ELECTROSTATIC STABILIZATION IN SPERM WHALE AND HARBOR SEAL MYOGLOBINS

Identification of Groups Primarily Responsible for Changes in Anchoring of The A Helix

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ABSTRACT The compact, largely helical structure of sperm whale and harbor seal myoglobins undergoes an abrupt one-step transition between pH 4.5 and 3.5 as monitored by changes in either the heme Soret band absorbance or circular dichroism probes of secondary structure, for which a modified Tanford-Kirkwood theory provides identification of certain dominant electrostatic interactions responsible for the loss of stability. A similar treatment permits identification of the electrostatic interactions primarily responsible for a process in which the anchoring of the A helix to other parts of the molecule is weakened. This process is detected with both myoglobins, in a pH range ~1 unit higher than the onset of the overall unfolding process, through changes in the circular dichroic spectra near 295 nm which correspond to the L_a O–O band of the only two tryptophan residues in these proteins, residues 7 and 14. In each case protonation of certain sites in neighboring parts of the molecule can be identified as producing destabilizing interactions with components of the A helix, particularly with lysine 16.

INTRODUCTION

The Tanford-Kirkwood theory (1, 2) as modified by Shire et al. (3–5) deals with interactions of point charges in a spherical model of a protein. From the crystallographic structural data the point charges are specified and the distances of all separations, r_{ij}, are tabulated. The algorithm of Tanford and Kirkwood (1, 2) then defines the interaction energy between pairs of point charges, W_{ij}, in terms of placement with respect to the low dielectric internal medium, the characteristics of the interface between the internal region and external solvent of high dielectric constant, and the ionic strength in the external solvent.

Shire et al. (3–5) introduced a modification incorporating static solvent accessibility, SA, that takes into account the relative exposure of each charge site to the external high dielectric solvent, using the method of Lee and Richards (6, 7). The fraction of the surface of the charged site that faces the interior of the protein is then given approximately by the solvent-exposed area in a model peptide less the exposed area in the protein divided by the exposed area in the peptide form, equivalent to (1-SA). The (1-SA) value for each charged

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site in the crystallographic protein structure can be used to compute (3, 8–11) a modulated value for the interaction energy

$$W'_{ij} = W_{ij}\,(1 - \mathrm{SA}). \tag{1}$$

Matthew et al. (8) showed that a direct adjustment of the effective dielectric constant applying to each pair of charged sites in place of the weighting factor, (1-SA), gave comparable results.

The effective pK at a given pH for the *i*th site is determined by first assigning to all groups the charge occupancy prescribed by their given intrinsic pK value, $\mathrm{pK_{int}}$. Values of $\mathrm{pK_{int}}$ were chosen from the results for small peptides and other model compounds (3, 8–10), with consideration given to solvent accessibility and hydrogen bonding as previously described (9–11). Following this initial assignment, an iterative procedure is used for adjusting the various charge loci to the occupancy of all others according to their respective W_{ij} values until equilibrium is reached (1, 3, 8–10). The pK for the *i*th site can be expressed as

$$\mathrm{pK}_i = (\mathrm{pK_{int}})_i - (1/2 \cdot 303kT) \sum_{ji} W'_{ij}\, z_i Z_j, \tag{2}$$

where W'_{ij} is the free energy of interaction for a pair of fully occupied point charges i and j on the modeled sphere, corrected for the accessibility at the jth site, with z_i maintained as a unit charge, and Z_j denoting the fractional occupancy of site j.

Calculation of the electrostatic free energy of stabilization or destabilization between two titratable sites in the presence of equilibrium occupancy of all other sites requires consideration of the static accessibilities, SA_i and SA_j, and charge fractions at both sites, Z_i and Z_j, so that

$$\Delta G''_d = (W_{ij})(1\text{-}\mathrm{SA}_j)(1\text{-}\mathrm{SA}_i)(Z_i)(Z_j). \tag{3}$$

$\Delta G''_{el}$ can obviously be summed for all jth sites to a given ith site, $\Delta G_{i,el}''$, or summed over the whole molecule, $\Sigma\Delta G_{i,el}''$ (11, 12).

The modified Tanford-Kirkwood treatment has been applied successfully to the prediction of pK_i values and summed electrostatic interactions in myoglobins (3, 4, 9–14), cytochrome *c* (9), hemoglobin (8, 9, 15), and ribonuclease.[1] Analysis of the summed electrostatic stabilization of sperm whale myoglobin as a function of pH predicted the stability maximum near pH 6.5 observed experimentally (16), well removed from the isoelectric point near pH 8.3 (11).

