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Protein–Protein Interactions: Nature of the Electrostatic Stabilization of Deoxyhemoglobin Tetramer Formation†

Stephen H. Friend, James B. Matthew,‡ and Frank R. N. Gurd*

ABSTRACT: The summed electrostatic free energy contributions to deoxyhemoglobin A_0 tetramer formation were computed at a series of pH and ionic strength values as the difference between the computed values for the tetramer and for the sum of the four individual chains. The electrostatic stabilization of each monomer is similar and close to that for myoglobin. At ionic strength 0.10 M the electrostatic contribution to the stability of the tetramer is approximately 35 kcal/mol at pH 6.0 and 18 kcal/mol at pH 9.6. The specific contribution to the stabilization of the tetramer, $(\sum \Delta G''_{i,el})_{tet}$, is obtained by difference and shows a broad plateau above 7 kcal/mol over the range from pH 6.0 to 8.0, which is nearly obliterated by pH 9.6. By examination of the contributions of individual sites under the above summation, it is found that sites in the α chains are responsible for virtually the entire stabilizing effects in tetramer formation. The major differences on tetramer formation are sensed at eight sites. The stabilization provided by four of these sites results simply from changes in solvent exposure of sites in the given monomers as the tetramer is assembled. They are offset in part by changes at three sites that sense the greatest destabilization and that are responsible for the near cancellation of effects among the β-chain sites. The general implications for the stabilization of molecular assemblies are considered.

Interactions between proteins are essential for most, if not all, biological processes. Protein complexes form the basis for structures needed for cellular integrity, motility, differentiation, and recognition (Friedman & Beychok, 1979; Frazier & Glaser, 1979). Allosteric mechanisms involved in homeostasis depend on quaternary interactions (Matthews & Bernhard, 1973). All these protein–protein relationships fundamentally depend on a common set of stabilizing and destabilizing forces which if understood for a particular case may be applicable to a range of examples.

The overall free energy of association, ΔG_{assoc}, may be taken as the sum of contributions as in eq 1. Here the terms within

$$\Delta G_{assoc} = \sum_{i}^{n} (\Delta G''_{i,el} + \Delta G_{i,h} + \Delta G_{i,conf} + \Delta G_{i,vw} + \Delta G_{i,hb}) + \Delta G_{buried} + \Delta G_{S\text{-}S} \quad (1)$$

the summation over all residues refer, respectively, to electrostatic, hydrophobic, conformational, van der Waals, and hydrogen bonding contributions. $\Delta G_{S\text{-}S}$, the contribution from

† From the Department of Chemistry, Indiana University, Bloomington, Indiana 47405. *Received May 5, 1980.* This is the 123rd paper in a series dealing with coordination complexes and catalytic properties of proteins and related substances. For the preceding paper see Matthew et al. (1981). This work was supported by U.S. Public Heath Service Research Grants HL-05556 and T01-GM-1046.

‡ Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520.

0006-2960/81/0420-0580$01.00/0

disulfide linkages (Levitt, 1976), is rarely an important term for protein–protein association (Friedman & Beychok, 1979). The contribution from the burial or exposure of titrating sites on association, ΔG_{buried}, is most easily assessed by the method of Tanford (1970).

The contribution from variation in the number of hydrogen bonds can be considered according to Rashin & Yudman (1979) or Nelder & Mead (1965). For the cases dealt with by Rashin & Yudman (1979), approximately 5–8 kcal/mol or 20% of the overall stabilization of association was assigned to preferential formation of hydrogen bonds. The contributions from van der Waals forces, $\sum_i^n \Delta G_{i,\text{vw}}$ (Levitt, 1976), were found by Rashin & Yudman (1979) to be about twice as important for stabilizing assoication as those from hydrogen bond formation.

