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Charge-Site Communication in Proteins: Electrostatic Heme Linkage of Azide Binding by Sperm Whale Myoglobin†

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ABSTRACT: The binding of azide ion to sperm whale ferrimyoglobin has been measured at 25 °C over the pH range 4.0–6.0 at ionic strengths 0.001, 0.002, 0.005, and 0.010 M. The pH dependence of the binding constant, k_L, was analyzed in terms of the modified discrete charge electrostatic theory in three ways. First, an "independent-site" model was constructed in which the charged sites that interact with the charged heme iron, and which lose that interaction on neutralization of the iron charge in the azide complex, are considered not to interact with each other. This unrealistic analysis of the heme linkage fails to conform to the experimental results. The subsequent analyses employed the full electrostatic treatment in which each site is taken to interact with each other as well as with the charge borne by the iron. Second, the increase, $\Delta\bar{\nu}$, in binding of hydrogen ions accompanying the azide binding was estimated from the experimental pH dependence of log k_L and compared with that computed in terms of the theoretical predictions of the changes in pK values accompanying binding. The agreement was satisfactory under all conditions studied, with divergence of about 25% at most. Third, the observed pH dependence of log k_L for each ionic strength was described within 0.10 unit in terms of the summed changes in electrostatic free energy for each charge site accompanying the azide binding as sensed at the iron binding site. The contributions attributed to the individual charged groups were most important where their static solvent accessibilities are low. In these terms, the effective electrostatic domain for interactions with the heme site corresponds to nearly the whole of the myoglobin molecule.

The Tanford–Kirkwood discrete-charge electrostatic theory (Tanford & Kirkwood, 1957), as modified by Shire et al. (1974a) allows calculation of the sites. interaction between charged sites on globular proteins as a function of pH and ionic strength. It has been possible to predict the titration curves of other globular proteins, including myoglobin from a number of mammalian species (Botelho et al., 1978), horse cytochrome *c* and human hemoglobin α chain (Matthew et al., 1978), human deoxy- and oxyhemoglobin A (Matthew et al., 1979a,b), and ribonuclease (J. B. Matthew, F. M. Richards, and F. R. N. Gurd, unpublished experiments). Work has also gone into testing the prediction of the hydrogen ion equilibria at individual sites on these molecules (Shire et al., 1974b, 1975; Botelho et al., 1978; Matthew et al., 1979a,b). Extending the application of the theory for sperm whale myoglobin has included prediction of the acid denaturation properties (Friend & Gurd, 1979a), calculation of the stabilizing and destabilizing interactions between specific groups and charge-site clusters as a function of pH and ionic strength (Friend & Gurd, 1979b), and prediction of hydrogen ion dependent conformational changes sensed in the region of the A helix (Friend et al., 1980).

This paper treats the equilibrium binding of azide ion by myoglobin as a sensitive probe of electrostatic interactions between charged sites. The binding at the heme has been selected so as to allow measurements at the functionally active site and because the low static accessibility of the heme pocket (Lee & Richards, 1971) increases the sensitivity to charge-site interactions (Shire et al., 1974a; Friend & Gurd, 1979b). Additionally, ligand binding to the heme is a long recognized property of heme proteins (Theorell & Ehrenberg, 1951) about which there is an abundance of theoretical models (George & Hanania, 1955; Wyman, 1964).

Azide has been used as the ligand in these studies for the following reasons. (1) It has a relatively high association constant, $\sim$100 mM^{-1}, for binding to myoglobin. (2) It forms a low-spin coordination complex with the iron atom, and the resultant cancellation of charge is easily treated by the electrostatic theory. (3) The X-ray crystallographic difference map for azide myoglobin indicates no major structural rearrangement upon azide binding (Stryer et al., 1964; Takano, 1977). (4) ^{1}H NMR (Botelho, 1975) and ^{13}C NMR

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(Wittebort, 1978; Wittebort et al., 1979; Neireiter, 1979) results indicate that azide and other low-spin complexes of myoglobins differ in only a few respects from aquoferrimyoglobins in terms of histidine titration and general structural properties in solution.

Azide binding has been studied in the pH region below 6 so as to extend previous measurements (Nakhleh, 1971) into the pH range just above that at which gross molecular unfolding occurs (Friend & Gurd, 1979a,b; Friend et al., 1980). This pH region has the advantage of being sufficiently removed from that of the hemic acid dissociation, with its pK of approximately pH 8.9 (Shire et al., 1974b), that the competing hemic acid equilibrium does not have to be considered.

Questions have been posed in the past about the charged-site environment of the heme, especially in the low pH region (Friend & Gurd, 1979b), about the nature of heme linkage (Coryell & Pauling, 1940; Wyman, 1964), and about electrostatic contributions of ligand binding to globular proteins (Tanford, 1961), which can now be addressed cautiously. Some of the questions dealt with in this paper include the following. (1) How suitable are previously formulated models of heme linkage for describing the azide binding to myoglobin in the low pH region? (2) How well can the pH dependence of the azide binding to myoglobin be represented by considering only the electrostatic contributions to heme linkage? (3) How closely does the functional domain of heme linkage correspond to the entire structure of myoglobin? (4) What might be inferred about the general nature of the electrostatic contribution to the association of ligands with proteins?

Theoretical Section

Discrete Charge Electrostatic Model. The assumptions and computational methods of Botelho et al. (1978) and Friend & Gurd (1979a) have been used along with their assignments of intrinsic pK values for the coordinate positions of sperm whale myoglobin as specified by Takano (1977). Detailed descriptions of the electrostatic calculations have appeared previously (Shire et al., 1974a; Matthew et al., 1979a). By specifying the protein radius, ion exclusion radius, temperature, external and internal dielectric constants, and ionic strength of the solution, it is possible to calculate W_{ij}, the work to bring two unit positive charges from infinite separation to any given distance apart on or within the prescribed protein sphere (Tanford & Kirkwood, 1957). W_{ij} has three components, an A_{ij} term for the work to bring these charges together through a low dielectric, D_i, medium, modified by a B_{ij} term which corrects for the high dielectric solvent, and a C_{ij} term sensitive to the solvent ionic strength. The solvent exposure of each site is taken into account (Shire et al., 1974a) by modulating the electrostatic interactions between an ith and a jth site according to the static acessibility of the jth site such that

$$W_{ij}' = W_{ij}(1 - SA_j) \quad (1)$$

The effective pK at an ith site, pK_i, is determined by summing the electrostatic contributions from all jth sites as

$$pK_i = (pK_{int})_i - \frac{1}{2.303kT}\sum_{j\neq i}(W_{ij}')(Z_j) \quad (2)$$

where $(pK_{int})_i$ is the intrinsic pK of the ith site (in the absence of any charged site interactions) and Z_j is the charge at the jth site (Matthew et al., 1979a).

