fragments by gel electrophoresis (21 pages). Ordering information is given on any current masthead page.

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Electrostatic Stabilization in Myoglobin. pH Dependence of Summed Electrostatic Contributions[†]

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ABSTRACT: The modified Tanford–Kirkwood theory of Shire et al. [Shire, S. J., Hanania, G. I. H., & Gurd, F. R. N. (1974) *Biochemistry 13*, 2967] for electrostatic interactions was applied to compute the free energy contributions from individual pairs of charge loci in sperm whale ferrimyoglobin. Such interaction energies depend not only on the fractional occupancy of each charge site but also on their static solvent accessibilities, in addition to the geometrical and other factors inherent to the treatment. The pH-dependent unfolding of the myoglobin in acid solution is strongly influenced by ionic strength in such a way that the native form is relatively destabilized by increased ionic strength. Since the titration behavior of the denatured form is less sensitive to ionic strength variation than that of the native form, it follows that the native form experiences net stabilization from intramolecular electrostatic interactions. The unfolded forms are likewise stabilized by ionic equilibria as a result of protonation of histidine residues that are masked in the native state but exposed in the denatured state. The summed electrostatic free energy of the native structure shows a broad maximum at about pH 6.5, in keeping with the observed thermal stability maximum [Acampora, G., & Hermans, J., Jr. (1967) *J. Am. Chem. Soc. 89*, 1543], with a net maximum stabilization of approximately 10.6 kcal/mol at 0.00 M, 10.0 kcal/mol at 0.01 M, and 8.6 kcal/mol at 0.10 M ionic strength. By difference it may be estimated that 5–6 kcal/mol of stabilization of the native protein structure can be ascribed to nonionic factors. The charge sites play a subtle dual role in both stabilizing and enhancing the water solubility of the protein.

Shire et al. (1974a,b, 1975) introduced into the Tanford–Kirkwood model of intramolecular electrostatic interactions (Tanford & Kirkwood, 1957; Tanford & Roxby, 1972) a term to take into account the degree of exposure to the solvent of each charged site. The treatment incorporates the static solvent accessibility values for each charged site derived from the crystallographic structural data (Lee & Richards, 1971) and employs a consistent set of intrinsic pK values. The first applications dealt with the overall titration curves of various ferrimyoglobins (Shire et al., 1974a,b, 1975), since then

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[†] From the Department of Chemistry and the Medical Sciences Program, Indiana University, Bloomington, Indiana 47405. *Received April 13, 1979.* This work was supported by U.S. Public Health Service Research Grant HL-05556. This is the 109th paper in a series dealing with coordination complexes and catalytic properties of proteins and related substances. For the preceding paper see Gurd & Rothgeb (1979).

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confirmed and extended to cytochrome *c* and the α chain of hemoglobin (Matthew et al., 1978b). Good agreement has been obtained between the theoretical p*K* values and experimental results for the NH_2-terminal residue, the iron-bound water, and all histidine residues in ferrimyoglobins from 12 distinguishable species (Shire et al., 1974a,b, 1975; Botelho et al., 1978). Most recently, the treatment has been applied to hemoglobin where similar comparisons with experimental observations have confirmed its predictive power (Matthew et al., 1979a,b).

The results mentioned above suggest that the modified Tanford–Kirkwood theory adequately describes the electrostatic energy of interaction between charged sites on several small globular proteins as a function of ionic strength and deals successfully with occupancy of the sites by protons as a function of pH. Such information should be applicable to numerous questions about protein structure and function. This paper addresses the importance of intramolecular electrostatic interactions in stabilizing the native structure of sperm whale myoglobin. The computed pH dependence of the net electrostatic stabilization is found to agree with observations of the pH dependence of thermal denaturation (Acampora & Hermans, 1967). The pH and ionic strength dependence of the denaturation transition in acid solution is first examined experimentally to establish the outline of the electrostatic contributions to the transition. From an examination of the pH dependence of the computed interaction energies of individual groups, it is possible to suggest the principal elements contributing to the destabilization at the lower pH values. The nature and roles of electrostatic interactions at individual sites are explored more fully in the following paper (Friend & Gurd, 1979).

The data base used here and in the following paper for the computations of electrostatic interactions in the native sperm whale ferrimyoglobin structure is identical with that employed in the test of the predictability of the p*K* values (Botelho et al., 1978), with the exception that contributions from the salt pair, residues 4 and 79, are included.

Two extensions of the treatment have been considered but are not incorporated in the stability calculations. The first is a relaxation of the rigid specification of charge loci to allow for their motions (Gurd & Rothgeb, 1979). For simplicity, we have elected to defer treatment of time dependence of the electrostatic interactions for the present purpose, although for other purposes it may prove essential to take it into account (Gurd & Rothgeb, 1979). The second extension was explored as described below and also was found generally negligible for the present purposes. It concerns the additive contributions of polarity resulting primarily from the helical arrangement of the peptide backbone. Such contributions have been considered by Orttung (1970), for example, in connection with the computation of the dipole moment of hemoglobin. Recently, Hol et al. (1978) dealt with the virtual charges that develop at the ends of an α helix of sufficient length and drew attention to the potential importance of such charges in ion binding. We include evidence that such virtual charges will rarely contribute to a practical extent in the present context. However, it is easy to conceive, as Hol et al. (1978) have indicated, that the dipole virtual charges will be important under other circumstances. Their possible role in directing the folding of myoglobin is discussed in the following paper (Friend & Gurd, 1979).

