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[†]

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ABSTRACT: The variations with pH and ionic strength of the NH_2-terminal valine, histidine, and iron-bound water ionization p*K* 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 p*K* 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 p*K* 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 p*K* 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 p*K* values, a further test of the theory is to compare the computed p*K* 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

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(Garner *et al.*, 1973) and for the titratable histidine residues from proton nuclear magnetic resonance titrations (Cohen *et al.*, 1972).[1] In both cases, reasonable agreement between theory and experiment is obtained (Shire *et al.*, 1974).

The present paper examines in more detail the behavior of the individual groups in terms of the variation of p*K* with pH and ionic strength. Since the theory is based on electrostatic interactions and the interactions are at a maximum at zero ionic strength, a further test of the theory would be to investigate the ionic strength variation of p*K* values at low ionic strength for individual groups in the protein. Available experimental data on histidine residues and the NH_2-terminal valine residue do not cover the appropriate range of low ionic strength. On the other hand, the iron-bound ionizing water molecule in ferrimyoglobin offers an excellent probe for this kind of study. The dissociation constant for this ionization has been extensively studied in a number of myoglobins covering a wide range of pH, protein concentration, and temperature, as well as of ionic strength variation (Hartzell *et al.*, 1968; Hanania and Irvine, 1970; Nakhleh, 1971).

Experimental Section

The materials, including the preparation of sperm whale ferrimyoglobin components, and the apparatus and procedure for potentiometric titrations, have been described (Shire *et al.*, 1974). Ionization p*K* values for the dissociation of the iron-bound water were determined by the method of mixtures (Nakhleh, 1971), except that Good's buffers were used (Good *et al.*, 1966). The stock buffer solutions at $I = 0.02$ M were Mes[2]-NaOH-KCl (pH 6.0), Bicine-NaOH-KCl (pH 8-10), and glycine-NaOH (pH 11.2). The buffers were diluted with boiled out deionized water to obtain the desired ionic strength.

Computations and treatment of data followed the methods of the preceding paper (Shire *et al.*, 1974), and employed the parameters given therein. The results are expressed in terms of pK_i for the *i*th group at a given pH and given average charge state of the protein, derived by adding a computed electrostatic correction term to the intrinsic p*K*. Use is also made of $pK_{1/2}$, the value of pK_i corresponding to the half-titration of the group in question. The static solvent accessibility factors are derived from the values of Lee and Richards (1971) as adapted by Shire *et al.* (1974).

Results and Discussion

Iron-Bound Water. The ionization constant for the iron-bound water in sperm whale ferrimyoglobin has been investigated in detail (Nakhleh, 1971; Hanania and Irvine, 1970). In particular the variation with ionic strength was measured with care, permitting a detailed comparison with theory of the behavior of a single dissociable group in the protein.

The intrinsic p*K* value was chosen such that the computed theoretical $pK_{1/2}$ value (as defined in Shire *et al.*, 1974) at 25° and zero ionic strength was in close agreement with the experimental value, 8.93, obtained by extrapolation of experimental data at finite salt concentrations in the region of ionic strength $I < 0.01$ M. The computations were then repeated at a series of finite ionic strength values for comparison with the observed results. All parameters were kept unchanged in each series of calculations. This in effect constitutes an evaluation of the C_{ij} terms in the Tanford-Kirkwood formulation (Tanford and Kirkwood, 1957) which give the contribution due to finite salt concentration.

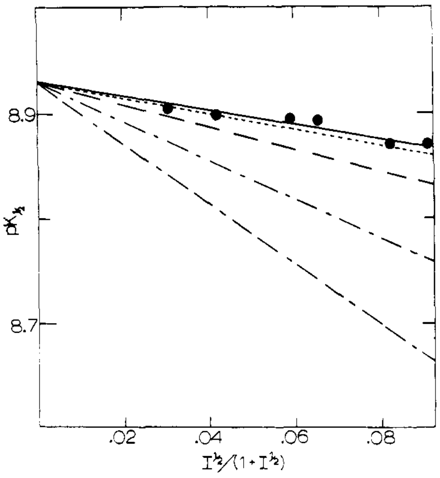

FIGURE 1: Plots of the p*K* value for half-titration, $pK_{1/2}$ against the ionic strength function, $I^{1/2}/(1 + I^{1/2})$, for the ionization of the iron-bound water molecule in sperm whale ferrimyoglobin (major component IV) at 25°. Experimental data are given in full circles, with an uncertainty of ±0.005 pH unit. Computations with solvent accessibility factors included, for $d = 0$ (——) and $d = 1.0$ Å (- - - -); solvent accessibility factors not included, for $d = 0$ (- — -) and $d = 1.0$ Å (- — — -); according to the Linderstrøm-Lang treatment described in the text (— —).

