the pK values is less at the higher ionic strength. This is to be expected, since electrostatic interactions are reduced by coulombic screening at higher counterion concentrations. Accordingly, carboxylic pK values are higher at the greater ionic strength, and thus result in a smaller contribution of negative charge, so that the protein molecule has a more positive net charge in the acidic region. In the basic region the opposite effect takes place. The net protein charge is negative, and the overall effect is to raise the arginine and lysine pK values; but at higher ionic strength interactions are reduced and thus the rise in these pK values is diminished. Accordingly, in this region the contribution to positive charge by these groups is reduced, and the overall charge on the protein drops. In the neutral pH region of the curve, both of the above effects are minimal because all the carboxylic groups are nearly fully ionized and all lysine and arginine residues are nearly completely protonated. The main groups contributing significantly in this region are the seven available histidines, and the computations show that ionic strength has a small effect on their pK values. Moreover, since the number of these groups is small compared to other ionizable groups in the protein their total contribution to this effect will be very small. Indeed at pH 8.25 the computed net protein charge is −0.14 at both I = 0.01 M and I = 0.1 M.

The computations were also repeated using different values of a, b, D_i, and D. The results were found to vary slightly with small changes in value for a, b, and D, and were found to be particularly insensitive to the value of D_i, as was observed previously in the computation for hemoglobin (Orttung, 1968).

Effective pK Values. One of the main features of these calculations is that they enable one to examine in detail the electrostatic behavior of individual groups in the protein. In this paper the computed pK_i values will be compared with experimental results where available. One can also obtain for each group the pH variation of the ionization constant, as well as the individual titration curve and its variation with ionic strength. These latter aspects are dealt with in more detail in the following paper (Shire *et al.*, 1974).

Since both the charge and the pK of every group vary with pH (as a result of electrostatic interactions), it is necessary to define a reference pH for comparison of pK_i values. An obvious choice is the pH at which 50% of the amino acid residue is dissociated, and at this pH we define p$K_{1/2}$ as equal to the corresponding effective pK_i. Table II collects such values for the terminal valine residue and the available histidine residues. The special and interesting case of the iron-bound water is dealt with in the following paper (Shire *et al.*, 1974).

N-Terminal Valine. We first consider the case of valine. From Table II the p$K_{1/2}$ is seen to be about 7.8. From studies of the kinetics of the reaction of cyanate with sperm whale myoglobin Garner *et al.* (1973) determined a pK of 7.96 in 0.2 M cyanate which agrees well with the computed value. The dif-

TABLE II: Comparison of Computed $pK_{1/2}$ Values with Experimental pK Values for Valine and Histidine in Sperm Whale Ferrimyoglobin, Major Component IV.

Residue	pK_{int}	$pK_{1/2}$ Ionic Strength, M 0	0.01	0.1	pK_{exp}
Val-1	7.7	7.8	7.8	7.8	7.96[a]
His-12	6.3	4.8	5.0	5.3	5.37[b]
His-48	6.8	6.6	6.7	6.8	6.83[b]
His-64	7.8	7.7	7.8	7.8	8.05[b]
His-81	6.3	6.1	6.3	6.3	6.65[b]
His-113	6.3	5.3	5.5	5.7	5.53[b]
His-116	6.3	5.8	6.0	6.1	6.44[b]
His-119	6.3	5.5	5.7	5.9	6.34[b]

[a] Garner *et al.* (1973). [b] Cohen *et al.* (1972) suggested a different assignment for the resonance of pK = 6.44. Very similar nmr results have been obtained by L. H. Botelho, M. H. Garner, and G. I. H. Hanania. The assignments made here are tentative.