Experimental Section

Materials. The major myoglobin from frozen sperm whale muscle was isolated according to Hapner et al. (1968) in the ferrimyoglobin form and characterized as described therein and in Botelho & Gurd (1978).

Potentiometric Titrations. The apparatus and methods for titration down to pH 4.0 have been described (Shire et al., 1974a). Below pH 4.0 the time-dependent proton uptake upon unfolding was taken into account by taking pH measurements uniformly 3 min after each addition of acid. The temperature of measurement was 25 °C. The protein concentration was 0.2 mM; at these concentrations the denatured protein aggregates to produce hysteresis effects as the pH approaches 5 during back-titration (Breslow & Gurd, 1962).

Optical Spectra. Absorbance spectra were recorded on a Cary Model 14 spectrophotometer. Concentration was calculated according to the extinction coefficients of Nakhleh (1971). Circular dichroic spectra were recorded on a Jasco optical rotatory dispersion recorder with a Sproul Scientific SS-10 CD modification. All data points represent the average of 12 scans at 25 °C.

Denaturation. Denaturation measurements from optical spectra were made at 25 °C by adding microliter amounts of concentrated freshly deionized myoglobin to 4-mL samples of acetate buffers of predetermined pH, made to the appropriate ionic strength with KCl or sodium acetate or sodium citrate, all of which gave similar results. The protein concentration was 1 μM. A period of 10–15 min was allowed for equilibration before the spectral measurements were begun. This time period was sufficient for substantially complete, reversible equilibration without significant formation of irreversible aggregates (Shen & Hermans, 1972; Breslow & Gurd, 1962). The pH value of each sample was rechecked after each experiment. The absorbance measurements in the near-ultraviolet region showed a strict isosbestic point down to below pH 3.50. Taken along with the concurrent measurements of circular dichroism changes reported below, these observations made it possible to treat the denaturation as a two-state cooperative process.

Computation of Electrostatic Interactions. The computational methods and assumptions of Botelho et al. (1978) were used along with their assignments of intrinsic p*K*, pK_{int}, values.[1] As previously set out (Shire et al., 1974a), each charge site senses a different dielectric environment which can be accounted for by inclusion of the appropriate static accessibility, SA, value (Lee & Richards, 1971; Matthew et al., 1978a). Here we use again a linear approximation for the resulting effective electrostatic interaction energy, W_{ij}' (Matthew et al., 1979a), defined by the relation

$$W_{ij}' = W_{ij}(1 - \mathrm{SA}_j) \qquad (1)$$

where W_{ij} is the free energy of interaction for a pair of unit point charges z_i and z_j denoting full site occupancy computed according to the spherical geometrical model for a given ionic strength and temperature.[2]

Since W_{ij}' is linear with charge occupancy at the *i*th site (Shire et al., 1974a; Botelho et al., 1978), and p*K* of *i* is determined by the derivative $\partial W_{ij}/\partial Z_i$ (Tanford, 1961; Matthew et al., 1979a), it follows that the changes in the pK_i of the *i*th site through its interactions with all other charged sites *j* are not determined by site occupancy of the *i*th group

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[1] The value of pK_{int} for the NH_2-terminal valine was taken as 8.00 (Matthew et al., 1978b). This value was employed by Botelho et al. (1978) but was omitted inadvertently from their listing.

[2] Following the usage of Matthew et al. (1979a), the lower case z_i or z_j is reserved for unit charge occupancy and the upper case Z_i or Z_j denotes actual site occupancy.

but only at the jth site, Z_j. The pK for the ith site can be expressed as

$$\mathrm{p}K_i = (\mathrm{p}K_{\mathrm{int}})_i - [1/(2.303kT)]\sum_{j\neq i}(W_{ij}'z_iZ_j) \qquad (2)$$

Here W_{ij}' is the free energy of interaction for a pair of fully occupied point charges i and j corrected for the accessibility of site j (eq 1) with z_i maintained as the unit charge and Z_j denoting the fractional occupancy of site j.