When calculating the electrostatic free energy of interaction between pairs of charged sites at equilibrium occupancy where ith and jth sites are treated identically, a product term, $(1 - SA_i)(1 - SA_j)$, has been used to take into account the solvent exposure at both sites (Friend, 1979). The free energy of electrostatic interaction between two sites with fractional charges of Z_i and Z_j (Friend & Gurd, 1979a) can then be represented as

$$\Delta G''_{el} = (W_{ij})(1 - SA_j)(1 - SA_i)(Z_j)(Z_i) \quad (3)$$

Models of Heme Linkage. The ligand binding reaction of azide ion with ferrimyoglobin may be represented by the overall chemical equilibrium

$$Fe^+(H_2O) + N_3^- = Fe\text{–}N_3 + H_2O \quad (4)$$

in which coordinate bond formation neutralizes the positive charge on the iron as the azide ion displaces the bound water molecule (Stryer et al., 1964). At a given temperature and ionic strength, this reaction will have an association equilibrium constant, k_L, defined below in terms of the molar concentrations of the reactant species. Two types of pH effects must be considered, specific acid dissociation equilibria for the reactants and general intramolecular heme-linked effects in the reactant and product protein molecules.

Specific Acid Dissociation Equilibria. The iron-bound H_2O in sperm whale ferrimyoglobin is known to undergo acid dissociation into the low-spin state, Fe–OH, with ionization pK_{Fe} near 8.9 (Hanania & Irvine, 1970; Nakhleh, 1971; Shire et al., 1974b). Similarly, the acid HN_3 is in equilibrium with its conjugate base, the ligand N_3^-, with an acid dissociation pK_a near 4.6 (Boughton & Keller, 1966).[1]

The experimentally measurable equilibrium constant, k_{obsd}, is defined and evaluated in component concentration terms which represent the sums of the concentrations of both acid and base conjugate forms involved for each of the reactants whereas only the $Fe^+(H_2O)$ and N_3^- species are in reality involved in the association reaction, as follows:

$$k_{obsd} = [Fe\text{–}N_3]/[([Fe^+(H_2O)] + [Fe\text{–}OH])([N_3H] + [N_3^-])] \quad (5)$$

The association constant for the formation of the ligand state that takes these equilibria into account, k_L, is related to k_{obsd} as

$$k_L = k_{obsd}(1 + K_{Fe}/H)(1 + H/K_a) \quad (6)$$

where H is the H^+ thermodynamic activity obtained from the measured pH. Since in the present work the measurements were confined to the range pH 4–6, the term K_{Fe}/H will have negligible effect.

General Heme-Linked Effects. Although the equilibrium constant k_L takes the above two acid dissociation equilibria into account, it nevertheless remains pH dependent, particularly so at low ionic strength. This is referred to as the heme-linked effect, attributable to the ionization of amino acid side-chain groups on the protein molecule (Coryell & Pauling, 1940). A group is said to be linked to the heme if its free energy of ionization is altered upon ligand binding to the iron atom, just as the free energy of ligand binding varies with the degree of ionization of the heme-linked group regardless of the mechanism of linkage.

From the viewpoint of the electrostatic theory, any of the 58 other titratable groups in myoglobin can be linked thermodynamically to the anion binding reaction at the iron site. Until recently, it was not possible to carry out the necessary computations. The introduction of the modified Tanford–Kirkwood discrete-charge theory (Shire et al., 1974a) now

[1] The results of Boughton & Keller (1966) for the pK_a^0 at $I = 0$ of 4.675 was adjusted by use of the Debye–Hückel expression (Barrow, 1973) to yield 4.66, 4.65, 4.64, and 4.62 at ionic strength values of 0.001, 0.002, 0.005, and 0.10 M, respectively.

enables one to consider the concomitant electrostatic interactions of all such groups. Moreover, the electrostatic contribution to heme linkage may be regarded simply as a special example of intramolecular charge-site interaction. In the case of anion binding, the positive charge at the heme iron is neutralized, so that there is an increase in the pK of all electrostatically heme-linked ionizing groups as the potential field becomes more negative. Heme linkage implies that the acid dissociation pK_a value for any given group will increase from pK_r in the reactant ferrimyoglobin to pK_p in the product, the ferrimyoglobin–azide complex. A quantitative measure of heme linkage is $RT(pK_p - pK_r)$, the free energy of interaction between the group and the heme iron, its magnitude depending on the distance, on solvent accessibility, and on the ionic strength of the medium.

This study deals first with a model in which the heme linkage of each site is treated as if these interactions were independent of the effects on any neighboring sites. These restrictions are unrealistic, yet not absurd, when, for example, only two groups are considered to be heme linked (George & Hanania, 1955). When, however, all groups are considered heme linked, as is done in this study, the results are shown not to agree with experiment.

The second model of heme linkage to be considered employs the theory of linked functions first developed by German & Wyman (1937) (Wyman, 1964; Hermans & Scheraga, 1961a,b; Tanford & Roxby, 1972) to interpret the Bohr effect in hemoglobin oxygenation. At constant temperature and ionic strength, it was shown that

$$(\mathrm{d} \log k_L/\mathrm{d}\ \mathrm{pH})_T = \Delta\bar{\nu} \qquad (7)$$

where $\Delta\bar{\nu}$ is the net average number of moles of hydrogen ion released upon ligand binding. This is how George & Hanania (1955) treated the heme-linked effect as observed in the kinetics and equilibria of anion binding to horse ferrimyoglobin. Anusiem et al. (1966) used this method to extend heme-linked analysis to the equilibria of azide and other anion binding reactions of human ferrihemoglobin.

The third model of heme linkage uses values for the free energy of electrostatic interaction between all charged sites to obtain directly the changes in the free energy of azide binding with pH and ionic strength.