Electrostatic free energy calculations for sperm whale ferrimyoglobin identified certain dominant interactions responsible for reducing the net electrostatic stabilization in acid solution (11, 17, 18), in agreement with the observed ionic strength dependent, two-state transition, according both to the heme Soret absorbance at 409 nm and to the circular dichroism (CD) of the α-helix conformation at 222 nm (11). Against this background it is possible to analyze the electrostatic basis for the process examined in this report, in which the anchoring of the A helix to other parts of the molecule is weakened, beginning at pH values above the range of the overall transition. This process is monitored by (CD) spectra at 295 nm which reflect the state of Trp-7 and Trp-14 (A5 and A10). The process is analyzed both for sperm whale myoglobin (13) and harbor seal myoglobin (19–21). In each case protonation of

[1]Matthew, J. B., F. M. Richards, and F. R. N. Gurd. Manuscript in preparation.

certain sites in neighboring parts of the molecule can be identified as producing destabilizing interactions with components of the A helix, particularly with Lys-16.

MATERIALS AND METHODS

The main fraction myoglobins from sperm whale and harbor seal muscle tissue were prepared as described previously (11). Materials, pH measurements, optical spectra and denaturation procedures have been described (11).

RESULTS AND DISCUSSION

Effects of pH on Conformational Probes

Fig. 1 for sperm whale ferrimyoglobin shows changes in the CD spectrum in the near ultraviolet range below pH 5.00. The L_a O–O tryptophan band at 295 nm, reflecting a relatively rigid nonpolar environment for the tryptophans (22), decreases in intensity as the pH is lowered. Since the band remains centered close to 295 nm, a change in hydrogen bonding is improbable (22). The tryptophan residues are over 14 Å from the heme centroid and over 10 Å from other aromatic groups, so that coupling with other groups is unlikely to be responsible for the observed changes. Possible causes of the observed changes would most likely be direct protonation of a nearby group or a more general, pH-dependent alteration in the tryptophan microenvironments (23). The relation of the percent change in tryptophan CD intensity with pH is plotted in Fig. 2 *A* (as open symbols) in conjunction with the percent overall conformational change (closed symbols) recorded at 409 nm by absorbance or

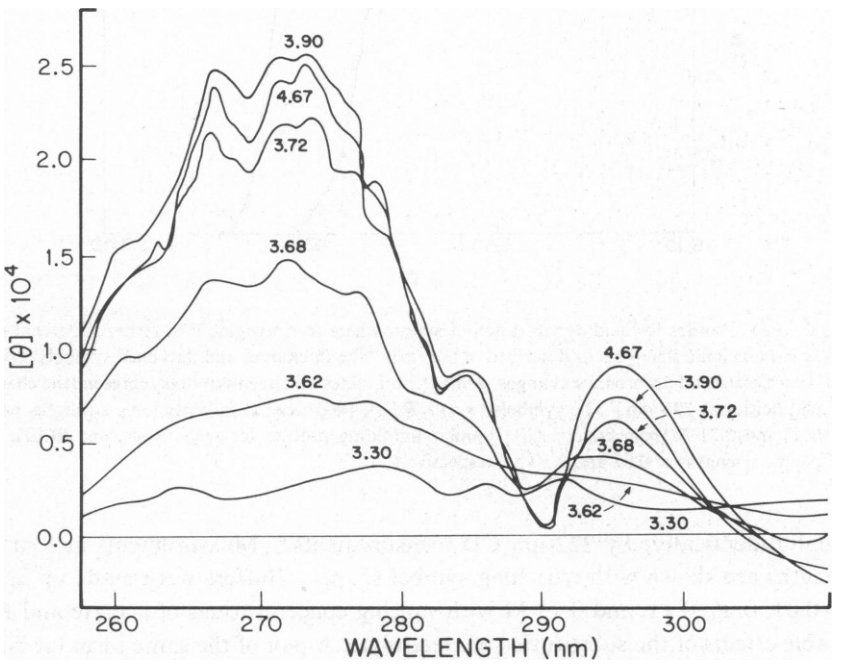

Figure 1 Near ultraviolet CD spectra of sperm whale ferrimyoglobin, 10 μM, at various pH values. The spectra were recorded in acetate buffers at an ionic strength of 0.01 M. Each curve is an average of 12 spectra.