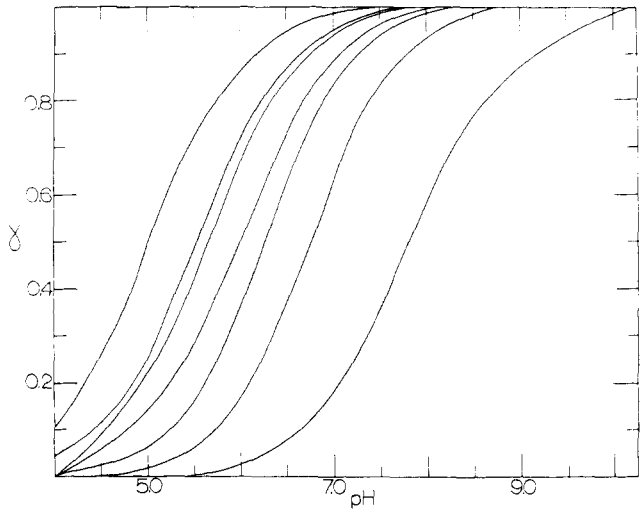

FIGURE 3: Theoretical dissociation curves, showing fraction dissociated α plotted against pH, for individual histidine residues in sperm whale ferrimyoglobin, major component IV, at 25° and I = 0.01 M. The computed $pK_{1/2}$ values at I = 0.01 M are listed in Table II, and the corresponding curves from left to right represent the histidine-12, -113, -119, -116, -81, -48, and -64.

ference in these pK values is appropriate in terms of the effect of ionic strength on the intrinsic pK. It is interesting to note that the computed $pK_{1/2}$ is higher than the intrinsic pK of 7.7 which implies that the terminal valine is in a predominantly negative environment. However, inspection of the molecular model shows that at pH = $pK_{1/2}$ the immediate surroundings (within 10 Å) contain more positively than negatively charged groups, which should lower the pK of the valine. Evidently, however, the total interactions with all the other charges on the molecule lead to an increase in the pK. This example illustrates the usefulness of the theoretical model in helping to avoid incorrect interpretations of the effects of intramolecular electrostatic interactions on the pK value of an individual site based solely on an examination of the immediate vicinity of the site.

Histidine Residues. The histidine residues form a particularly interesting class of groups. Their protonation equilibria cover the important physiological pH range and control the middle portion of the titration curve. The results of the calculations for these pK values are shown in Table II which also includes the nmr data (Cohen *et al.*, 1972), the latter being obtained at higher ionic strengths.[1] Note that the calculations are based on two fitted abnormal pK values (histidine-48 and -64) and a normal pK_{int} of 6.3 for the other groups, no *ad hoc* assumptions being made for these other histidines. Figure 3 shows the individual titration curves for each of the residues and shows the wide spread of $pK_{1/2}$ values, the pattern being similar to that found by nmr. The experimental values in Table II are tabulated to correspond as closely as possible to the calculated values and are not meant as definite assignments. For the reasons already discussed, more attention is to be paid to the spread of experimental pK values than to the actual values obtained in D_2O.

Histidine-12 is a special case because it has been tentatively assigned an effective pK of 6.44 on the basis of comparison of proton nmr spectra of sperm whale and horse myoglobins (Cohen *et al.*, 1972). Our calculations show that histidine-12 has the effective $pK_{1/2}$ of 5.3 at I = 0.1 M, an effect which is due mainly to its close proximity to lysine-16 (3.3 Å) in the X-ray crystallographic structure. If this group is to be matched with a pK of 6.44, it is necessary to find a strongly negative environment or a special structural feature (such as hydrogen bonding) in solution. Neither of these possibilities seems very likely, since the nearest negative group is over 10 Å away and the histidine-12 residue protrudes well into the solution. It is possible that a pK of 6.4 could be calculated by raising the intrinsic pK to about 6.4 and moving the group far enough away from lysine-16 to minimize the interactions. However, if we change the pK_{int} for histidine-12 we must do the same to the other four normal histidines and then the fit of the titration curve in the neutral region is not as good as before. Since all the experimental nmr assignments are tentative it is necessary to postpone a final interpretation of this apparent conflict.

Minor Component. A further test of the theory would be its applicability to another protein. The various components of sperm whale myoglobin, which are closely related, afford an excellent model system. Hanania and Nakhleh (1970) have shown that the individual components of sperm whale myoglobin have significantly different thermodynamic properties. Component II has been shown to have two major charge changes relative to component IV (Garner *et al.*, 1974). The two asparagine residues at positions 122 and 132 in component IV are replaced by aspartic acid residues in component II, with normal pK_{int} of 4.0. The calculations were repeated using the same sperm whale component IV model, the only changes being the above replacements. Figure 4 shows the comparison of the theoretical titration curve with experiment at ionic strength 0.01 M and 25°. As can be seen, agreement is fairly good. The computed pI is 7.25 which is to be compared to the experimental pI of 7.27. Small deviations do occur but these may be due to slight differences between the structures of components II and IV. The dashed curve in Figure 4 is the corresponding curve for component IV. For comparative purposes we have included in Table III the computed NH_2-terminal and histidine pK values for component II.