To determine the work required to bring two unit point charges from an infinite distance to their respective locations, which will define the electrostatic free energy of stabilization or destabilization between the two of them in the protein molecule in the presence of equilibrium occupancy of all other sites, it is necessary to consider the static accessibility at both sites. All sites are concomitantly treated as both ith and jth sites, as the interactive free energy of any pair is reciprocally identical. The linear approximation for W_{ij}' used in computation of H^+ ion equilibria applies exactly as before for the electrostatic interaction between the two unit charge sites and can now be represented by

$$W_{ij}'' = W_{ij}[(1 - \mathrm{SA}_j)(1 - \mathrm{SA}_i)] \qquad (3)$$

The individual W_{ij}'' term defines the free energy contribution from the isolated interaction between the two unit charge loci under given conditions of constant temperature and pressure. To obtain the free energy contribution for the actual site occupancies, it is convenient to define

$$\Delta G_{\mathrm{el}}'' = W_{ij}''Z_iZ_j \qquad (4)$$

The value of $\Delta G_{\mathrm{el}}''$ depends on both the fractional occupancy of the two charge sites following the reintroduction of Z_i and Z_j to replace the unit occupancy assumed in the calculation of W_{ij} and W_{ij}'' and their static accessibilities applying to a given W_{ij} value. Note that both these occupancy factors and the static accessibility factors vary over wide ranges for given sites or pairs of sites as treated in this and the following paper (Friend & Gurd, 1979). Hence, a given $\Delta G_{\mathrm{el}}''$ may be diminished with respect to the maximum W_{ij} for a given distance of separation, r_{ij}, by a considerable factor on the basis of the effects of the individual static accessibility and occupancy factors at each site in any combination. Site occupancy is obtained from W_{ij}' as expressed through eq 2 in which the 1 − SA_i term does not appear. It is clear from eq 3 that a pair of sites that have high solvent exposure will interact effectively only at relatively short distances of separation, r_{ij}. On the other hand, if one or both sites are relatively inaccessible to solvent, the interaction will be potentially effective over substantial distances (Matthew et al., 1979a).

The summed electrostatic free energy terms used here reflect the experimental match of theory and observation with respect to individual pK values, eq 2, and the overall titration behavior as previously reported (Botelho et al., 1978; Shire et al., 1974a,b, 1975). This match of theory with observation gains strength from the good fit obtained for computed and observed histidine pK values for a wide variety of myoglobins (Botelho et al., 1978). The data underlying the application of eq 2 in the computation of the pK values included the 1 − SA term as required in eq 1. The added step required for the electrostatic free energy computations, eq 3 and 4, involves necessarily the second 1 − SA factor to treat the pairwise summations and so weights the contributions of the individual static accessibilities somewhat more. This is done without any manipulation of the data base.

Unless otherwise stated, all values presented here refer to an ionic strength of 0.01 M.

Table I: Dipole Virtual Charge Placements for Sperm Whale Myoglobin

no.	residue	helix ref	nearest atom	charge	1 − SA
4	Glu	2A	N	0.5	0.95
34	Lys	15B	O	−0.5	0.95
58	Ser	1E	N	0.5	0.95
78	Lys	1EF	O	−0.5	0.95
84	Val	7EF	CA	0.5	0.95
95	Thr	10F	O	−0.5	0.95
102	Lys	3G	N	0.5	0.95
120	Pro	2GH	CA	−0.5	0.95
150	Gly	1HC	CA	−0.5	0.95

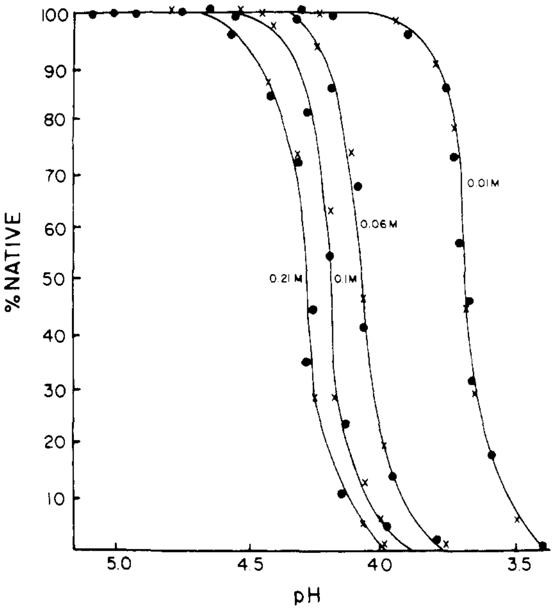

FIGURE 1: Dependence of unfolding transition of sperm whale ferrimyoglobin on pH. The ordinate shows the percent of the native form found for the substantially two-state process (see text). The curves show results for ionic strength values of 0.21, 0.10, 0.06, and 0.01 M, respectively: (●) 409 nm; (×) 222 nm.

Virtual Charges of α-Helix Dipoles. Hol et al. (1978) suggested simulating the presence of an overall dipole moment associated with an α helix by placing 0.5 positive and negative charges, respectively, at the amino-terminal and carboxyl-terminal ends of each helix formed of seven or more residues that is more than two turns. Since the charge placement appropriate for the computations is loosely defined, we have chosen to locate the virtual charges on the distal atoms along the helix axis of each appropriate helix as determined by use of computer graphics and a large skeletal model of the myoglobin molecule. The locations chosen and the signs of the virtual charges are listed in Table I. In the computations reported below the corresponding values of SA have been set at 0.05 to assure full emphasis of the effect, and these charged points at half-occupancy have been included in calculations of individual pK_i values according to eq 2. The precise geometrical placement of the virtual charges is critical only for the groups immediately at the ends of the helices; otherwise, a variation in position of as much as 2 Å produces comparable results.