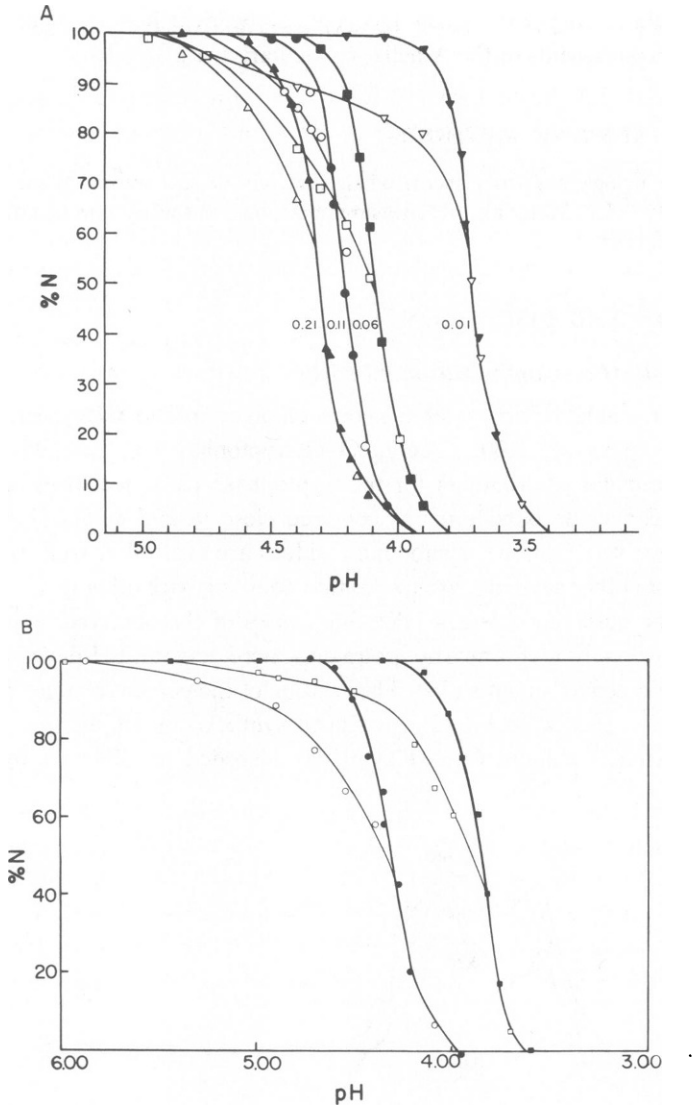

Figure 2 (*A*) Profiles for acid denaturation of sperm whale ferrimyoglobin in terms of percent native values at various ionic strengths as described in the text. The thick lines and darkened symbols represent the 409-nm Soret band absorbance changes, while the thin lines and open symbols represent the change in molar ellipticities at 295 nm. The symbols (▼,▽), (■,□), (●,○), (▲,△) indicate ionic strengths of 0.01, 0.06, 0.11, and 0.21 M respectively. (*B*) Similar unfolding profiles for seal myoglobin; (■,□), (●,○) indicate ionic strengths of 0.01 and 0.10 M, respectively.

(substantially identically) by 222-nm CD measurements.[2] Measurements at four different ionic strengths are shown with matching symbol shapes. Buffers were made up at the ionic strengths 0.01, 0.06, 0.11, and 0.21 M with varying concentrations of acetate and KCl with no detectable effects of the substitution of electrolyte. A plot of the same form for harbor seal

[2]The heme band centered around 275 nm (Fig. 1) undergoes a slight increase in intensity at low ionic strength, but not above 0.01 M ionic strength. This effect may be related to a tyrosine-heme interaction, and hence the course of changes at 275 nm has not been included in Fig. 2.

ferrimyoglobin at 0.01 M ionic strength (square symbols) and 0.10 M ionic strength (circles) is shown in Fig. 2 *B*.