Four sets of calculations were done in this way, corresponding to the inclusion or exclusion of the static solvent accessibility factors (eq 8 in Shire *et al.*, 1974), and with the burial parameter *d* set at 0 or 1.0 Å. The results are shown in Figure 1, relating $pK_{1/2}$ with the ionic strength function $I^{1/2}/(1 + I^{1/2})$. Although the appropriate form of the extrapolation function is not known with certainty, it can be shown that the theory agrees with experiment when the experimental points and computed values are plotted using the same ionic strength function even if it differs from the one we chose. The reason for the particular choice of function is given later in this paper. The filled circles are the experimental results (Nakhleh, 1971; Hanania and Irvine, 1970), which fit well the curves for either assumption for the value of *d* with the static solvent accessibility factors included in the comparison, but not the curves obtained with the exclusion of the latter correction terms. The results clearly favor the use of the static solvent accessibility factors.

Included in Figure 1 as a dashed line is the variation of the p*K* value with ionic strength as calculated by the Linderstrøm-Lang equation based on a smeared charge model (Linderstrøm-Lang, 1924). The electrostatic correction parameter *w* was calculated from the structural parameters used in the discrete charge model computations, that is, $b = 18$ Å and $a = 20$ Å. The average charge $\bar{Z}$ at each ionic strength was obtained from the Tanford-Kirkwood calculations for $d = 0$ and including the static solvent accessibility factors. Agreement at zero ionic strength is good, provided that a p*K* value computed with the modified Tanford-Kirkwood theory at zero net charge is used as the intrinsic p*K*. This result is not surprising since the Linderstrøm-Lang intrinsic p*K* is defined as the p*K* of the ionizing group at zero net protein charge. Instead of a value of 8.93 a value of 8.90 was computed and thus for comparative purposes all the p*K* data at the different ionic strengths were shifted up by a constant 0.03 value. Interestingly enough, the slope of the Linderstrøm-Lang plot is closer to experiment than the Tanford-Kirkwood plots without the static solvent accessibility factors. This may be interpreted to mean that the effect of spreading the charge on the surface of the sphere is to

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[1] L. H. Botelho, M. H. Garner, and G. I. H. Hnania, work in progress.

[2] Abbreviations used are: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine.

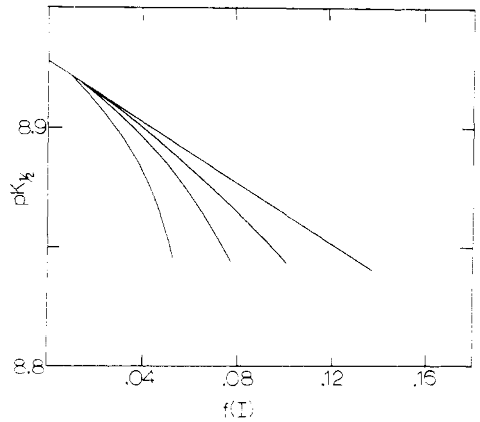

FIGURE 2: Computed values for the ionization pK for iron-bound water of sperm whale ferrimyoglobin (major component IV), expressed as the value for half-titration, p$K_{1/2}$. The described model was used with static solvent accessibility factors at 25°, and the ionic strength function $f(I) = I^{1/2}/(1 + BaI^{1/2})$. Reading from the top down the values of the product Ba are 1, 3, 6, and 12, respectively.

minimize the strong interactions of sites which are close to each other. The solvent accessibility factors perform the same minimizing function provided the sites under question are well exposed to solvent.

Choice of Ionic Strength Function. It has been customary to plot the pK data *vs.* a general ionic strength function $f(I) = I^{1/2}/(1 + BaI^{1/2})$. Here B and a have their usual significance in the Debye–Hückel treatment in which B is 0.329×10^8 cm^{-1} for water at 25° and a is the so-called distance of closest approach of counterions.