The most important destabilizing contribution $\sum_i^n \Delta G_{i,\text{conf}}$, represents the effect of freezing out translational and rotational motions. Following the method of Page & Jencks (1971), Chothia & Janin (1975) estimate this contribution to be in the range of 20 to 30 kcal/mol in the formation of the insulin dimer, the trypsin–pancreatic inhibitor complex, and the hemoglobin α,β dimer. The main counterbalancing contribution is the hydrophobic free energy of association, $\sum_i^n \Delta G_{i,\text{h}}$. This most important term for protein–protein association is estimated in terms of the extent of burial of protein surface on association (Lee & Richards, 1971; Chothia, 1974; Chothia & Janin, 1975).

The electrostatic contribution to association may be estimated from the change in summed electrostatic free energy terms for all charge sites, $\sum_i^n \Delta G''_{i,\text{el}}$, according to the modified Tanford–Kirkwood theory (Matthew et al., 1981; Friend & Gurd, 1979a,b). The following application to the association of subunits to form deoxyhemoglobin A_0 tetramer explores the electrostatic contribution for this important system and points toward a general assessment of the nature of charged-site interactions in protein–protein association. Since the model used requires a spherical geometry, the computations are most appropriately applied to hemoglobin by comparing the four monomers, $2\alpha + 2\beta$, with the resulting tetramer, $\alpha_2\beta_2$. The treatment of charged-site interactions and how they influence the effective pK values of groups in both monomers and the deoxyhemoglobin tetramer (Matthew et al., 1978b, 1979a,b), was applied in the immediately preceding paper (Matthew et al., 1981) to the binding of allosteric effectors. Here we assess the changes in charge-site interactions brought about by binding between subunits.

Electrostatic Computations

The procedures have been extensively described (Shire et al., 1974; Matthew et al., 1979a,b, 1981; Friend & Gurd, 1979a,b) and need not to be detailed here. The free energy of electrostatic interaction between any two charged sites i and j depends on W_{ij}, the work required to bring the charges Z_i & Z_j to their respective positions on a model impenetrable sphere once the radius of the sphere, ion exclusion radius, internal and external dielectric constants, ionic strength, and temperature are specified (Tanford & Roxby, 1792). Following Friend & Gurd (1979a,b), the inclusion of factors allowing for the effect of exposure of each site to solvent is made as follows

$$\Delta G_{\text{el}} = W_{ij}(1 - \text{SA}_i)(1 - \text{SA}_j)Z_iZ_j = W''_{ij}Z_iZ_j \quad (2)$$

where Z_i and Z_j are fractional occupancies with respect to the respective charge sites under given conditions and SA is the static accessibility with respect to each given site (Lee & Richards, 1971; Shire et al., 1974; Matthew et al., 1978a).

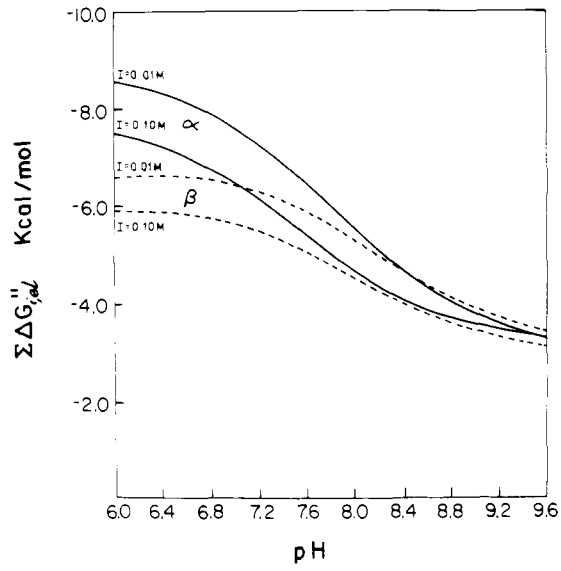

FIGURE 1: The summed electrostatic free energy, $\sum \Delta G''_{i,\text{el}}$, in kcal/mol, compted for an isolated deoxyhemoglobin A_0 α chain (—) and β chain (---) as a function of pH for both I = 0.01 M and I = 0.10 M at 25 °C.