Results and Discussion

Effects of pH and Ionic Strength on Stability. The conformational transition of sperm whale myoglobin in acid solution is an example of a poised process in which the effects of variation of pH and ionic strength clearly show the importance of electrostatic interactions for stability of the native structure. Figure 1 shows the effect of pH on the percentage of the native structure present at four different values of ionic strength. The two measures of the native and denatured states were absorbance at 409 nm (Breslow & Gurd, 1962) and molar ellipticity at 222 nm (Puett, 1973). These variables detect respectively the loss of the characteristic environment of the heme (Theorell & Ehrenberg, 1951) and the loss of helical folding of the polypeptide backbone elements (Breslow et al., 1965). The changes in these two variables are clearly concurrent, indicating a cooperative process involving reversibly the whole protein molecule.

Figure 1 shows that the dependence on ionic strength is marked, indicating that the native state is destabilized relative to the denatured state as the electrostatic interactions are lessened with increasing ionic strength. Note that the variation in the effect is most marked at the lower ionic strengths, as expected from the modified Debye–Hückel treatment (Tanford & Kirkwood, 1957; Shire et al., 1974a,b). As shown previously (Breslow & Gurd, 1962) and confirmed with separate experiments in the present study, the proton titration curve for the denatured state is much less dependent on ionic strength than that for the native state. It follows that intramolecular electrostatic effects are more important for the native structure. Furthermore, since the increase in ionic strength serves to reduce the absolute magnitude of the electrostatic interaction terms, W_{ij}, it follows that the net effect of these terms is to contribute to the stability of the native structure under the conditions of the experiments reported in Figure 1. Since the total number and distribution of bound hydrogen ions among the binding sites are dependent on ionic strength at a given pH, quantitative computations were made as reported below to confirm this conclusion.

The results in Figure 1 are substantially unchanged by varying the electrolyte between KCl, sodium acetate, and sodium citrate, which indicates the absence of specific ion effects. Potassium perchlorate, on the other hand, appears to have a specific destabilizing effect.

The conversion to the denatured state not only alters structural variables such as solvent accessibility and separation of charged groups but also alters the effective composition of the myoglobin by causing exposure of several fully buried histidine residues that do not enter the hydrogen ion equilibria of the native protein (Breslow & Gurd, 1962; Gurd, 1970). These histidine residues are at positions 24, 82, 93, and 97 (Takano, 1977; Botelho et al., 1978). They have been shown to be uncharged in the native structure and to take up protons in the usual way following denaturation (Breslow & Gurd, 1962). The change in effective composition with respect to histidine residues produces a hysteresis effect as illustrated in Figure 2. The experimental points at an ionic strength of 0.01 M are shown, starting from pH 7.0 and titrating along the lower limb into the region of the acid transition where a sharp rise in hydrogen ions bound, $\bar{Z}$, occurs. The extent of this sharp rise is attributed (Breslow & Gurd, 1962) to the diminished electrostatic interaction term that applies to any group in the less compact denatured form as well as to the release of the buried histidine residues. In the unmodified protein (Clark & Gurd, 1967), the re-formation of the native structure is sluggish under the conditions of the experiment, which leads to the distinct upper limb in Figure 2 observed during the back-titration to higher pH.

The five curves in Figure 2 are computed as follows. The solid curve 1 bearing the experimental points down to pH 4.0 represents the theoretical behavior computed from the electrostatic theory as previously defined. The dashed curve 2 results from including in the theoretical calculation the four buried histidine residues 24, 82, 93, and 97, residue 64 being already included in the treatment (Botelho et al., 1978). The broken curve 3 shows the effect of assuming that the internal dielectric constant (Tanford & Kirkwood, 1957; Matthew et al., 1979a) is set equal to that of water. Additionally, for approximation of the decreased interactions between charged sites due to the larger distances between groups (r_{ij}) upon unfolding, the W_{ij} values, which are directly a function of r_{ij}, were arbitrarily assigned half the values used for the native structure in the computation shown in curve 4 to match the experimental points in the back-titration. The manipulations performed in curves 3 and 4 go beyond the specific restrictions placed on the Tanford–Kirkwood model of a large difference in internal and external dielectric constants and are included here simply to show how limited electrostatic interactions are in the denatured forms and not as a specific modeling of denatured electrostatic interactions. The top curve 5 corresponds to the condition in which the pK_i values of the individual groups are taken equal to their pK_{int} values, which amounts to eliminating the summation term in eq 2.