Approximating the protein molecule to a sphere allows one to estimate a from the diameters of the protein, counterions, and water molecules. This procedure has led to functions such as $f(I) = I^{1/2}/(1 + 12I^{1/2})$ for hemoglobin (Beetlestone and Irvine, 1968) and $f(I) = I^{1/2}/(1 + 6I^{1/2})$ for myoglobin (Hanania and Irvine, 1970). In the present work the parameters of the spherical model lead to $Ba = 6$.

The p$K_{1/2}$ value of the iron-bound water molecule is plotted in Figure 2 utilizing different values of Ba in the ionic strength function. It is evident from the plots that only the ionic strength functions $I^{1/2}$ and $I^{1/2}/(1 + I^{1/2})$ are anywhere near linearity up to $I = 0.02$ M. We have chosen to use the latter function.

This choice is supported by three observations. From the theoretical viewpoint, the first term of the sum of the polynomials which determine the ionic strength factor in the Tanford–Kirkwood formalism is $\kappa a/D(1 + \kappa a)$, where D is the solvent dielectric constant and κ is the Debye–Hückel parameter which is proportional to $I^{1/2}$. It has also been shown (Tanford and Kirkwood, 1957) for a model very similar to the one used in the present calculations that this is the dominant term in the ionic strength polynomical series, and that this holds up to an ionic strength of 0.04 M. In addition to this point, the use of the above function is supported by the experimental evidence based on analysis of the ionic strength variation of the observed pK values for iron-bound water in sperm whale ferrimyoglobin (Nakhleh, 1971). The analysis makes use of an error function calculated for a general ionic strength function $f(I) = I^{1/2}/(1 + BaI^{1/2})$, and was found to be a minimum at $Ba = 1$. In addition to this experimental observation on myoglobin preliminary measurements on hemoglobin at very low ionic strength (Hanania and Nakhleh, 1974) indicate that the extrapolation function $f(I) = I^{1/2}/(1 + 12I^{1/2})$ will result in a pK value at zero ionic strength which is greater than the true value.

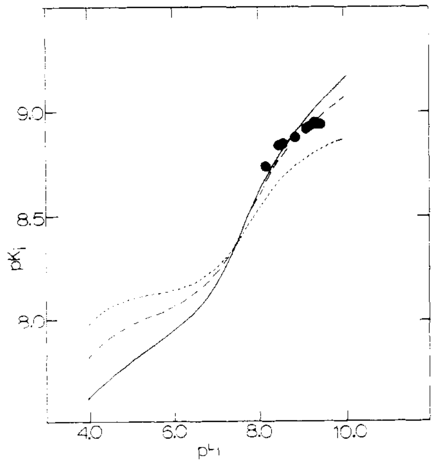

FIGURE 3: Variation with pH of the computed pK_i for the iron-bound water in sperm whale ferrimyoglobin (major component IV) at 25°. Ionic strength 0 (——), 0.01 (— —), and 0.10 M (- - -); full circles represent experimental values obtained at approximately 0.001 M ionic strength.

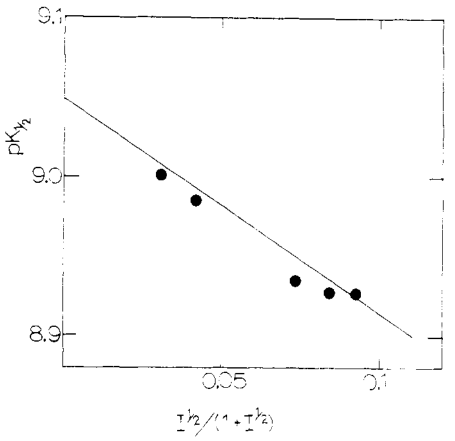

FIGURE 4: Plot of the form of Figure 1 for the minor component II of sperm whale ferrimyoglobin. The curve is computed for $d = 0$ with the static solvent accessibility factors taken into account. The filled circles are experimental points.