In Figs. 2 *A* and 2 *B* the changes in CD of the tryptophan residues begin at appreciably higher pH than those in the overall conformation which, however, overtake them so that at lower pH the two measures coincide. Note that the tryptophan changes for the seal protein initiate at higher pH values than those for the sperm whale protein. In seal myoglobin, the ionic strength dependence of the tryptophan changes follows that of the overall unfolding process (24). In sperm whale myoglobin, where the tryptophan changes occur at a slightly lower pH value on going from $I = 0.01$ M to $I = 0.11$ M, and then at a slightly higher pH value on going from $I = 0.11$ M to $I = 0.21$ M, there is little similarity to the overall unfolding changes. Electrostatic calculations can be used to sort out the basis for such pH and ionic strength dependent tryptophan changes.

Electrostatic Interaction Between A Helix and GH Corner

The effective pK values and computed electrostatic free energy contributions, $\Delta G_{i,el}''$, in calories per mole for all individual sites in sperm whale myoglobin as functions of ionic strength and pH have been reported (11, Table II; 12, Fig. 3). None of the histidine or acidic residues which protonate in the pH region between pH 6.0 and 3.5 are within 12 Å of either of the tryptophans in these two proteins. The tryptophan changes therefore suggest a conformational change resulting from destabilizing electrostatic interactions. There are only three groups, His-64, His-113, and His-119, whose site occupancy produces significant destabilization between pH 6.0 and 3.5 (12). Protonation of His-64 has been shown to contribute substantially to the overall unfolding process occurring at lower pH values than the independent tryptophan changes (12). The $pK_{1/2}$ value (at half-titration) of His-113 (14G) is 5.35 at 0.01 M ionic strength and 25°, and it senses a destabilization, $\delta\Delta G_{i,el}''$, of almost 650 cal/mol in going from pH 6.0 to pH 4.0. This destabilization comes about almost entirely from its close proximity to Arg-31 (12B), 3.44 Å away. For the destabilization involving these two sites to be sensed by the tryptophan residues in the A helix, major conformational changes would have to be propagated from the B and G helices which would no doubt be sensed at the heme.

In contrast, His-119 (1GH) occurs at the GH corner where the A helix is anchored (12). This anchor consists mainly of the interaction between Lys-16 (14A) and Asp-122 (4GH), with the uncharged form of His-119 possibly being involved in a hydrogen bonding interaction with Lys-16 (13). The $pK_{1/2}$ of His-119 is 5.42 (12), which enables it to participate in the uncharged state under conditions of the crystallographic analysis (13). Conversely, on protonation of His-119, a strong destabilizing interaction with Lys-16 will occur.

Fig. 3 plots the interactive free energy, $\Delta G''_{el}$ between Lys-16 and all other charged sites labeled according to the helices represented. Values for pH 7.0 down to 4.0 are shown for $I = 0.01$ M in calories per mole. White bars represent stabilizations, especially Asp-122, and solid bars, destabilizations, especially His-119, Arg-118, and His-12. Since the tryptophan changes are initiated independently of changes in secondary structure (Fig. 2), the role of the His-119 interaction with Lys-16 is probably more significant at this stage than the His-12 interaction, which could in principle destabilize the A helix overall.[3] The changes reported by Trp-7 and

[3]The nearest histidine residue in seal myoglobin corresponding to His-12 ($pK_{1/2} = 5.70$; 1-SA = 0.15) is His-8 ($pK_{1/2} = 5.84$; 1-SA = 0.10), more distant from Lys-12 (10). Since the change in question initiates at a distinctly higher pH in the seal protein (Fig. 2), it is unlikely that the interaction of Lys-16 with either of these residues is important for the process affecting the tryptophan rigidity, in contrast with its probable importance in the general unfolding.