Discussion of Assumptions. The above calculations have been based on a number of assumptions which should now be restated. The assumptions are dealt with in terms of structural geometry, assignment of charge state, and intrinsic pK values. The structure of the myoglobin molecule in solution was assumed to be the same as that of the crystallographic structure. Although in general this is thought to be true, minor perturbations may occur in solution and thus some of the pK calcula-

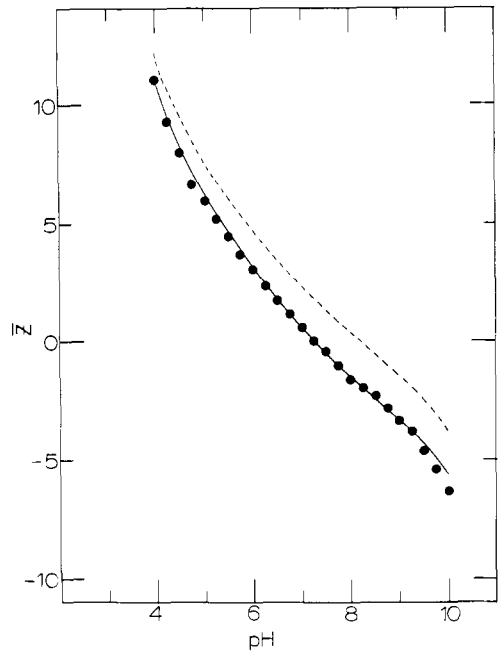

FIGURE 4: Comparison of titration curves for two components of sperm whale ferrimyoglobin at 25° and I = 0.01 M. Theoretical curve for major component IV (- - - - -); theoretical curve for minor component II (——); full circles, experimental points for minor component II.

tions may not be completely reliable. For example, the role of histidine-36 is different in solution and in the crystalline state (Hugli and Gurd, 1970; Nigen and Gurd, 1973). The theoretical model assumes spherical symmetry, which for myoglobin is a good first approximation, and a uniform dielectric medium which ignores local fluctuations. It also assumes a smooth boundary between internal and external media which we have attempted to correct for by introducing a static solvent accessibility factor.

In addition to the above assumptions we have used the same crystallographic structure for component II as for component IV of sperm whale myoglobin. The fact that the pI and the titration curve can be adequately accounted for suggests that the tertiary structure is the same in both proteins. Consequently it may be assumed that minor perturbations which may occur in solution are very similar. Any effect of ion binding has been assumed to be the same for the two components.

The assignment of groups according to charge state and intrinsic pK values presents questions of choice that will affect directly the fit of the curve. An effort has been made to hold special assumptions to a minimum while borrowing evidence from various sources. The evidence of chemical reactivity of histidine residues is useful (Hugli and Gurd, 1970; Gurd, 1970; Nigen and Gurd, 1973) but any given case of restricted reactivity need not apply to proton binding. The assignment of histidine-97 to the uncharged class may well prove incorrect. Special behavior of the lysine and carboxyl residues is largely unexplored. The formal choice to withhold the arginine-45 charge from the calculation amounts to an assumption either that this is the region of an anion binding site (Cameron *et al.*, 1966; Watson, 1969) under the conditions of our experiments, or that some group with an aberrant pK has not been recognized in the examination of the structure. If one or two more lysine residues were assigned lowered intrinsic pK values, it would be possible to allow for the inclusion of a corresponding number of additional positively charged sites. The uncertainty in the location of one or two charges may require later revisions

TABLE III: Computed $pK_{1/2}$ Values for Terminal Valine and Histidine in Sperm Whale Ferrimyoglobin, Minor Component II.