Variation of pK with pH. The sensitivity of the ionization of iron-bound water in ferrimyoglobin is shown by marked effects of pH on the absorption bands associated with the heme (Hanania *et al.*, 1966). Even small changes in ionic strength have some effect on the Soret band (Hanania *et al.*, 1966). Chemically modified myoglobins may show appreciable changes in the intensity of the various absorption bands (Ray and Gurd, 1967). Figure 3 shows the pH dependence of the computed pK_i values for the iron-bound water in sperm whale ferrimyoglobin major component IV at 25°. The curves represent ionic strengths of 0, 0.01, and 0.1 M. For comparison, experimental values obtained at $I \approx 0.001$ M are included as filled circles. The sensitivity of the pK_i to pH is very large, even at $I = 0.1$ M. For demonstrating sensitivity to electrostatic effects the plot of the form of Figure 3 is more striking than the usual titration curve format of charge plotted against pH. In keeping with the good fit of the observed values in Figure 1, the experimental points in Figure 3 are in reasonable agreement with the computed behavior over the range shown between pH 8 and 10.

Application to Ferrimyoglobin Component II. A further test of the applicability of the theory is to use a closely related protein such as the minor component II of sperm whale ferrimyoglobin. The computations employed the same model as was applied to the major component IV (Figure 1), with $d = 0$ and static solvent accessibilities taken into account. The only

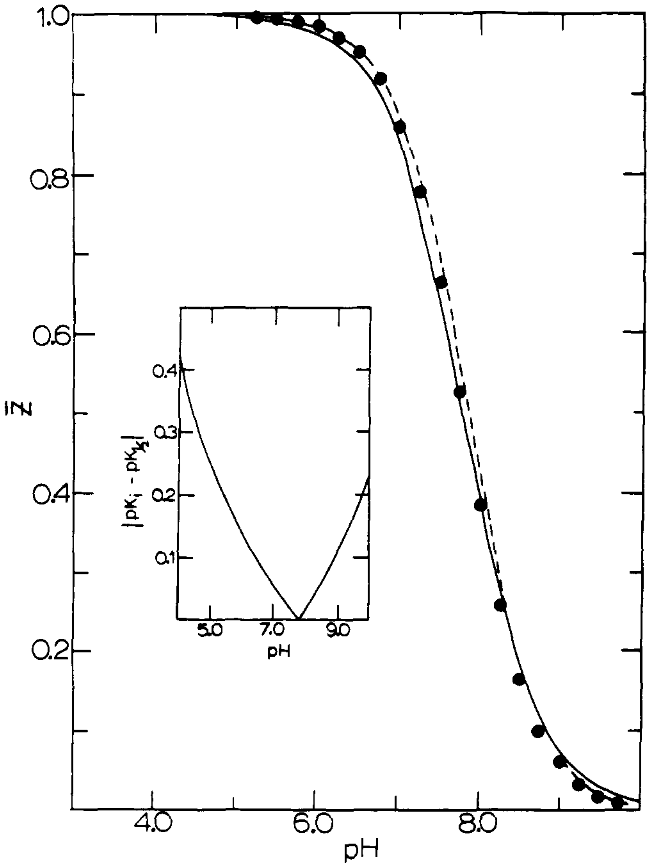

FIGURE 5: Computed titration curves for the NH_2-terminal valine residue in sperm whale ferrimyoglobin component IV. Ionic strength 0 (——), 0.01 M (- - -); filled circles represent simple titration without electrostatic effects included. Inset: plot of $|pK_i - pK_{1/2}|$ against pH, as described in the text.

changes were the adjustments for the replacement of asparagine-122 and -132 with aspartic acid residues (Garner *et al.*, 1974).

The experimental p*K* values, determined in this laboratory, are included as full circles in Figure 4. The agreement is very good. Note by comparison with Figure 1 that the extrapolated value was higher in the case of the minor component as was also the slope of the ionic strength dependence. As shown in the preceding paper (Shire *et al.*, 1974), the effect of the change in charge characteristics of the two sites produced a noticeable change in the calculated and observed titration curves.

NH_2-Terminal Valine. A second unique group whose p*K* can be determined by direct measurement is the amino group of the terminal valine residue (Garner *et al.*, 1973). The experimental p*K* value was 7.96 at 25° at an ionic strength of 0.2 M. The observed value for the pentapeptide corresponding to the first five residues of the protein was 7.87 under the same conditions (Garner *et al.*, 1973). Since this pentapeptide, L-valyl-L-leucyl-L-seryl-L-glutamylglycine, contains negatively charged groups, the intrinsic p*K* was chosen as the somewhat lower value of 7.70 (Shire *et al.*, 1974) which is in reasonable agreement with other model compound values (Tanford, 1962).