The overall electrostatic free energy contribution is represented as

$$\sum \Delta G''_{i,\text{el}} = \tfrac{1}{2}\sum_{i=1}^{n}\sum_{j=1}^{n} W''_{ij}Z_iZ_j \quad (3)$$

The radii of the α and β subunits were assumed to be identical at 18 Å (Matthew et al., 1978a) and that of the tetramer to be 27 Å (Matthew et al., 1979a). The electrostatic free energy of tetramer formation, $(\sum \Delta G''_{i,\text{el}})_{\text{tet}}$, is obtained by subtracting the individual values of $\sum \Delta G''_{i,\text{el}}$ for the four monomer units from that for the tetramer. The coordinates of atomic positions within subunits were taken to be identical in the free monomers with those in the tetrameric structure (Fermi, 1975), i.e., no tertiary rearrangements were considered. The chloride ion binding sites incorporated in the treatment of the deoxyhemoglobin tetramer were not considered as available sites for the separate monomer chains (Matthew et al., 1979b, 1981).

Results and Discussion

Electrostatic Stabilization of Monomers. The summed electrostatic free energy, $\sum \Delta G''_{i,\text{el}}$, according to eq 3 over the pH range from 6.0 to 9.6 for the monomeric α (full curves) and β chains (broken curves) are shown in Figure 1 for the values of ionic strength 0.01 and 0.10 M. As was observed with sperm whale myoglobin (Friend & Gurd, 1979a) and the deoxyhemoglobin tetramer (Matthew et al., 1981), the computed pH of maximum electrostatic stabilization for each monomer falls well below the isoionic point, which is near pH 8.3 and 7.2 for α and β chains, respectively (Bucci et al., 1968). Both hemoglobin monomers contain histidine residues with pK_i values greater than their intrinsic pK values because of their negative electrostatic environments (Matthew et al., 1979a). These negative environments contribute to the stabilization of the histidine sites as they become protonated. Note that for the α chain the computed maximum stabilization occurs below pH 6.0 whereas for the β chain this maximum occurs between pH 6.0 and pH 6.5.[1]

[1] In most cases the crystallographic structure was taken to be valid over the pH range from approximately 6.0 to 9.6, with some computations included down to pH 5 for illustrative purposes.

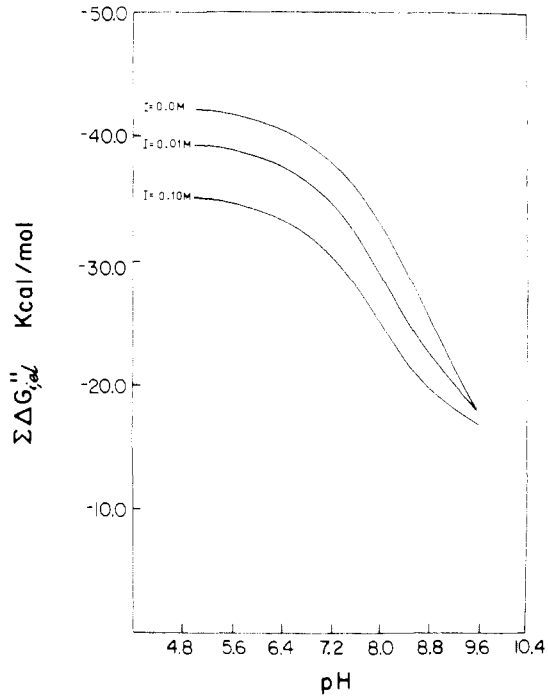

FIGURE 2: The summed electrostatic free energy, $\sum\Delta G''_{i,el}$, in kcal/mol, computed for the deoxyhemoglobin A_0 tetramer as a function of pH for I = 0.00, 0.01 M, and 0.10 M.