		$pK_{1/2}$		
		Ionic Strength, M		
Residue	pK_{int}	0	0.01	0.1
Val-1	7.7	8.0	7.9	7.9
His-12	6.3	5.1	5.3	5.5
His-48	6.8	6.7	6.7	6.8
His-64	7.8	7.8	7.8	7.8
His-81	6.3	6.4	6.4	6.3
His-113	6.3	5.4	5.7	5.8
His-116	6.3	6.1	6.2	6.2
His-119	6.3	6.0	6.1	6.2

of the computations but will not have large overall consequences. It should be borne in mind that Breslow and Gurd (1962) found the equivalent of six residues with the properties of histidine masked in the basic form, but with an inherent uncertainty in the estimate of at least one residue. The treatment reported here will be used as a guide in future experimentation, and has proved useful for more detailed analyses presented in part in the accompanying paper (Shire *et al.*, 1974).

Despite the limitations discussed above, the electrostatic treatment of myoglobin appears to be successful, particularly in comparing closely related molecules. The advantage of using myoglobin is that the assumption is justified that the major perturbation on the pK_{int} is electrostatic in nature. An example of a protein where other effects are prominent is lysozyme where, unlike myoglobin, the neutral and basic regions of the titration curve are not affected by ionic strength (Tanford and Roxby, 1972). In this respect myoglobin is a good model for the study of electrostatic effects.

The present work is being extended to other myoglobins and their chemically modified derivatives, and to other aspects of electrostatic interactions, with particular reference to structure-reactivity relationships.

Acknowledgments

Helpful discussions with Professor Donald McQuarrie and Mr. Richard Bogardt are acknowledged with thanks. We are grateful to Professor Charles Tanford for advice on several occasions.

References

Bradbury, J. H., and Brown, L. R. (1973), *Eur. J. Biochem. 40,* 565.

Breslow, E., and Gurd, F. R. N. (1962), *J. Biol. Chem. 237,* 371.

Cameron, B. F., Hanania, G. I. H., and Tayim, H. (1966), *in* Hemes and Hemoproteins, Chance, B., Estabrook, R., and Yonetani, T., Ed., New York, N. Y., Academic Press, p 263.

Cohen, J. S., Hagenmaier, H., Pollard, H., and Schechter, A. N. (1972), *J. Mol. Biol. 71,* 513.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions, New York, N. Y., Reinhold.

Edmundson, A. B., and Hirs, C. H. W. (1962), *J. Mol. Biol. 5,* 663.

Garner, M. H., Garner, W. H., and Gurd, F. R. N. (1973), *J. Biol. Chem. 248,* 5451.