Experimental Section

Materials. The major myoglobin component from frozen sperm whale muscle was isolated, purified, and characterized according to Hapner et al. (1968). The ferrimyoglobin was lyophilized and stored at −20 °C until ready for use, at which time it was deionized on a Rexyn I300 (Fisher) column. All chemicals were of reagent grade.

pH Measurements. The apparatus consisted of a water-jacketed titration vessel with a Radiometer G222B glass electrode connected to a water-jacketed calomel reference (Radiometer K497) by an agar bridge, all enclosed within a grounded Faraday box and connected to a Radiometer pHM4 meter (Shire et al., 1974a).

Optical Spectra. Absorbance spectra were recorded on a Zeiss PMQII spectrophotometer by using a water-jacketed cell at 25.0 ± 0.1 °C. Myoglobin concentration was calculated according to the extinction coefficients of Nakhleh (1971).

Equilibrium Studies of Azide Binding. All reactions were carried out at 25.0 ± 0.1 °C in acetate buffers with thermostating. Stock solutions of NaN_3 made up at 20 mM were diluted to 2 mM in acetate buffers at the needed pH and ionic strength for each measurement. Concentrated, deionized myoglobin was made up as a stock solution of 0.1 mM in acetate buffer at the appropriate pH and ionic strength for each set of readings. A 1-mL aliquot of the stock myoglobin and varying quantities of the NaN_3 working solution were brought to 10 mL in volumetric flasks with the appropriate acetate buffers. Enough NaN_3 solution was added to bring the solutions to 0.01–0.06 mM NaN_3 so as to have a series of solutions with varying concentrations of azide at set values for concentration of myoglobin, pH, and ionic strength. For each set of readings, the absorbance values at 409 nm were first obtained without any azide and then with saturation with respect to azide. From these values, the concentrations of myoglobin complex and in turn free total azide and free myoglobin at equilibrium were determined to obtain k_{obsd} values. The absorbance was measured after 30 min of equilibration, and the pH value of each separate reaction was determined at this time. At least six k_{obsd} values were determined for a range of azide concentrations and were averaged for each pH and ionic strength value.

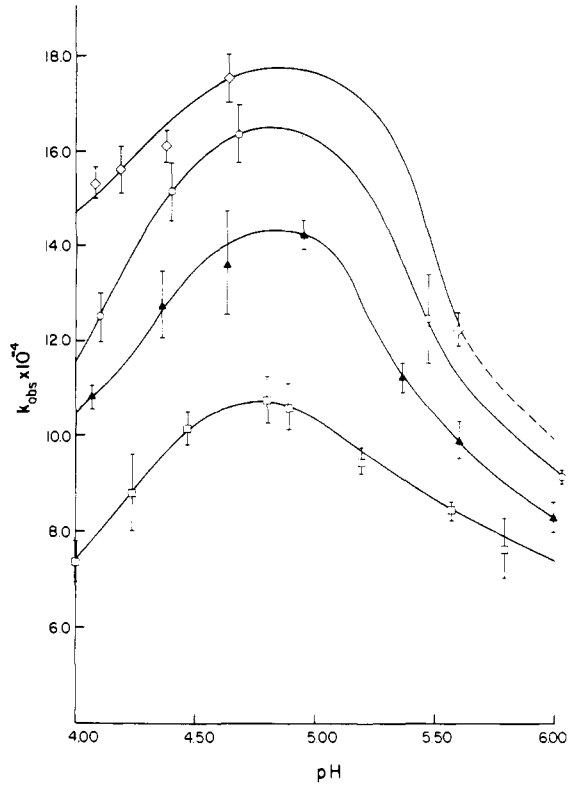

FIGURE 1: pH variation of k_{obsd} (eq 5) for azide binding to sperm whale ferrimyoglobin, in the range pH 4–6 at T = 25.0 °C and ionic strengths I = 0.010 (■), 0.005 (▲), 0.002 (○), and 0.001 M (◇).

Results and Discussion

Experimental Observations. Figure 1 is a plot of k_{obsd} measured at pH values between 6 and 4, at 25 °C and ionic strength I = 0.010, 0.005, 0.002, and 0.001 M. Smooth curves are drawn through the experimental points to help show the trend. The limits of uncertainty are shown by error bars, each representing the standard deviation derived from six determinations, and are generally ±5% of the experimentally determined values. These measurements are extremely sensitive to temperature, reflecting the highly exothermic nature of the reaction (Bailey et al., 1969; Nakhleh, 1971). The bell-shaped curves of k_{obsd} at each ionic strength rise between pH 6 and 5 due to the increased positive charge sensed at the iron as the titratable groups protonate. The k_{obsd} values shift to lower values below pH 4.80 due to protonation of the ligand (pK_a = 4.6 for HN_3).

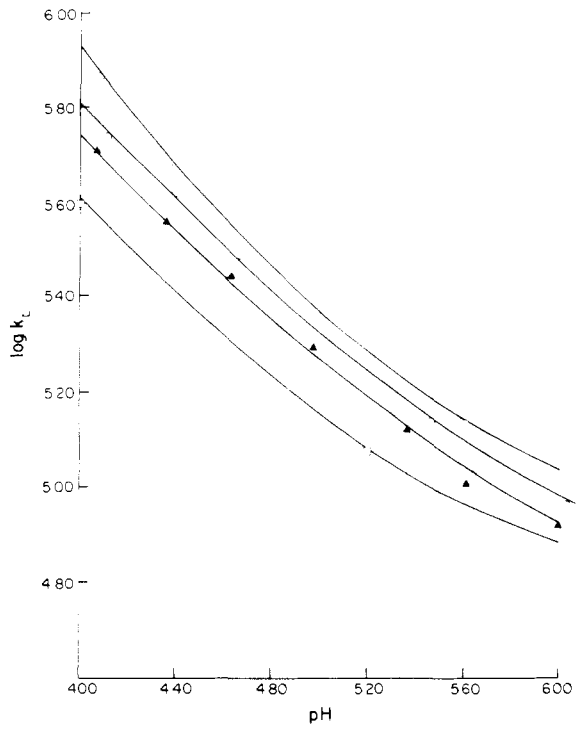

FIGURE 2: pH variation of log k_L. Slopes of the curves yield the net proton uptake accompanying azide binding as a function of pH (eq 7) for the conditions specified and coded in Figure 1.

The observed association constant, k_{obsd} for azide binding to myoglobin is converted to k_L by correcting for the fraction of azide in the protonated unreactive form (eq 6).[1] The log k_L values derived from the data of Figure 1 are plotted in Figure 2 with curves drawn through the data points. Values at the four ionic strengths form a family of smooth curves sloping upward at low pH values without evidence of any discontinuity.