The computed titration curve for this group at zero ionic strength is shown as the solid curve in Figure 5, with a dashed line to indicate the values obtained for $I = 0.01$ M. The effect of the increase in ionic strength is small and has the form of steepening the curve slightly. To bring out the effects of the electrostatic interactions even in this case the simple Henderson-Hasselbalch behavior (Mahler and Cordes, 1971) assuming no electrostatic interactions is indicated by the filled circles. The inset of Figure 5 is a plot of the absolute value of the difference at any pH between the pK_i and the $pK_{1/2}$ value as computed by the Tanford-Kirkwood procedure and used in the Henderson-Hasselbalch calculation, and illustrates more clearly the asymmetry in the titration curve due to electrostatic interactions.

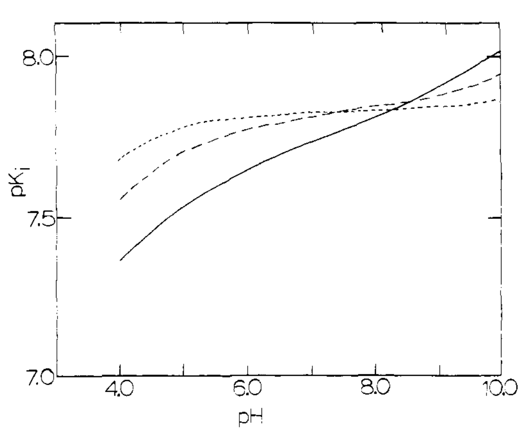

FIGURE 6: Variation with pH of the computed pK_i for the NH_2-terminal valine residue in sperm whale ferrimyoglobin component IV. Ionic strength: 0 (——), 0.01 (— —), and 0.10 M (- - -).

The computed dependence of pK_i on pH is shown in Figure 6 for ionic strength values of 0, 0.01, and 0.10 M. The effect of higher pH in increasing p*K* is diminished as the ionic strength rises. Experimental measurements[3] indicate that the p*K* observed in the range of pH near 8 is about 0.1 unit higher at $I = 0.05$ than at $I = 0.20$. Computed $pK_{1/2}$ values at $I = 0.05$ and 0.1 M are 7.89 and 7.84, respectively. These values include the appropriate corrections of intrinsic p*K* values for ionic strength. Even though the trend is in agreement with experiment it must be kept in mind that computations at $I = 0.1$ M with a theory based on the Debye-Hückel approximation may not be very accurate.

Histidine Residues. The histidine residues dominate an important region of the myoglobin titration curve, and are the subject of intensive study by various methods (Breslow and Gurd, 1962; Cohen *et al.*, 1972; Nigen and Gurd, 1973). Comparison of theory with experiment will become more certain after completion of nmr studies following the approach of Cohen *et al.* (1972). At this point it is instructive to extend the description of the computed titration behavior of the *i*th histidine residue (Shire *et al.*, 1974) by showing the dependence in each case of pK_i on the pH. These results are shown in Figure 7 for 25° and zero ionic strength, covering the pH range 4–10.

The curves in Figure 7 show the usual trend upward in pK_i with increasing pH. However, the effect of pH is not the same for all the groups, which is a reflection of the fact that their electrostatic environments are different. The form of the pH dependence is largely determined by the given electrostatic environment, whereas the position on the ordinate scale is primarily determined by the choice of intrinsic p*K*, as listed in Table I of Shire *et al.* (1974).

A particularly interesting case is that of histidine-64 which shows a very marked perturbation in the pH range of 8–10. The dominant factor in the computations contributing to this effect is the ionization of the iron-bound water molecule which has a p*K* value near 9. This effect may be compared with the results of proton nmr studies of histidine residues in sperm whale ferrimyoglobin (Cohen *et al.*, 1972) which also show a strong perturbation in the titration curve of a histidine group having an ionization p*K* near 8. The same nmr component is

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[3] M. H. Garner and J. S. Morrow, unpublished observations.

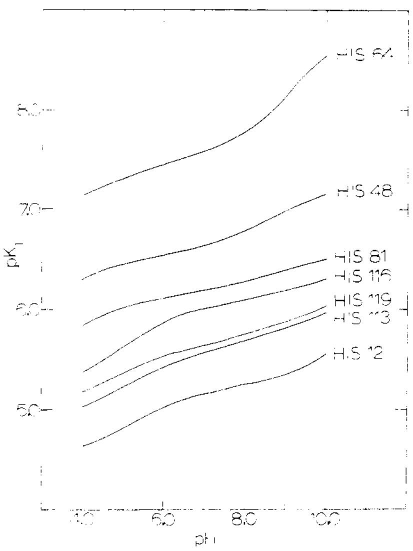

FIGURE 7: Variation with pH of the computed pK_i for the various histidine residues marked, at 25°, and zero ionic strength.