Garner, M. H., Garner, W. H., and Gurd, F. R. N. (1974), *J. Biol. Chem. 249,* 1513.
Gillespie, J. M., Hapner, K. D., Hartzell, C. R., and Gurd, F. R. N. (1966), *J. Mol. Biol. 21,* 399.
Gurd, F. R. N. (1970), *in* Physical Principles and Techniques of Protein Chemistry, Part B, Leach, S., Ed., New York, N. Y., Academic Press, p 3831.
Gurd, F. R. N., Lawson, P. J., Cochran, D. W., and Wenkert, E. (1971), *J. Biol. Chem. 246,* 3725.
Gurd, F. R. N., Morrow, J. S., Keim, P., Visscher, R. B., and Marshall, R. C. (1974), *in* Protein-Metal Interactions, Friedman, M., Ed., New York, N. Y., Plenum, in press.
Hanania, G. I. H., and Irvine, D. H. (1970), *J. Chem. Soc. A,* 2389.
Hanania, G. I. H., and Nakhleh, E. T. (1970), *8th Int. Congr. Biochem., Interlaken.*
Hapner, K. D., Bradshaw, R. A., Hartzell, C.R., and Gurd, F. R. N. (1968), *J. Biol. Chem. 243,* 683.
Hartzell, C. R., Bradshaw, R. A., Hapner, K. D., and Gurd, F. R. N. (1968), *J. Biol. Chem. 243,* 690.
Hugli, T. E. (1968), Ph.D. Thesis, Indiana University, Bloomington, Ind.
Hugli, T. E., and Gurd, F. R. N. (1970), *J. Biol. Chem. 245,* 1939.
Janssen, L. H. M. (1970), Ph.D. Thesis, University of Nijmegen, Nijmegen, Netherlands.
Kendrew, J. C., and Parrish, R. G. (1957), *Proc. Roy. Soc., Ser. A 238,* 305.
Kirkwood, J. G. (1934), *J. Chem. Phys. 2,* 351.
Kirkwood, J. G., and Westheimer, F. H. (1938), *J. Chem. Phys. 6,* 506.
Lee, B., and Richards, F. M. (1971), *J. Mol. Biol. 55,* 379.
Linderstrøm-Lang, K. (1924), *C. R. Trav. Lab. Carlsberg 15,* 7.
Nakhleh, E. T. (1971), Ph.D. Thesis, American University of Beirut, Beirut, Lebanon.
Nigen, A. M., and Gurd, F. R. N. (1973), *J. Biol. Chem. 248,* 3708.
Nozaki, Y., and Tanford, C. (1967), *J. Biol. Chem. 242,* 4731.
Orttung, W. (1969), *J. Amer. Chem. Soc. 91,* 162.
Orttung, W. (1970), *Biochemistry 9,* 2394.
Schoenborn, B. P. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 569.
Shire, S. J., Hanania, G. I. H., and Gurd, F. R. N. (1974), *Biochemistry 13,* 0000.
Stryer, L., Kendrew, J. C., and Watson, H. C. (1964), *J. Mol. Biol. 8,* 96.
Tanford, C. (1957a), *J. Amer. Chem. Soc. 79,* 5348.
Tanford, C. (1957b), *J. Amer. Chem. Soc. 79,* 5340.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, Chapter 8.
Tanford, C. (1962), *Advan. Protein Chem. 17,* 69.
Tanford, C., and Kirkwood, J. G. (1957), *J. Amer. Chem. Soc. 79,* 5333.
Tanford, C., and Roxby, R. (1972), *Biochemistry 11,* 2192.
Watson, H. C. (1969), *Progr. Stereochem. 4,* 299.

Electrostatic Effects in Myoglobin. pH and Ionic Strength Variations of Ionization Equilibria for Individual Groups in Sperm Whale Ferrimyoglobin†

S. J. Shire, G. I. H. Hanania,‡ and F. R. N. Gurd*

ABSTRACT: The variations with pH and ionic strength of the NH_2-terminal valine, histidine, and iron-bound water ionization pK values for the major component of sperm whale ferrimyoglobin were computed with a modified Tanford–Kirkwood theory which includes a set of static solvent accessibility factors. Where possible experimental values were compared to the theoretical results and good agreement was obtained. Exclusion of the newly introduced parameters yielded poor agreement with experiment for the ionic strength variation of the ionization pK value for the iron-bound water molecule. The computations with the modified theory were also performed for a minor sperm whale ferrimyoglobin component. The theoretical ionic strength variation of pK value for the water molecule was again in agreement with experiment.

The preceding paper (Shire *et al.,* 1974) deals with the computation of titration curves of sperm whale myoglobin with the Tanford–Kirkwood treatment (Tanford and Kirkwood, 1957; Tanford and Roxby, 1972) adapted by the introduction of static solvent accessibility factors (Lee and Richards, 1971). The inclusion of the static solvent accessibility factors allows good overall fit to experimental data with the choice of individual intrinsic pK values for various groups in myoglobin that are generally reasonable in the light of other evidence.

The model allows computation in good agreement with experiment of the net charge as a function of pH for both the major component IV and the minor component II of sperm whale ferrimyoglobin (Shire *et al.,* 1974). Since the computations also yield information about individual pK values, a further test of the theory is to compare the computed pK values with experimental ionization constants for individual groups in the protein. Experimental data of this kind are available for the NH_2-terminal groups from the kinetics of the cyanate reaction

† From the Department of Chemistry, Indiana University, Bloomington, Indiana 47401. *Received March 11, 1974.* This is the 60th paper in a series dealing with coordination complexes and catalytic properties of proteins and related substances. For the preceding paper, see Shire *et al.* (1974). This work was supported by Public Health Service Research Grant HL-05556.

‡ Permanent address: Department of Chemistry, American University of Beirut, Beirut, Lebanon.