The significant heme linkage in myoglobin is evident from the pH dependence of the k_L values. In going from pH 6.0 to 4.0, k_L values at all four ionic strengths increase by approximately 0.7 unit, implying that the free energy of azide binding is about 1 kcal/mol more favorable at pH 4.0 than it is at pH 6.0. Three models for treatment of the data are discussed below.

Independent-Site Model of Heme Linkage. The independent-site model (Theorell & Ehrenberg, 1951) assumes that each group interacts with the heme independently of all other groups. In order to compare the experimentally observed pH variation of the equilibrium constant k_L with that predicted by this model, it is necessary to obtain the acid dissociation constants K_r and K_p for each heme-linked group. Usually K_r and K_p values are chosen by numerical analysis of experimental data which makes it possible to obtain adequate fit of the data over a limited pH range by suitable choice of one or two sets of K_r and K_p values (George & Hanania, 1955).

Equation 6 may be taken to refer to a standard state in which all the heme-linked groups are in their uncharged form. The pH dependence of k_L is then viewed as due to concentration terms involving all arrays of charge-site occupancies at heme-linked residues. Two treatments arise. For a cationic acid ($HX^+ = H^+ + X$), the standard reaction involving uncharged forms of heme-linked groups will deal with the conjugate base form

$$X\cdots Fe^+(H_2O) + N_3^- = X\cdots Fe{-}N_3 + H_2O \quad (8)$$

Every group of the HX^+ type will have different acid dissociation constants, K_r and K_p, in the reactant and product, respectively. When the two ionization equilibria are introduced into eq 8, it follows that

$$k_L = k[K_r(K_p + H)/K_p(K_r + H)] \quad (9)$$

where k is the pH-independent equilibrium constant for the reaction represented by eq 8 and k_L is the ligand binding constant defined in eq 6. The term $K_r(K_p + H)/K_p(K_r + H)$, the heme-linked pH function for positively charged acids, is symbolized as f_+. If two or more such groups are heme linked independently, eq 9 will contain a product of i terms, one for each of the positive groups, so that

$$k_L = k\prod_i (f_+)_i \quad (10)$$

The standard reaction for the neutral acid groups is written in the uncharged form

$$HX\cdots Fe^+(H_2O) + N_3^- = HX\cdots Fe{-}N_3 + H_2O \quad (11)$$

Corresponding to the above, every group of the HX type will have acid dissociation constants K_r and K_p, and when the two ionization equilibria are introduced into eq 11, it follows that

$$k_L = k[(K_r + H)/(K_p + H)] \quad (12)$$

where k is the pH-independent equilibrium constant for the reaction in eq 11, k_L is as defined above, and the term $(K_r + H)/(K_p + H)$ is the heme-linked pH function for neutral acids, symbolized f_-. For two or more independent groups, a product of i terms is taken. Considering both types of acidic groups in an independent-site model, the overall heme-linked pH effect can be represented by an equation of the form

$$k_L = k\prod_i [(f_+)(f_-)]_i \quad (13)$$

which gives the theoretical variation of the equilibrium constant k_L under the influence of independent heme-linked ionizations in the protein.[2]

By allowing calculation of individual pK_i values for all titratable sites under the influence of their mutual electrostatic interactions, the discrete charge electrostatic theory provides a theoretical method for determining K_r and K_p values for all groups without any reference to experimental data. It was applied to azide binding according to the independent-site model as follows. The pK_i values calculated for ferrimyoglobin are taken to represent pK_r values for all the groups. The corresponding calculation for ferrimyoglobin azide cannot be taken to provide pK_p values to conform to the independent-site model since the change from K_r to K_p would then take into account compensating interactions and the altered distribution of protons among the charged sites. Instead, it is necessary to evaluate the change in the pK_r at each site resulting solely from the loss of interaction between the given site and the iron atom following azide binding. This "independent" change in pK_r then gives the corresponding pK_p value from the relation

$$(pK_p)_i = (pK_r)_i - (\Delta pK_{j=Fe})_i \quad (14)$$

where $(\Delta pK_{j=Fe})_i$ represents only the contribution from interaction with the iron atom to the net ΔpK at the ith site in ferrimyoglobin, which is the heme-linked effect $(pK_r - pK_p)_i$ according to this model.

Given the appropriate K_r and K_p values for each titratable group, the heme-linkage pH function, F, is computed from eq 13 where

$$F = \prod[(f_+)(f_-)]_i \quad (15)$$

[2] Values of f_+ apply uniquely to positively charges sites and values of f_- uniquely to negatively charged sites.

Table I: Heme-Linkage pH Function, F, Calculated According to the Independent-Site Model at the Ionic Strengths Indicated[a]

pH	ionic strength (M) 0.010	0.005	0.002	0.001
4.00	11.60	14.65	19.70	22.28
4.50	4.42	5.36	6.75	7.55
5.00	1.89	2.23	2.67	2.96
5.50	1.01	1.15	1.33	1.47
6.00	0.68	0.75	0.84	0.91
pH ($F = 1$)	5.51[b]	5.65	5.88	5.85
$k \times 10^{-4}$ ($F = 1$)	9.5[c]	10.9	10.6	11.2

[a] $T = 25.0$ °C. [b] pH at which $F = 1$ is obtained by interpolation (eq 15). [c] k (eq 13) is the pH-independent azide binding constant: k ($F = 1$) is its value at pH ($F = 1$).

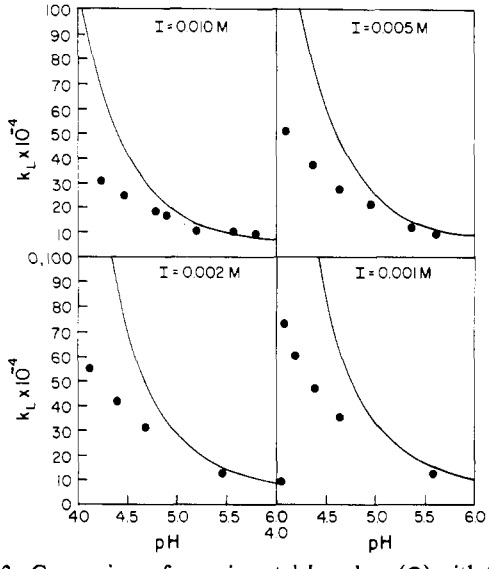

FIGURE 3: Comparison of experimental k_L values (●) with the theoretical variation of k_L as a function of pH according to the independent-site model. Experimental conditions are as in Figure 1.