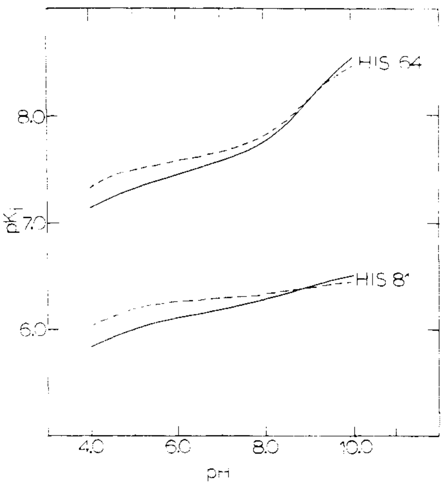

FIGURE 8: Variation with pH of the computed pK_i for histidine-81 and -64 at two values of ionic strength: 0 (——) and 0.01 M (- - - -).

uniquely sensitive to alteration of the ligand charge by fluoride ion.[1] On this basis it seems likely that the abnormally high pK value is that of the distal histidine-64 (see also Shire *et al.*, 1974).

The influence of ionic strength on the pH dependence of the pK_i value is illustrated in Figure 8 for the case of histidine-64 and -81. The effect of increasing ionic strength is similar to that shown in Figure 6 for the case of the NH_2-terminal valine. The other histidine residues show similar behavior. However, the pH where the curves at the two ionic strengths cross, hereafter called the crossover point, is different for all the residues. Inspection of the detailed calculations shows how this effect comes about. For a given group at fixed pH, the net effect of ionic strength on the pK value is the result of the effect of ionic strength on the electrostatic interactions of this group with each of the other charged groups in the protein. In some cases it is found that the net effect is the sum of many small contributions. Examples of this behavior are shown by the NH_2-terminal valine residue (Shire *et al.*, 1974) and histidine-81 (Figure 8). In other cases one or two groups may have dominant interactions with a given group, and thus greatly influence the behavior of this specific group. As discussed above, histidine-64 is an example of such behavior. Histidine-12 is likewise an example of a group which is affected in this manner; the lysine-16 which is 3.2 Å away (Watson, 1969) contributes about 70% of the total effect (Shire *et al.*, 1974). Lysine-16 will contribute greatly as long as the pH is far enough below the pK value for the lysine. At higher ionic strength the interaction with the positively charged lysine will be smaller and thus the pK of histidine-12 will rise. An opposite effect will be observed at higher pH values where the protein molecule has a net negative charge and the lysine plays a less dominant role. The computations show that histidine-12 has a crossover point between pH 10.75 and 11.00 whereas that for histidine-81 has a value between pH 8.50 and 8.75. Experimental data at different ionic strengths are not yet available.

Heme-Linked Effects. In this report the pH and ionic strength dependence of the ionization of the iron-bound water is related to the electrostatic behavior of the particular ferrimyoglobin in terms of all the charged groups in the protein. The importance of the so-called heme-linked ionizations has been recognized for some time for the ferri form of myoglobins (George and Hanania, 1955, 1957) and hemoglobins (Beetlestone and Irvine, 1963a,b). The present adaptation of the Tanford–Kirkwood treatment should be tested further to see how well effects of this kind can be explained on the basis of intramolecular electrostatic interactions. Myoglobin, in contrast to hemoglobin, shows only slight dependence of its oxygenation equilibrium on pH, which implies that large changes in intramolecular electrostatic interactions do not accompany the local conformational change around the heme that probably goes with the binding of O_2 (Rossi-Fanelli and Antonini, 1958). In keeping with the previous discussion (Shire *et al.*, 1974), it is obvious that the form $Fe(III)O_2^-$ (Weiss, 1964) would not show enough charge separation to contribute important electrostatic interactions with charged groups in the molecule. In contrast, large ionization effects probably related to conformational transitions have been observed in the kinetics of ligand binding reactions of myoglobin (George and Hanania, 1955; Goldsack *et al.*, 1966; Ver Ploeg *et al.*, 1971) or in equilibrium observations with certain ligands (Hanania and Nakhleh, 1974). Such effects are seen to accompany major conformational changes of the native-denatured type (Marks *et al.*, 1971).