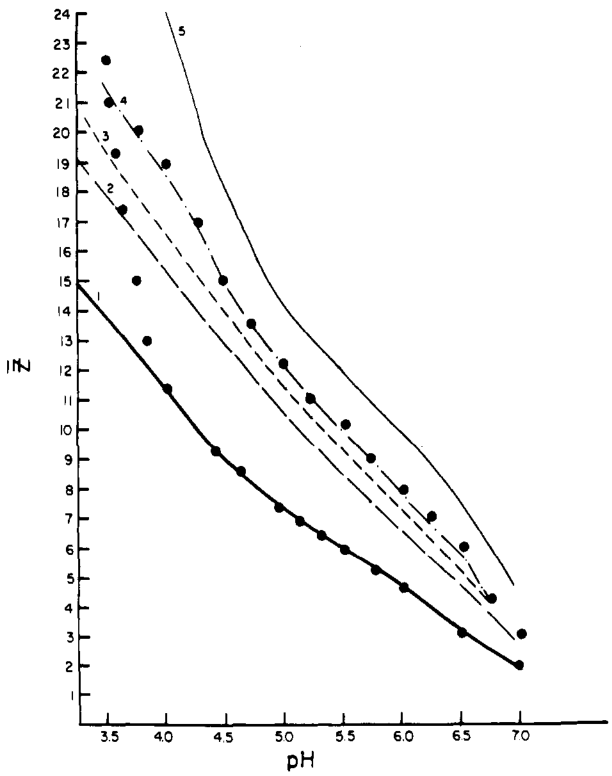

FIGURE 2: Dependence of the average net positive charge borne by sperm whale ferrimyoglobin (●) on pH. Curve 1 is computed according to eq 2 for the native protein structure. Curves 2–5 represent computed results according to a series of assumptions described in the text for the back-titration of the denatured protein in which the normally buried histidine residues 24, 82, 93, and 97 are exposed to solvent and capable of proton uptake.

The placement of the observed back-titration points in Figure 2 relative to the various computed curves indicates that, although the internal electrostatic interactions are considerably diminshed relative to the native structure, the charged sites are not completely isolated electrostatically from each other. It seems that the unfolded structure is loosely packed, in agreement with the large conformational motility observed, for example, with another model for the denatured protein, the apomyoglobin derivative containing fully carboxymethylated histidine residues (Nigen et al., 1973). These

Table II: Titrating Groups in Sperm Whale Myoglobin[a]

						$pK_{1/2}$	
group	residue no.	helix ref	atom	1 − SA	pK_{int}	formal charges only	including dipole virtual charges
carboxyl terminal	153	HC	OE1	0.10	3.60	3.02	3.02
propionic acid	155	heme	O1A	0.70	4.00	2.87	2.89
	156	heme	O1D	0.50	4.00	2.88	2.92
aspartic acid	60	3E	OD2	0.60	3.50	1.78	1.69
	141	18H	OD1	0.80	3.50	1.99	1.97
	20	1B	OD1	0.50	3.50	2.20	2.16
	27	8B	OD2	0.35	4.00	2.28	2.29
	122	4GH	OD1	0.45	4.00	2.29	2.22
	44	2CD	OD1	0.60	4.00	2.67	2.71
	126	3H	OD1	0.30	4.00	3.55	3.24
glutamic acid	38	3C	OE1	0.90	4.00	1.77	1.83
	4	2A	OE1	0.50	4.00	2.34	1.89
	6	4A	OE1	0.50	4.00	2.71	2.52
	18	16A	OE2	0.40	4.00	2.87	2.84
	105	6G	OE1	0.35	4.50	3.16	3.01
	109	10G	OE1	0.30	4.50	3.36	3.30
	52	2D	OE2	0.30	4.50	3.39	3.43
	136	13H	OE1	0.50	4.50	3.45	3.36
	54	4D	OE1	0.25	4.00	3.48	3.41
	41	6C	OE2	0.50	4.50	3.54	3.59
	85	8EF	OE2	0.75	4.50	3.49	3.38
	83	6EF	OE1	0.40	4.50	3.78	3.68
	148	25H	OE1	0.20	4.50	3.79	3.81
	59	3E	OE1	0.30	4.50	4.06	3.90
histidine	64	7E	Nπ	0.95	5.00	4.20	4.17
	113	14G	Nτ	0.35	6.60	5.35	5.39
	119	1GH	Nπ	0.80	6.00	5.42	5.35
	12	10A	Nπ	0.15	6.00	5.70	6.63
	116	17G	Nτ	0.05	6.60	6.36	3.22
	48	6CD	Nτ	0.30	6.60	6.53	6.45
	81	4EF	Nτ	0.05	6.60	6.55	6.67
	36	1C	Nτ	0.85	6.60	7.77	7.68
amino terminal	1	1NA	N	0.05	8.00	7.87	7.80
iron	154	heme	Fe	0.95	8.40	8.93	8.94
tyrosine	151	2HC	OH	0.40	10.00	10.20	10.78
	103	4G		0.60	10.00	10.61	10.60
	146	23H		0.95	10.00	11.90	11.80
lysine	98	3FG	NZ	0.20	10.40	10.38	10.57
	147	24H		0.50	10.40	10.50	10.57
	34	15B		0.15	10.40	10.72	10.52
	87	2F		0.95	10.40	10.75	10.67
	62	5E		0.20	10.40	10.82	10.71
	56	6D		0.65	10.40	10.84	10.78
	63	6E		0.05	10.40	10.95	10.91
	50	8CD		0.40	10.40	10.96	10.97
	96	1FG		0.05	10.40	11.04	11.21
	77	20E		0.45	10.40	11.05	11.05
	16	14A		0.90	10.40	11.17	11.09
	102	3G		0.40	10.40	11.18	11.04
	78	1EF		0.60	10.40	11.23	11.15
	145	22H		0.50	10.40	11.29	11.27
	79	2EF		0.45	10.40	11.31	11.17
	42	7C		0.75	10.40	11.42	11.51
	133	10H		0.60	10.40	11.53	11.42
	140	17H		0.45	10.40	11.77	11.75
	47	5CD		0.55	10.40	11.89	11.90
arginine	31	12B	NT1	0.40	12.00	12.91	12.85
	139	16H	NT1	0.95	12.00	13.20	13.10
	45	3CD	NT1	0.75	12.00	>13.5	>13.5
	118	19G	NT2	0.70	12.00	>13.5	>13.5