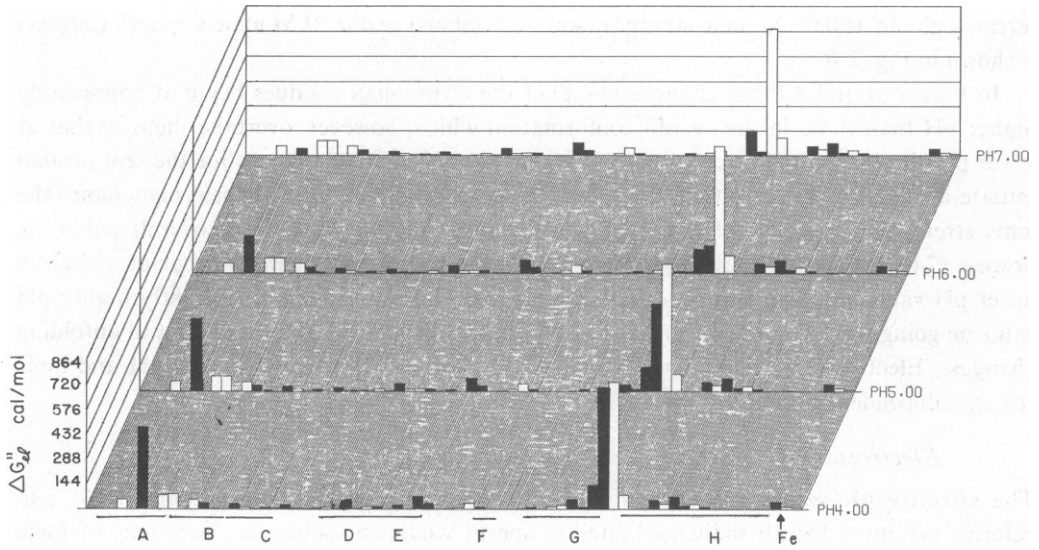

Figure 3 Computed free energy of electrostatic interaction between Lys-16 and each of the other charge sites, $\Delta G''_{el}$, in calories per mole for sperm whale myoglobin. The diverse charge sites are indicated by their helix references; the iron site is marked on the right hand end and is followed by the propionic acid sites. Negative (stabilizing) free energies are denoted by open bars, and positive (destabilizing) free energies are denoted by solid bars. The results are shown for an ionic strength of 0.01 M and 25°C.

Trp-14 could most simply result from destabilizing interactions between Lys-16, His-119, and Arg-118, with the stabilizing influence of Asp-122 unchanged (12). The role of Lys-16 is particularly important, since the anchoring of the A helix depends largely on three charge pairs, Glu-4 with Lys-79, Glu-6 with Lys-133, and Lys-16 with Asp-122, apart from nonpolar interactions (12).

The primary focus on residues Lys-16, His-119, Glu-122, and Lys-118 applies also to the corresponding process in harbor seal myoglobin (14). Here two of these residues are substituted, Glu-122 for Asp-122, and Lys-118 for Arg-118 (21). Out of 24 other substitutions, 8 involve the gain or loss of a charge (21). Comparisons of the computed net charge and individual histidine pK values with experimental values (10, 14) indicate a comparably satisfactory basis for the computation of charge site interactions from crystallographic data (19, 20). Table I shows comparisons of the computed interactive free energies in calories per mole and r_{ij} values in angstroms between Lys-16 and the charge sites at positions 118, 119, and 122 for the two proteins at $I = 0.01$ M and 0.10 M at a series of pH values. The (1-SA) values applying to the sperm whale and seal myoglobins, respectively, are 0.90 and 0.70 for Lys-16, 0.70 and 0.25 for position 118, 0.80 and 0.20 for His-119, and 0.45 and 0.35 for position 122. Similarly the $pK_{1/2}$ values are, respectively, 5.42 and 5.24 for His-119 at $I =$ 0.01 M and 2.29 and 3.24 for residue 122; the other residues do not undergo $pK_{1/2}$ changes that will affect the results in the pH range 4.0–6.0. Note the effect of the substitution at 122.

The differences between $\Delta G''_{el}$ values for the two proteins are shown in summed form in Fig. 4 for the two ionic strengths. Note that the destabilization shown by the net positive values takes effect at higher pH values for the seal protein, and also that the different ionic strength trends found for the two proteins (Figs. 2 *A* and *B*) are reflected in the computed curves in Fig. 4. The ionic strength effects are especially interesting since they reflect differences in site occupancy (Eq. 2) controlling Z_i and Z_j values (Eq. 3). Note also the substantial differences in r_{ij} values in Table I, reflecting in part the residue substitutions

TABLE I

INTERACTIVE ELECTROSTATIC FREE ENERGIES IN CALORIES PER MOLE BETWEEN POSITION 16 and POSITIONS 118, 119, and 122 IN SPERM WHALE AND SEAL MYOGLOBIN AS A FUNCTION OF pH AT I = 0.01 M and I = 0.10 M at 25°