Relation to Observed pK Values. Figures 3, 6, 7, and 8 illustrate the considerable dependence of pK_i on pH and ionic strength. Clearly a meaningful value for the iron-bound water dissociation (Figure 3) is difficult to define without taking the strong electrostatic effects into account. A case may be made for giving most weight in a limited study to the determination of p$K_{1/2}$ at various ionic strength values (Figures 1 and 3). Obviously, invariance of a pK_i over a range of pH cannot be used as a test of the reliability of the experimental observations.

Figure 3 illustrates the important point that the effective pK_i for a given group may vary widely enough that finite proportions of a given species may be present over a much wider range of pH values than might be expected. This effect may result in enhanced activity of some highly reactive group in a pH range well removed from its usual pK value, with consequences for potential catalytic roles and the like.

An important variable that may affect comparison with experiment is the concentration of protein. The computations are strictly appropriate only for infinitely dilute protein solutions. Changes in concentration of protein may affect intermolecular interactions as well as the degree of binding of solute ions. In addition, very dilute protein solutions are often unstable. Nakhleh (1971) has observed differences in the results for the ionization of iron-bound water as the protein concentration was varied from 1 to 100 μM.

References

Beetlestone, J. G., and Irvine, D. H. (1963a), *Proc. Roy. Soc., Ser. A 277,* 401.

Beetlestone, J. G., and Irvine, D. H. (1963b), *Proc. Roy. Soc., Ser. A 277,* 414.

Beetlestone, J. G., and Irvine, D. H. (1968), *J. Chem. Soc. A,* 951.

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

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

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.

George, P., and Hanania, G. I. H. (1955), *Discuss. Faraday Soc. No. 20,* 216.

George, P., and Hanania, G. I. H. (1957), *Biochem. J. 65,* 756.

Goldsack, D. E., Eberlein, W. S., and Alberty, R. A. (1966), *J. Biol. Chem. 241,* 2653.

Good, N. E., Winget, G. D., Winter, W., Connolly, T., Izawa, S., and Singh, R. M. M. (1966), *Biochemistry 5,* 471.

Hanania, G. I. H., and Irvine, D. H. (1970), *J. Chex. Soc. A,* 2389.

Hanania, G. I. H., and Nakhleh, E. T. (1974), *Ann. N. Y. Acad. Sci.* (in press).

Hanania, G. I. H., Yeghigyan, A., and Cameron, B. F. (1966), *Biochem. J. 98,* 189.

Hartzell, C. R., Clark, J. F., and Gurd, F. R. N. (1968), *J. Biol. Chem. 243,* 697.

Lee, B., and Richards, F. M. (1971), *J. Mol. Biol. 55,* 379.

Linderstrφm-Lang, K. (1924), *C. R. Trav. Lab. Carlsberg 15,* 7.

Mahler, H., and Cordes, E. H., (1971), Biological Chemistry, 2nd ed, New York, N. Y., Harper & Row, p 12.

Marks, R. H. L., Cordes, E. H., and Gurd, F. R. N. (1971), *J. Biol. Chem. 246,* 466.

Nakhleh, E. T. (1971), Ph.D. Thesis, American University of Beirut.

Nigen, A. M., and Gurd, F. R. N. (1973), *J. Biol. Chem. 248,* 3708.

Ray, D. K., and Gurd, F. R. N. (1967), *J. Biol. Chem. 242,* 2062.

Rossi-Fanelli, A., and Antonini, E. (1958), *Arch. Biochem. Biophys. 77,* 478.

Shire, S. J., Hanania, G. I. H., and Gurd, F. R. N. (1974), *Biochemistry 13,* 0000.

Tanford, C. (1962), *Advan. Protein Chem. 17,* 69.

Tanford, C., and Kirkwood, J. (1957), *J. Amer. Chem. Soc. 79,* 5333.

Tanford, C., and Roxby, R. (1972), *Biochemistry 11,* 2192.

Ver Ploeg, D. A., Cordes, E. H., and Gurd, F. R. N. (1971), *J. Biol. Chem. 246,* 2725.

Watson, H. C. (1969), *Progr. Stereochem. 4,* 299.

Weiss, J. J. (1964), *Nature (London) 202,* 83.