[a] Ionic strength of 0.01 M, 25 °C.

results are in keeping with the previously observed limited effects of ionic strength on the potentiometric titration curve of denatured myoglobin (Breslow & Gurd, 1962) and confirm the ascription made above of the primary electrostatic stabilization to the native structure. The major pH-dependent contribution to the stability of the denatured form at low pH, therefore, is not the electrostatic configuration per se but the protonation of the newly exposed histidine residues. This conclusion agrees with the picture put forward by Hermans & Acampora (1967), who combined the evidence from the earlier results (Breslow & Gurd, 1962) with observations of apparent heats of reaction.

Overall Electrostatic Stabilization of Native Myoglobin. Table II lists for each charged site in sperm whale myoglobin the position in the sequence, the helix reference, the value of SA for the atom to which the charge is ascribed, pK_{int}, and $pK_{1/2}$, showing the value of pK_i at the pH of half-titration (Botelho et al., 1978). The conditions refer to an ionic strength of 0.01 M at 25 °C. Within each chemical class of residue, the ranking is according to ascending order of $pK_{1/2}$. These data were employed in the computation of pK values in sperm whale myoglobin (Botelho et al., 1978). The last column shows the corresponding values of $pK_{1/2}$ if the virtual charges of α-helix dipoles are introduced as listed in Table I.

The effect of including the α-helix dipole contributions was computed in the same manner as chloride ions were included in the computations of pK_i values for hemoglobin (Matthew et al., 1979a), making allowance for the fact that the charged sites introduced in this way are invariant with pH. The negative charge at the carboxyl terminus of the A helix falls within 2 Å of the positive charge at the amino terminus of the B helix, so that they were deemed to cancel each other (Table I). Table II shows that the inclusion of the virtual charges very rarely affects $pK_{1/2}$ by more than 0.20 unit. The principal exceptions are found with residues Glu-4, Asp-126, and Tyr-151. The value for $pK_{1/2}$ of Glu-4 is shifted down over 0.4 unit to 1.89 by the proximity of the virtual positive charge at the beginning of the A helix (Table I). Asp-126 likewise is shifted down by 0.31 unit by proximity to the virtual charge at the beginning of the H helix. The value for Tyr-151 at the end of the H helix is shifted upward by 0.58 unit. The observed value of 10.6 for this tyrosine (Wilbur & Allerhand, 1977) is in good agreement with the new value, so that in this one case the virtual charge of the α dipole may be said to make an important contribution. However, the virtual charges were introduced with an unrealistically low value of SA of 0.05 in each case to enhance the effect as much as possible. For the purposes of this and the following paper in this issue, we deem the effect to be sufficiently small to be negligible and have omitted the virtual charges of the α helices from all other computations.

Summation of all the computed electrostatic interactions felt by individual residues in sperm whale myoglobin according to W_{ij}'' values yields the overall electrostatic free energy contribution to the stability of the native structure, $\sum \Delta G_{el}''$, according to the equation

$$\sum \Delta G_{el}'' = \left(\sum_{i=1}^{n} \sum_{j=1}^{n} W_{ij}''\right)/2 \qquad (5)$$

The divisor is required to allow for double counting of interactions between i and j in the summation process over the n charged sites.

Figure 3 shows the results of the calculation made for the three conditions of ionic strength, 0.00, 0.01, and 0.10 M. Here $\sum \Delta G_{el}''$, expressed in kilocalories per mole, is plotted as the ordinate against pH as the abscissa. Over the range of pH involved, the free energy change resulting from the full constellation of charge point interactions is negative, indicating substantial stabilization ascribable to this source. As would be expected for a net stabilizing effect, the result of increasing the ionic strength is to reduce the stabilization. Experimentally, this is what is generally found (Rothgeb & Gurd, 1978). Increasing the ionic strength from 0.00 to 0.10 M decreases the stabilization rather generally by approximately 20% (Figure 3). The result at an ionic strenth of 0.15 M would place the curve slightly below that for 0.10 M. The relative effect of ionic strength on W_{ij} is distance dependent, and the overall ionic strength effect observed here fits roughly with the effect to be found when the charged groups are on the average 6 Å apart and average values of SA are taken into account (Matthew et al., 1979a). As discussed in detail in the following paper in this issue, the stronger interactions in myoglobin occur at less than 6 Å separation whereas the more numerous weak interactions involve greater distances.