Table I lists the resulting F values between pH 4.0 and 6.0 at each of four ionic strengths. As eq 13 shows, $k_L = k$ at $F = 1$. Under these conditions, the net electrostatic heme-linked effect will be zero, and this value of k represents the pH-independent equilibrium constant for the azide binding reaction according to the independent-site model. The pH at which $F = 1$ is found by interpolation of the data in Table I. The corresponding $k_L = k$ value at each ionic strength is obtained from the data portrayed in Figure 2 and is listed along with the pH at which $F = 1$ at the end of Table I. At each ionic strength, k_L is then calculated as a function of pH from k and F according to eq 13. The results are plotted in Figure 3.

Figure 3 compares the experimental k_L data (closed circles) with the theoretical curves for pH variation of k_L according to the independent-site model. The agreement is good near pH 6.0 where F is close to unity. At progressively lower pH values, however, the theoretical curves diverge increasingly while showing the same trend as the experimental points. This overestimation of the pH effect is inherent in the model for the following reason. Neutralization of the positive charge on the iron as a result of azide binding shifts the effective pK of each group to a higher value and increases the positive charges, or reduces the negative charges, on the groups that titrate in the given pH region. The extra net positive charges so accumulated have a compensating effect by reducing the rise in pK values, a feature not accounted for in the independent-site model. Accordingly, the theoretical estimate of the K_r/K_p ratio, and consequently of k_L, is too high. In this

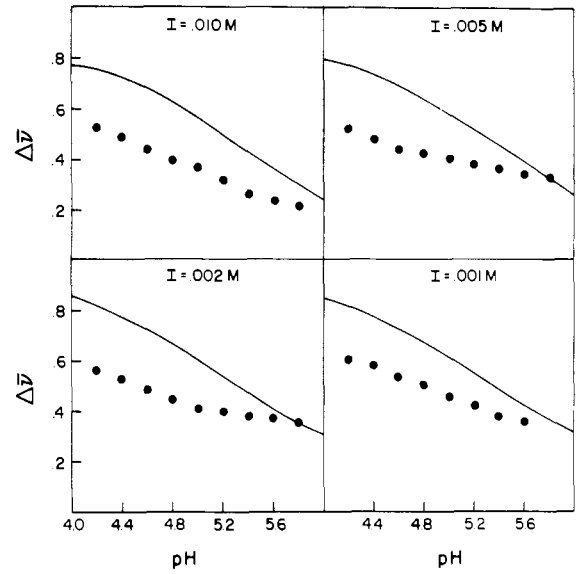

FIGURE 4: Comparison of the net proton uptake, $\Delta\bar{\nu}$, obtained from the experimental data in Figure 2 (eq 7) (●) with that predicted by the discrete charge model (—) for each ionic strength. Experimental conditions are as in Figure 1.

respect, the degree of overestimation by the independent-site model gives an indirect measure of the compensation effect.

Net Proton Uptake and the Electrostatic Discrete Charge Model. The modified Tanford–Kirkwood discrete charge model (Shire et al., 1974a) provides a more realistic approach to the calculation of electrostatic effects since the theory takes into account the mutual intramolecular interactions of all charged sites. As mentioned above, it may be applied to prediction of both $\Delta\bar{\nu}$ and $\Delta G''_{el}$ accompanying the ligand binding. The calculations yield pK_i values for each titratable group (see eq 2). In ferrimyoglobin, these are $(pK_r)_i$ values. In ferrimyoglobin azide, where the charge at the iron site is taken to be zero, the pK_i values are $(pK_p)_i$ for the product. The corresponding effective charges on the groups, $(Z_r)_i$ and $(Z_p)_i$, are then calculated for each group. It follows that the total change in protons bound to the protein accompanying azide binding is given by

$$\Delta\bar{\nu} = \Sigma(Z_p - Z_r)_i \qquad (16)$$

Figure 4 shows theoretical curves for variation of $\Delta\bar{\nu}$ with pH over the range from pH 6 to 4 at each of four ionic strengths. A net uptake of protons upon azide binding is predicted, varying from about 0.2 equiv at pH 6 to 0.8 equiv at pH 4.0, with a minimal ionic strength effect. This increase in bound protons, especially at low pH, would largely compensate for the loss of charge at the iron atom upon azide binding. Such a factor is not taken into account by the independent-site model.

The theoretical estimation of the heme-linked effect by this means may be compared with the experimental $\Delta\bar{\nu}$ values obtained from the slope of the log k_L vs. pH plots which appear in Figure 2 (eq 7). The experimental values are shown as solid circles at 0.2 pH intervals in Figure 4. Agreement between theory and experiment is seen to be excellent near pH 6.0. This is encouraging because the data have not been normalized and because the electrostatic calculations are completely independent of assumptions about heme linkage. At lower pH values, the trend toward higher $\Delta\bar{\nu}$ is found for both theory and experiment, but theory predicts the heme-linked effect to be somewhat greater at pH 4.0. This is a significant improvement over the predictions made by the independent-site model.

Table II: Electrostatic Free Energy Contribution to Heme Linkage, $(\Delta G''_{el})_L$ (cal/mol), Calculated According to the Discrete Charge Model at the Ionic Strengths Indicated[a]

pH	ionic strength (M) 0.010	0.005	0.002	0.001
4.00	−550	−570	−590	−600
4.50	−170	−180	−190	−200
5.00	+140	+130	+120	+100
5.50	+330	+320	+310	+300
6.00	+430	+430	+420	+410
pH $[(\Delta G''_{el})_L = 0]$	4.76[b]	4.77	4.78	4.80
$\log k_L^0$	5.25[c]	5.37	5.42	5.46

[a] $T = 25.0$ °C. [b] pH $[(\Delta G''_{el})_L = 0]$ is the pH at which the free energy contributions as sensed at the iron atom sum to zero. [c] Log k_L at this pH value equals log k, the pH-independent binding constant.