Ionic Strength	Sperm whale myoglobin				Seal myoglobin			
	pH	$\Delta G''_{el}$ 16–118	$\Delta G''_{el}$ 16–119	$\Delta G''_{el}$ 16–122	pH	$\Delta G''_{el}$ 16–118	$\Delta G''_{el}$ 16–119	$\Delta G''_{el}$ 16–122
r_{ij} (*ångströms*)		10.25	5.21	3.41		10.42	2.30	6.74
I = 0.01 M	4.00	138	720	−734	4.00	66	310	−130
	4.50	138	662	−734	4.50	66	220	−160
	5.00	138	542	−734	5.00	66	120	−170
	5.50	138	360	−734	5.50	66	51	−180
	6.00	138	173	−734	6.00	66	19	−200
I = 0.10 M	4.00	63	662	−619	4.00	39	320	−77
	4.50	63	567	−631	4.50	39	250	−110
	5.00	63	475	−637	5.00	39	160	−120
	5.50	63	331	−648	5.50	39	71	−130
	6.00	63	173	−648	6.00	39	26	−130

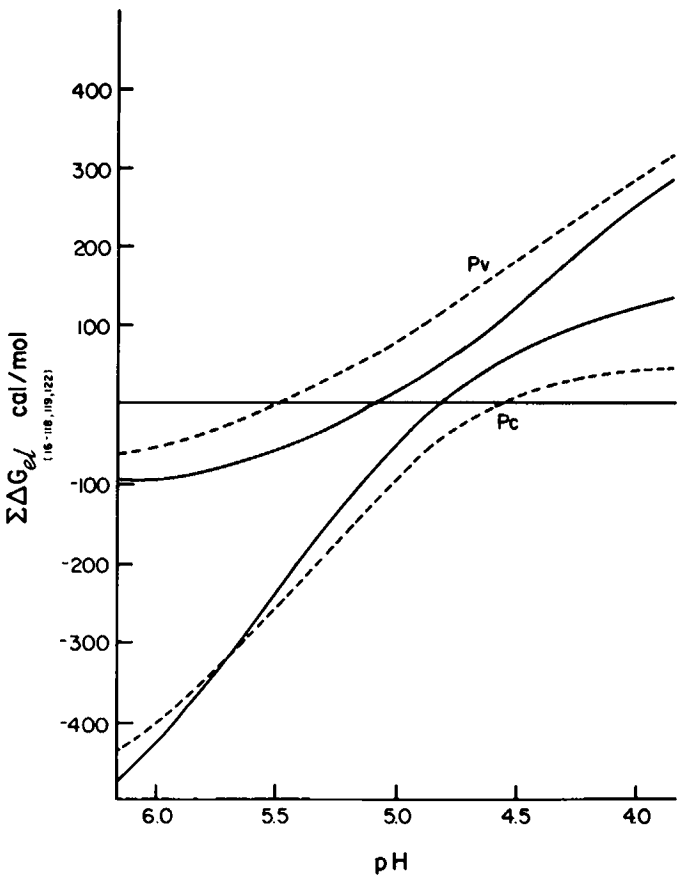

Figure 4 Summed electrostatic free energy between Lys-16 and Arg-118, His-119, and Asp-122, $\Sigma\Delta G''_{el\,(16\text{–}118,\,119,\,122)}$, in sperm whale myoglobin (Pc) and seal myoglobin (Pv) as a function of pH between 6.0 and 4.0. Computed values at 25°C are shown for I = 0.01 M (——) and I = 0.10 M (----).

described above. Lastly, as mentioned above, the computed (1-SA) values applying to the residues in the two proteins differ substantially. A slightly more open structure for the seal protein may be reflected in a generally more rapid rate of carboxymethylation (25).

Comment

The analysis applied to the group of four residues most involved in anchoring the A helix to the GH corner (Table I, Fig. 4) fits the observed differences between the sperm whale and seal myoglobins (Fig. 2). The inversion for the tryptophan CD points between 0.06 and 0.11 M ionic strength for the sperm whale protein (Fig. 2 *A*) reflects an interplay among electrostatic interactions affecting both the pK and the ionic strength shielding, which is also illustrated in Fig. 4. The quantitative accuracy of the predictions from the theory will be more searchingly tested when crystallographic analyses in the pertinent ranges of pH and ionic strength have been made. One would expect for the seal protein, for example, that some of the positive free energy associated with protonation of His-119 would be relieved by a movement of the average position of the flexible Lys-16 side chain. Further relief would require a movement of the A helix as a whole to increase the distance between Lys-16 and His-119, sufficient to loosen the tryptophan side chains. This movement is reminiscent of the modeling of helix movements by Ptitsyn (26) and Richmond and Richards (27).