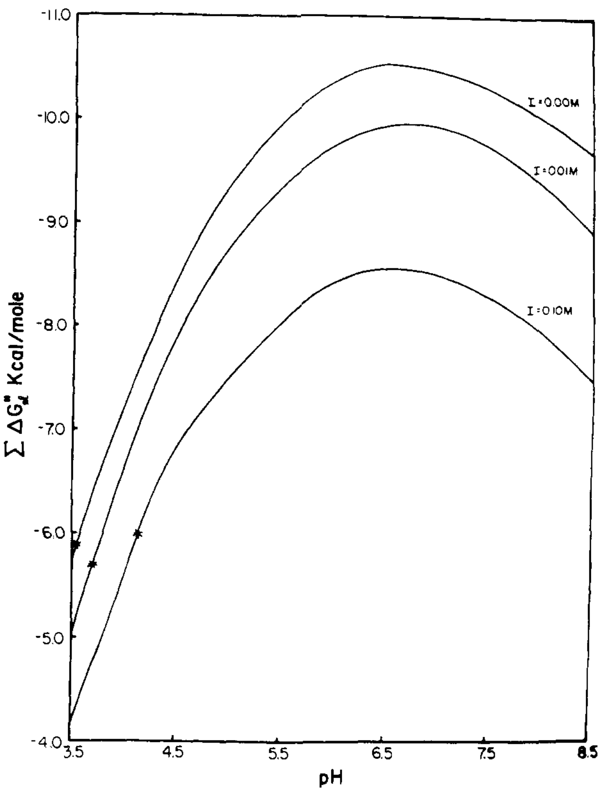

FIGURE 3: Dependence of summed electrostatic free energy terms, in kilocalories per mole, between individual charged sites as a function of pH at ionic strength values of 0.00, 0.01, and 0.10 M. The asterisked point on each curve at low pH represents the midpoint pH for the unfolding transition, extrapolated or interpolated from Figure 1, at the particular ionic strength.

The maximum stabilization at each ionic strength in Figure 3 occurs near pH 6.5, well below the isoionic point near pH 8.30. Substantially the same pH profile was obtained by Acampora & Hermans (1967) when they plotted the midpoints of thermal denaturation transitions against pH. This observation provides very strong support for the general correctness of the electrostatic treatment applied here. This result is significantly different from what is predicted for an even distribution of a similar magnitude of net charge according to the smeared-charge model (Linderstrøm-Lang, 1924).

The maximum stabilization falls in a range, pH 5.5–7.5, where histidine residues are much the most prominently titrating class. The breadth of the maximum reflects in part the fact that the majority of the titratable histidine residues has a wide range of $pK_{1/2}$ values complicated by a considerable range of SA values. The electrostatic fields sensed by histidine residues of higher $pK_{1/2}$ (e.g., His-36, Table II) are relatively negative at pH 6.5 whereas those of lower $pK_{1/2}$ (including residue 64 of very low $pK_{1/2}$) sense predominantly positive environments. Protonation of the first set with their higher $pK_{1/2}$ values generally promotes preferential stabilization in the relatively negative environments and produces the rise in overall stabilization seen in Figure 3. Conversely, protonation of histidine residues in the relatively positive environments occurs at the lower pH values, and it is upon the protonation of these histidines below pH 6.5 that the summed electrostatic free energy values first become less stabilizing. The resultant of these effects produces the maximum stabilization pattern observed in Figure 3. The wide stability range centered around pH 6.5 presumably has adaptive advantage for a molecule that functions in the environment of a muscle cell.

Since the electrostatic interactions depend not only on the state of charge of a given group under consideration but also on those of all other groups in the molecule, the stabilization or destabilization sensed by each group varies over the entire pH range and must be defined separately for each pH value. The following paper in this issue (Friend & Gurd, 1979) illustrates the importance of considering changes in interaction patterns among charged sites that do not themselves titrate as well as among those sites that do undergo changes in proton occupancy within the pH range in question. Further variation in the effects of charged sites comes about from the wide range of static accessibility values. The patterns of charge site distribution, proton occupancy, and static accessibility help mold the total sum of electrostatic interactions in such a way that the individual W_{ij}'' contributions can vary over at least 2 orders of magnitude even when pH, temperature, ionic strength, internal and external dielectric constants, and distance between all the groups are specified.