Electrostatic Free Energy of Heme Linkage. The heme-linked effect can be considered in terms of the summed electrostatic free energy of interaction between charged groups and the iron site expressed as the difference between the free energy of azide binding in the presence of charged-site interactions with the iron and the corresponding free energy of reaction in the absence of these interactions (Tanford, 1961). This electrostatic free energy difference, sensed at the iron due to interaction with all charged sites, is given by the relation derived from eq 3

$$(\Delta G''_{el})_L = -\sum_j (W_{Fe,j})(Z_{Fe})(1 - SA_{Fe})(Z_j)(1 - SA_j) \quad (17)$$

where the minus sign reflects anion binding, $W_{Fe,j}$ is the electrostatic interaction between iron and a unit positive charge at the jth site, and Z_{Fe} is +1 in ferrimyoglobin. By definition, electrostatic interactions with the iron site are zero in the absence of electrostatic heme linkage, and they are zero in ferrimyoglobin azide which has zero charge at the iron site. Consequently, $(\Delta G''_{el})_L$ is computed directly from interactions of charged sites with the iron in ferrimyoglobin. The resulting values of $(\Delta G''_{el})_L$ are listed in Table II for the range pH 4–6 at four ionic strengths. The corresponding values of the equilibrium constant k_L (eq 13) are obtained from

$$\log k_L = \log k_L^0 - (\Delta G''_{el})_L / 2.303RT \quad (18)$$

The values for the logarithm of the pH-independent binding constant, $\log k_L^0$, at each ionic strength can be determined as $\log k_L$ at the pH where $(\Delta G''_{el})_L$ is equal to zero, that is, where the iron senses a summed null electrostatic environment. Both these values are listed at the bottom of Table II for each ionic strength.

Figure 5 shows the experimentally determined k_L values as a function of pH for $I = 0.010$, 0.005, 0.002, and 0.001 M, along with the theoretical curves computed from the values of $\log k_L^0$ and $(\Delta G''_{el})_L$ shown in Table II according to eq 18. Throughout the pH range from pH 6 to 4, at all four ionic strengths, the theoretical curves are within 0.10 unit of the experimentally determined $\log k_L$ values. Such a good correlation suggests that the pH-dependent changes in azide binding affinity due to heme linkage can be almost entirely accounted for in electrostatic terms. The treatment serves as a sensitive probe of electrostatic interactions at a particular site in the myoglobin molecule.

The individual contribution of each charged site to the overall $(\Delta G''_{el})_L$ of azide binding to the iron is plotted in Figure 6 for $I = 0.01$ M. Individual values at pH 6.0 are shown as thick lines, and values at pH 4.00 are shown as thin lines. Residue positions are indicated according to the helixes on which they occur, and the groups which contribute more than

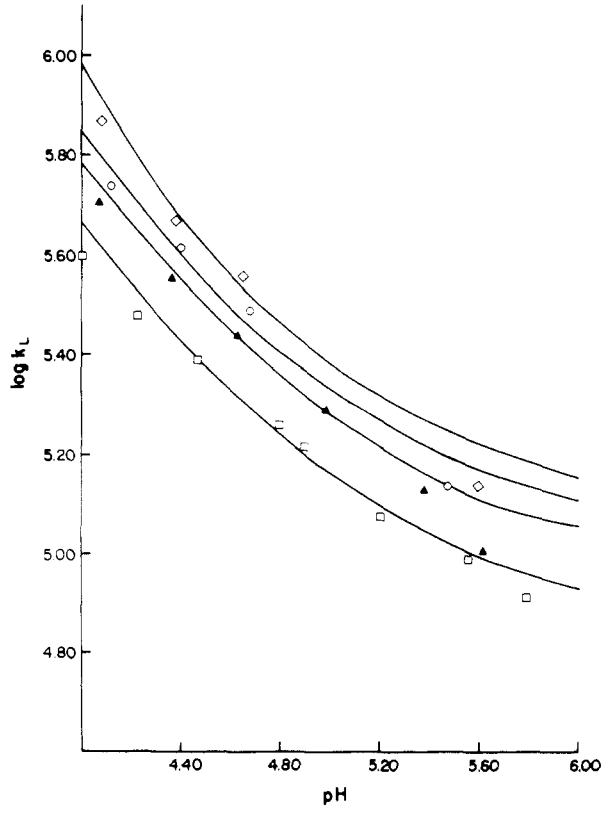

FIGURE 5: Computed pH variation of log k_L for azide binding to sperm whale ferrimyoglobin. Experimental data are as in Figure 2 for the conditions specified in Figure 1. The curves show the theoretical variation according to the discrete charge model.

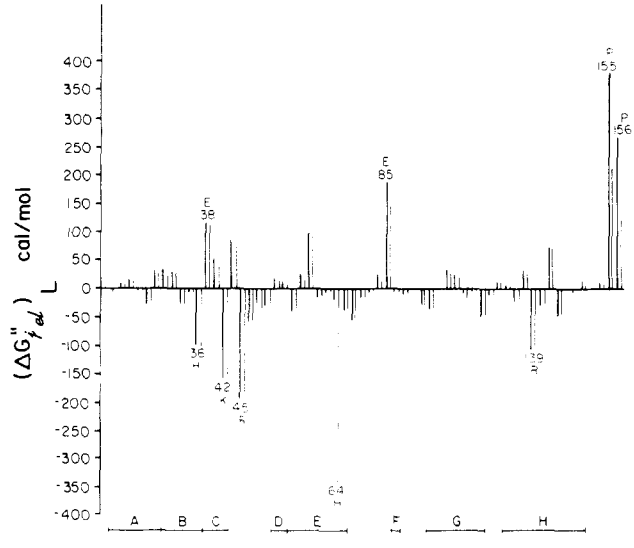

FIGURE 6: Individual-site free energy contributions to heme linkage, $(\Delta G''_{j,el})_L$ (cal/mol); $T = 25.0$ °C; $I = 0.010$ M; pH 6.00 (thick lines), pH 4.00 (thin lines). Charged-site locations are labeled according to their helix reference. The nine most strongly heme-linked groups are specifically labeled. Negative $(\Delta G''_{j,el})_L$ values indicate stabilization of azide binding.

100 cal/mol have been specifically labeled by their residue position in the sequence and the one-letter amino acid code. It is obvious that there are many electrostatically heme-linked groups that contribute significantly, several by more than 200 cal/mol. In going from pH 6.0 to 4.0, the groups which titrate in this pH range vary in the degree of their heme linkage. The cationic groups provide more stabilization and the negative groups contribute less destabilization to the azide binding reaction.