The indication that electrostatic effects may be specific and localized is important in itself, especially in view of the involvement of a major helix that can move as a unit to transmit effects over appreciable distances (28–31). The present case is an example of a selective effect depending on all electrostatic interactions within the molecule but largely attributable to interaction of a small number of primary charged sites. Other examples in ferrimyoglobin, involving different sets of primary charged sites, include the gross acid unfolding process and azide binding to the heme iron (12). The electrostatic domain for the localized process discussed here is appreciably more restricted within the myoglobin molecule than that involved in the stabilization of azide binding (32).

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DISCUSSION

Session Chairman: V. Adrian Parsegian *Scribe*: Preston Hensley

SCHUSTER: Do you think that there is a single crucial group involved in the destabilization of the protein, or do you think that there are just a lot of carboxyl groups titrating at once?

GURD: The overall destabilization, the two state kind of change seems to be related to a lot of groups. The change

process parallels the distal histidine titration very well, as was studied by you and Sandy Asher. The pH dependence of the protonation of that distal histidine parallels exactly the overall change at different ionic strengths, but we compute that it contributes perhaps a tenth of the total effect.

KAHN: We have an observation in support of your use of the tryptophan CD working with hemoglobin. Doing exactly the same kind of titration, we see a distinct loss of Soret CD preceding the loss of ellipticity in the far U.V. So you can monitor separate regions that way. We can't see the tryptophan because it's swamped by the heme transition which you don't have.

SIMON: The only other hemoglobin or myoglobin-like protein that I know of that has a different spatial relationship between the A-helix and the G-H region is human fetal hemoglobin in which the A-helix is pulled unusually tightly towards the G-H region. I'm wondering if the substitutions in that protein can be treated similarly. Do your calculations confirm the resultant reorientation of the A-helix for the human fetal protein?

GURD: We have done some modeling of the human fetal hemoglobin, but not enough to give you an answer. It would be very desirable to do that analysis in detail.

BLOOMFIELD: When you think about calculating the magnitudes of these electrostatic effects, you do have to choose a dielectric constant for the water near the surface of a protein, or inside a protein. This dielectric constant may be different from the bulk dielectric constant of water because of saturation and immobilization effects.

GURD: There are motional effects when one compares the relaxation behavior of protonated and unprotonated groups, possibly indicating appreciable changes in hydration states with changes in formal change.

PARSEGIAN: I would like to point out that if you look at the electric field near any ion you find fields already strong compared to breakdown fields, or what would be a breakdown field if applied at that same strength between the plates of a capacitor. Somehow this huge field gets built into the energetics.

One normally looks at self energies of solvation via methods like the Born theory where you use the fiction of a macroscopic dielectric constant right up to the ionic surface. But, then, as they found 40 or 50 years ago, in order to explain the solvation data you have to add to the ion radius, ~0.65 Å to make solvation energies of all the alkali cations fit with this fictional continuum dielectric. I think that's the sort of self-consistent picture one would use in this protein situation. One would use continuum ideas to get first estimates about the energetics and idealize geometry accordingly.

Another point struck me when I read this paper. Why do you neglect the self energy of the ion? That always seems to be the leading term, especially when you're talking about removing solvent from a body. Self energies typically look like interactions between two charges across a distance the size of the particle, which is always going to be smaller than the distance between the centers of any two particles. Self energy is typically the leading term in any energetics involving dielectric constants or anything else. Why neglect that leading term?

GURD: Of course, we don't know how to handle the self energy. We have to get the difference between what it is in the model compound, determining the pK there, and what it is in the protein. Most of the information we have when we're making a severe test of this treatment has to do with protons going onto imidizole groups, and such uncharged sites. Those groups are notoriously less sensitive to this kind of effect. For example, if you do titrations of the cation going on to an uncharged group, that type of titration done in a variety of solvents of different dielectric constants shows relatively little difference compared to the combination of charged species. The intrinsic pK that we are using is a function of the nature of the amino acid so we have a consistent set of intrinsic pKs for a whole lot of different proteins that we've studied. We look after the histidines with one kind of intrinsic pK, and the carboxyls of the different kinds (Asp and Glu and terminal) with others, and the lysines and the terminal amino with other pK intrinsic values, and so on. It depends on the nature of the amino acid. In the case of histidines, there is an inherent pK difference between the two ring nitrogens. We let the static accessibility measurement tell us which one to use. One has a pK = 6.6 in our usage and the other is 6.0. Furthermore, the nature of the flanking residues obviously should be affecting what happens to the pK of an individual residue. Experimentally, we know very well from the Molday and Englander studies that exchange of N-H protons depends on the flanking residues. Finally, the effect of the dielectric constant is not put into the intrinsic pK at present. Probably what we should do is find some way of allowing the degree of exposure to solvent to modulate our intrinsic pK. This factor is probably inherent in the model.