The large net electrostatic stabilization illustrated in Figure 3 could not result from random placement of charge sites in the myoglobin. Elegant geometrical modeling of various charge placements (Tanford, 1961; Hill, 1956) has previously illustrated the sensitivity of electrostatic stabilization on charge placement. The staggered charge array required to achieve the net stabilization is examined in detail in the following paper in this issue (Friend & Gurd, 1979) where its main features are shown to be highly conserved in other species of myoglobins.

Charge sites, therefore, play a dual role in maintaining the native structure [see Cohn (1943)]. In part they act to stabilize, anchor, or constrain the highly mobile, fluid protein structure (Gurd & Rothgeb, 1979; H. Frauenfelder, G. A. Petsko, and D. Tsernoglou, unpublished experiments) and in part they provide the needed polarity at the solvent interface. This latter role is played at the expense of diminishing the electrostatic interactions with other charged sites on the molecule. The subtle balancing of the two roles is accomplished through wide variations in static accessibility of charged sites within certain geometrical arrays.

Stabilization of the Native and Acid-Denatured Forms. The points on each curve in Figure 3 corresponding to the pH of the midpoint for the acid-denaturation transition (Figure 1) were estimated by interpolation and extrapolation of the denaturation results and are entered as asterisks in Figure 3. They indicate that in each case the midpoint of the acid transition occurs under conditions in which the net electrostatic stabilization of the native structure amounts to 5.7–6.0 kcal/mol. At these pH values under the respective conditions of ionic strength, half of the myoglobin molecules are in the unfolded state in which they are presumably stabilized by the substantially complete protonation of the four buried histidine residues, 24, 82, 93, and 97. Assuming an effective pK of 6.60 (Reynolds et al., 1973; Botelho et al., 1978) for the four newly exposed histidine residues, at an ionic strength of 0.10 M where the midpoint is close to pH 4.2, the protonation of these histidine residues would collectively stabilize the denatured form by slightly over 11 kcal/mol (Tanford, 1970; Hermans & Acampora, 1967; Puett, 1973).[3]

Conclusion

In summary, the electrostatic contribution to the stabilization of the native structure, at an ionic strength of 0.10 M, is found by computation to be approximately 8.6 kcal/mol at the pH of maximum stability. The maximum stabilization is computed to occur near pH 6.5, in agreement with experimental observations (Acampora & Hermans, 1967). At higher pH values stabilization terms involving positively charged histidine residues are lost. At lower pH values positive charges on other histidine residues contribute sufficient net destabilization through interactions with other positively charged groups to reduce the stabilization to approximately 5.5–6 kcal/mol at the pH of 4.2 where conversion to the acid-denatured form is half-completed at an ionic strength of 0.10 M.

The denatured form gains its pH-dependent stabilization largely through the exposure and concurrent protonation of four histidine residues, and the constellation of charge site interactions in this case is less important than that for the native conformation. The approximate value of the free energy contribution from protonation of the newly exposed histidine residues is 11 kcal/mol. Hermans & Acampora (1967) utilized the results of an earlier smeared-charge treatment implying the release of six buried histidine residues (Breslow & Gurd, 1962) to calculate appropriately a higher free energy contribution of 14 kcal/mol.

At the transition midpoint pH, therefore, where the net free energy change between the native and denatured forms is zero by definition, the contribution of ionic processes to the stabilization of the denatured form is substantially greater than that to the stabilization of the native form. This result requires the conclusion that a substantial net contribution to the stability of the native protein structure at this pH relative to the denatured structure, roughly of the order of 5 or 6 kcal/mol, derives from nonionic sources. The sources of the net nonionic stabilization of the native structure are presumably changes in internalization of hydrophobic groups, heme iron coordination and other heme interactions with the protein moiety, and hydrogen bonds or other dipolar interactions (Kendrew et al., 1961; Kendrew, 1962; Hermans & Acampora, 1967; Tanford, 1970; Richards, 1974; Richmond & Richards, 1978; Hol et al., 1978; Gurd & Rothgeb, 1979). These net nonionic contributions now can be more clearly set off against the quantitative description of the pH-dependent discrete charge site interactions.

The distinction between the pH of the maximum stability at pH 6.5 and that of the isoionic point at pH 8.3 is related to the properties of the charge array determined by both geometrical location and solvent accessibility. Matthew et al. (1979b) have shown that this array responds to variation in ionic strength in a more classical manner than does the array in human hemoglobin. The pH of maximum stability of the oxygen complex in terms of autoxidation is close to the isoionic point (Yamazaki et al., 1964), however, and presumably depends on other factors including the pH dependence of motions and other time-dependent phenomena in the protein (Gurd & Rothgeb, 1979).

Acknowledgments

The advice and encouragement of Professor G. I. H. Ha-

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[3] The precise value for the electrostatic free energy change will depend on the given pH of 4.2 and the appropriate pK_i for the unmasked histidine residues which will be governed in part by the relatively small but not necessarily negligible electrostatic interaction terms in the unfolded molecular forms.

nania, Dr. J. B. Matthew, and Dr. T. M. Rothgeb are gratefully acknowledged.

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