Table III: Strongly Heme-Linked Groups in the Azide Binding Reaction of Sperm Whale Ferrimyoglobin at pH 4 and 6[a]

heme-linked groups[b]	$(1 - SA_j)$[c]	r_{Fej}[d]	pH 6.00		pH 4.00	
			Z_j	$(\Delta G''_{j,el})_L$	Z_j	$(\Delta G''_{j,el})_L$
His-36	0.85	15.6	+0.98	−99	+0.99	−101
Glu-38	0.90	15.5	−1.00	+115	−0.80	+114
Lys-42	0.75	11.5	+1.00	−160	+1.00	−160
Arg-45	0.75	10.4	+1.00	−191	+1.00	−191
His-64	0.95	5.8	+0.03	−18	+0.58	−341
Glu-85	0.75	10.4	−0.99	+189	−0.75	+143
Arg-139	0.95	16.4	+1.00	−103	+1.00	−103
Prop-155[e]	0.70	6.3	−0.98	+381	−0.56	+216
Prop-156[e]	0.50	8.6	−0.98	+170	0.59	+103

[a] $T = 25.0\,^{\circ}C$, $I = 0.01$ M. [b] Residue and position of the heme-linked jth sites. [c] Burial parameter of the jth site. [d] Distance between the jth and iron sites (Å). [e] Heme propionic acid side chains.

Table III lists the nine most significant groups labeled in Figure 6 according to the residue position, the factor $(1 - SA_j)$, the distance from the iron ($r_{Fe,j}$), and the free-energy values computed at pH 6.0 and 4.0, respectively, along with the equilibrium charge occupancies, Z_j, of the group in question. As shown in the second column, all the strongly heme-linked groups in this pH range are 50% or more buried. The largest values of $(1 - SA_j)$ allow the more distant groups to be sensed strongly at the iron. Three of the nine groups are significantly heme linked at a distance of over 15 Å (His-36, Glu-38, and Arg-139), which is nearing the molecular radius of 18 Å. It would therefore appear that almost any charged group with restricted solvent exposure on a protein molecule of this size and shape may be significantly linked. Furthermore, it can be seen that the heme linkage of groups is pH dependent.

Comparison of Heme Linkage Models. Each model tested treated the change in interaction between each protonation site and the iron site after nullifying the charge on the latter through the specific azide binding. In the independent-site model, the protonation sites were treated individually without allowing for any redistribution of protons between binding groups in response to the change expressed at the iron site. Since this model forbids compensating redistributions, it is not surprising that it fits the observed results relatively poorly (Figure 3, Table I).

In the examination of the net proton uptake in its relation to heme linkage, the discrete charge electrostatic theory was applied through eq 16 to the relation in eq 7. The underlying concept is that the distribution of site occupancies will determine the free energy of the protein in solution (Hermans & Scheraga, 1961a,b). The fit with experiment within about 25% is quite satisfactory (Figure 4). The iterative procedure that is central to the full computation (Tanford & Kirkwood, 1957; Matthew et al., 1979a) allows for equilibration of the proton distribution between sites. The present case is analogous to the inclusion of bound chloride ions in the treatment of the titration behavior of hemoglobin (Matthew et al., 1979a,b). Note that satisfactory results are obtained in the present case where $(1 - SA)$ for the iron atom is taken as relatively large at 0.95. The difficulty of evaluating the slopes (Figure 2) required in eq 7 introduces some uncertainty in these calculations. It is instructive to compare in similar terms the present system and treatment with the pioneering anion binding studies of Scatchard & Black (1949) which made use of the smeared-charge electrostatic model (Linderstrøm-Lang, 1924) and the subsequent extension by Halfman & Steinhardt (1971).

The computation of the electrostatic free energy of heme linkage extends the treatment by summation of individual pairwise interaction terms (Friend & Gurd, 1979a,b) to the process of azide binding according to eq 17 (Table II) with evaluation of the pH-independent binding constant, k_L^0, according to eq 18. The agreement between theory and experiment is excellent, as shown in Figure 5. This agreement complements the good prediction of changes in the summed free energy upon azide binding to myoglobin as it was used to identify the effects of the complex formation on the acid stability of the molecule (Friend & Gurd, 1979b). Note that the summation in eq 17 does not provide for the ionic strength dependence of the azide binding process itself (eq 4), which primarily accounts for the trend of log k_L^0 values in Table II. The actual mechanism of binding could involve the penetration of either N_3^- or HN_3 with expulsion of H^+ (Gurd & Rothgeb, 1979).

The pH dependence of k_L is satisfactorily explained in Figure 5 in terms of the electrostatic interactions between charged sites. Table III shows that the nine most prominent heme-linked groups make different relative contributions at the two pH values of 6.00 and 4.00. This is especially true of His-64, but prominent changes are shown for the two propionic acid side chains and Glu-85. The summed positive terms in $(\Delta G''_{j,el})_L$ at pH 6.00 amount to 850 cal/mol for the groups listed in Table III out of a total of 1360 cal/mol if all positive terms for the whole structure are included.[3] Similarly, negative terms at pH 6.00 for the groups listed in Table III sum to −570 cal/mol out of a total of −970 cal/mol. The corresponding values at pH 4.00 are 576 and 765 cal/mol for the positive terms and −896 and −1300 cal/mol for the negative terms. Note that the changes among the negatively charged groups responsible for positive contributions in $(\Delta G''_{j,el})_L$ are more evenly distributed than those among the positively charged groups.

The prominently heme-linked groups suggested to be involved in the hemic acid dissociation of pK = 8.9 (Hartzell et al., 1968; Nakhleh, 1971; Hanania & Irvine, 1970) may not correspond to the set for azide binding in the lower pH range studied here. The present study of the azide binding reaction has been limited to the lower pH range to avoid competition with the hemic acid dissociation reaction. However, k_{obsd} values for azide binding to sperm whale myoglobin in phosphate buffer from pH 6.2 to pH 8.2 (Nakhleh, 1971) connect well with the present values at pH 6.0.

Botelho (1975) observed that the ^1H NMR resonance assigned to the C-2 proton of His-36 does not titrate in the azide derivative. From the NMR evidence, it is not possible to establish whether this residue remains protonated or unprotonated over the pH range explored in the present work. We have chosen to retain for this residue the pK of 7.97

[3] $(\Delta G''_{j,el})_L$ represents the free energy value for the sum of all electrostatic interactions between an ith site, in this case the iron site, and all the jth sites.

reported for the aquoferrimyoglobin (Botelho & Gurd, 1978) which amounts to assigning a substantially invariant positive charge for the present purpose. If, instead, this residue should be assigned a very low pK value,[4] the effect would be to reduce to zero its Z_j and its contribution to $(\Delta G''_{el})_L$ in Table III. The effect of thereby omitting His-36 from the calculations would be to produce small alterations in the titration behavior calculated for other charged sites but would not affect appreciably the fit of the pH dependence of log k_L. A small reduction in log k_L^0 for each ionic strength would be seen (see Table II). In keeping with the treatment given here (eq 2, 7, and 16), Botelho (1975) observed after azide ligation that the pK values of the other histidine residues increased by an average of 0.17 unit.