PARSEGIAN: After reading your paper, I calculated what two charges would see in each other if both were sitting at an interface, namely your SA = 0.5 case. I made one side of the interface water, with a macroscopic dielectric constant of 80, and the other side the dielectric constant you used in your paper. The calculated energy of interaction was 2 or 3 times what your approximate expression gives. This was just pure but rigorous electrostatics. Why do you think your calculation should be so different? If your calculation is so different from an exact solution, what are you testing when you get your data to fit this approximate model?

GURD: It could have something to do with the time dependence of what's happening, the effect of the dielectric constant as it really is, as you've brought out. We're not sure how to allow for transients. The treatment we have maintained in the same form since Steve Shire initiated it 7 or 8 years ago gives very nice fits to titration curves (see Matthew et al., 1978, *Biochem. Biophys. Res. Commun.* **81**:416–420). If we take away the electrostatic effect as influencing the pK values we obtain a much steeper curve which does not fit the data. If we take the Tanford treatment without the (1-SA terms), i.e., ignoring the effect of the exposure to the solvent, the curve fits only for a very small range of pH. So we've stuck to our guns here in the sense that satisfactory overall treatment is producing satisfactory predictions. If we find fault with one aspect of the treatment, we shall want to evaluate some compensating shortcoming. I guess that's an accurate way to put it.

KALLENBACH: Does your treatment depend on the total amount of other charges that are around? Also the pH range that you showed in Matthew et al. is ~4–6. Can you extend out to other pH's?

GURD: We can, in principle, compute the free energy of charging under equilibrium conditions over our whole pH range. We find, for example, that we can predict the maximum stability of the protein, which occurs a long way from the isoionic point, because the effect of various discrete changes are by no means equal to each other. The charge distribution makes large differences. Yes, we can follow the treatment through for various particular applications. For example, a different set of groups, a fairly large set, would be the major ones involved in the interaction that see a change when the charge on the iron is neutralized. If we put azide on the ferric ions as a function of ionic strength and pH, we can measure the equilibria and also predict by computation the stability of the azide complex as a function of pH and ionic strength (Friend et al., 1980, *Biochemistry*, **19**:3039).

TELLER: I have some data on the dimerization of a chymotrypsin at low pH, where it's highly charged. Measurement of the free energy of association as a function of pH and of the hydrogen ions released (T. A. Horbett and T. C. Teller, 1972, *Biochemistry* **13**:5490–5495) can be explained without electrostatic theory, only specific pK's. So I am somewhat puzzled. One can use individual pK's. Why worry about the fact that they are attached to proteins?

GURD: In the paper with the azide equilibria we treated the interaction as though there were two groups that were specifically and uniquely being affected. This amounts to the old classical approach of looking at one or two heme-linked groups. That treatment does not work as well as the complete interactive redistribution model. It will be a complex interaction, and very interesting to study. It is a difficult job to deviate from the sphere model in our treatment.

JENTOFT: I was wondering if you could tell me if your treatment would be useful for looking at a small pocket in a protein, to calculate what a pK_a of an active site group would be. What size area do you need to consider for your calculations to work?

GURD: We've been very happy with the interaction of, say, DPG with deoxyhemoglobin where one need not look at all the groups. The primary effects are taken into account by a dozen or so groups. This binding analysis is very ionic strength-dependent, of course.

ACKERS: One comment about the data that Dave Teller mentioned. It seems to me that the finding of correspondence between the number of protons released and apparent number released as a result of measurements of the pH dependence of the dimer-tetramer equilibria merely means that there are no additional effects of pH upon the protein association constant other than those which are affecting the proton equilibria. If that's true, correspondence will always be observed experimentally. You're left with the question of whether the description of both processes in terms of pK's is really unique. It seems to me that there's no information in those data which allows you to make a decision.