The pH range examined here is limited at the acid extreme by the onset of the gross unfolding transition described previously in light of the discrete charge electrostatic treatment (Friend & Gurd, 1979a,b). The present study has been limited to sufficiently low ionic strength values such that the onset of the transition disrupting the heme pocket and the secondary structure would occur below pH 4.0. Some loosening of the structure in the region of the A helix below pH 5.0 is indicated by changes in circular dichroism assignable to tryptophan chromophores at residue positions 7 and 14 (Friend et al., 1978) and is compatible with observations of low-angle X-ray scattering in solution (Stuhrmann, 1973; Fedorov & Denesyuk, 1978). There is no evidence that such modest, apparently localized structural changes influence the pH-dependent variation in log k_L. Indeed, an analysis of the electrostatic interactions most probably involved in the loosening of the moorings of the A helix points to a different set of charge-bearing sites from those listed in Table III (Friend et al., 1980).

General Comments. The first question posed in the introductory section dealt with the suitability of various models for describing the azide binding by myoglobin in the pH region in question in light of the discrete site electrostatic treatment. The treatment was first adapted to the restrictions of an independent-site model. The poor correspondence with experiment is easily understood because the different charged groups interacting with the heme iron are in reality far from independent of each other and frequently interact with each other as strongly as they do with the iron site (Friend & Gurd, 1979b). The standard treatment was then applied directly to the net proton uptake to be compared through the thermodynamic linkage function of German & Wyman (1937) with the observed pH dependence of azide binding. Both this application and the direct computation of the summed electrostatic free energy of heme linkage yielded satisfactory correspondence with the experimental results. Note that these two methods utilize relationships involving, respectively, one (eq 2) and two (eq 3) factors of the form $(1 - SA)$. The last method in particular describes the pH and ionic strength dependence without difficulty. These findings answer the second question posed concerning the degree of success achievable.

The pattern of the distances of separation between the iron site and other charge sites, the pertinent pK_{int} values, and the static accessibility values is such that the pH at which the summed electrostatic free energy equals zero is approximately 4.8 (Table II). Note that in the ionic strength range involved at 25 °C, the average net charge attributable at this pH to noniron sites in myoglobin is approximately +7.0 (Breslow & Gurd, 1962). This finding reflects the specific structural details of the protein and is completely incompatible with a smeared-charge model. Because of the lack of comparable applications of the treatment to proteins up to the present and the difficulty of making comparisons with nonprotein heme model systems (Wang et al., 1979), it is premature to comment on the precise values for log k_L^0 listed in Table II. Note, however, that the treatment given here is readily adapted to the computation of k_L under any conditions to which the discrete charge model properly applies, including those at higher pH where competition with Fe–OH formation (eq 5) would interfere with direct measurement.

The third question deals with the degree to which the functional electrostatic domain for heme-linkage corresponds to the entire structure of the protein. Figure 6 and Table III show that the most important groups of the molecule that modulate the binding, apart from the propionic acid groups of the heme itself and His-64 at the lowest pH, are found 10–15 Å distant from the iron. These groups are distributed widely over the surface of the molecule in the C helix, CD region, E helix, F helix, and H helix. Only the A, B, and G helices are sparsely represented. Because of the nature of the treatment (see eq 17), the results just quoted establish that the residues in these last regions in particular are little influenced by the charge borne by the iron in aquoferrimyoglobin. The range of the distribution of important heme-linked sites is particularly striking when Lys-42 and Arg-139, for example, are recognized to be on directly opposite sides of the myoglobin molecule. It follows that a large part of the molecule, if not all, corresponds to the pertinent functional domain of anion binding by the heme.

The fourth question about what may be inferred about the general nature of the electrostatic contribution to the association of ligands with proteins is in the process of being answered. First, there will be a limitation on the size of a protein or, more specifically, on the size of a functional electrostatic domain within the protein. The myoglobin structure is close to that limit, especially when it is considered that at the physiological ionic strength of 0.15 M the strength of electrostatic interactions will be approximately three-quarters of that at 0.01 M for this protein (Matthew et al., 1979a,b; Friend & Gurd, 1979a,b). Second, once distance from the binding site is established, the nature and number of charged residues contributing to the ligand association will depend on their pK values, which are modulated by electrostatic interactions, and their static accessibility values. These variables allow the selection of certain residues to control the ligand association, an aspect of adaptive significance. The SA is minimized by surrounding structures that exclude direct contact with the high-dielectric water; in this way, the electrostatic interactions are strongly modulated by uncharged as well as charged components of the protein structure. A case in point is provided by Arg-139 (Table III), and indeed, the most important heme-linked residues in aquoferrimyoglobin have low values of SA_j resulting in large values of the factor $(1 - SA_j)$.

The SA factor is particularly sensitive to contact with external structures, which provides a mechanism for amplified allosteric mediation. The free energy of electrostatic interaction of a given group with other groups wiill be augmented by the binding of any component that reduces its SA value. The effect will be particularly strong when numerous sites are involved, as in the association with one or more other protein subunits (Matthew et al., 1979a,b; Friend, 1979).

[4] Assigning a low pK value to His-36 in the azide derivative of ferrimyoglobin puts this residue in the class with residues 24, 82, 93, and 97 that do not titrate in the native protein (Botelho & Gurd, 1978) and which are masked in the conjugate base form (Breslow & Gurd, 1962).

All these general points are well emphasized by the recognition of functional domains in proteins (Visser et al., 1975), the widespread occurrence both of binding of ions at primary active sites and of allosteric modulators at secondary sites that themselves usually contribute positive or negative charges (Matthew et al., 1979b), and the recognized regulatory role of association in quaternary structures (Friedman & Beychok, 1979). The results of the present study help answer the original questions about the attractions of proteins for small molecules and ions posed 30 years ago by Scatchard (1949): "How many? How tightly? Where? Why?". The deeper question, "What of it?", added later (Scatchard et al., 1954) may now be more easily assessed.

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