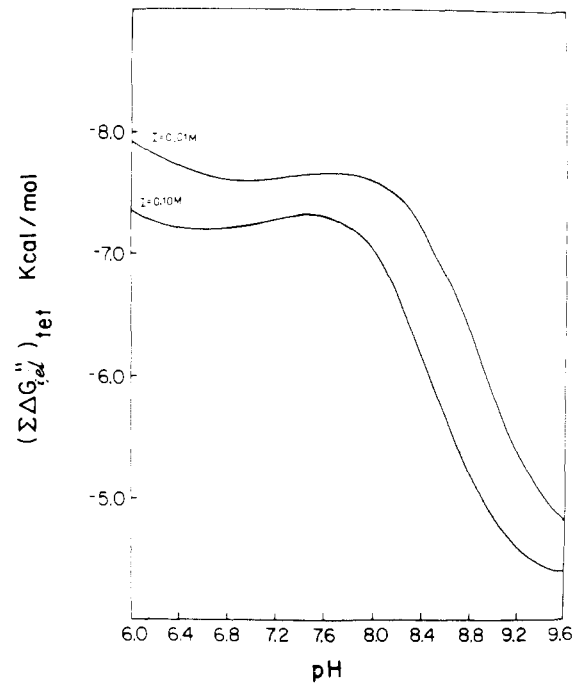

FIGURE 3: The electrostatic contribution to tetramer formation, $(\sum\Delta G''_{i,el})_{tet}$, in kcal/mol, calculated as the additional net stabilization between the overall charged site interactions in the tetramer compared to the four isolated chains as a function of pH at 25 °C for I = 0.01 M and I = 0.10 M.

The 41 charge sites of the α-chain monomer provide 8.6 kcal/mol of stabilization at pH 6.0, I = 0.01 M. The 45 charge sites of the β-chain monomer under the same conditions provide 6.8 kcal/mol of stabilization. Such values, particularly for the α chain, are similar to that for the 59 charged sites on sperm whale myoglobin, which under the same conditions provide 9.5 kcal/mol of stabilization (Friend & Gurd, 1979a). The similarity among the three proteins suggests the conservation of electrostatic stabilization characteristics.

Electrostatic Stabilization of Tetramer. The summed electrostatic stabilization in kilocalories/mole in the deoxyhemoglobin tetramer is shown in Figure 2 for I = 0, 0.01, and 0.10 M. Close to 39 kcal/mol of stabilization are computed for I = 0.01 M at pH 6.0, dropping to less than 20 kcal/mol at pH 9.6. As in the previous paper (Matthew et al., 1981), chloride ions are assumed to be bound at the Val-lα and His-117β sites at I = 0.1 M. Calculations including alternative Cl^- ion binding at β cleft positions 1 and 2 as described in the previous paper did not alter the $\sum\Delta''G_{i,el}$ values throughout the pH range being considered. The computations are shown down to pH 5.0 to bring out the strong stabilizing effect of the proximity of acidic groups to the histidine residues. This is in contrast with sperm whale myoglobin for which the computed and observed stability maximum falls closer to pH 6.5 (Friend & Gurd, 1979a; Acampora & Hermans, 1967). The contrast with the charge configuration of myoglobin also produces a distinct difference in the ionic strength dependence of titration properties (Matthew et al., 1979b).

Electrostatic Stabilization of Tetramer Formation. Throughout the pH range shown, the charge site interactions in the tetramer (Figure 2) are more stabilizing than the sum of the four monomer values (Figure 1). The difference is denoted by $(\sum\Delta G''_{i,el})_{tet}$, shown in Figure 3 in kilocalories/mole per tetramer as a function of pH at I = 0.01 M and 0.10 M. Figure 3 shows that between pH 6.0 and 9.6 the electrostatic interactions favor tetramer formation. The maximum stabilization at I = 0.10 M is centered directly over the physiological pH range from pH 7.2 to 7.8. This characteristic will tend to maximize in vivo tetramer levels.

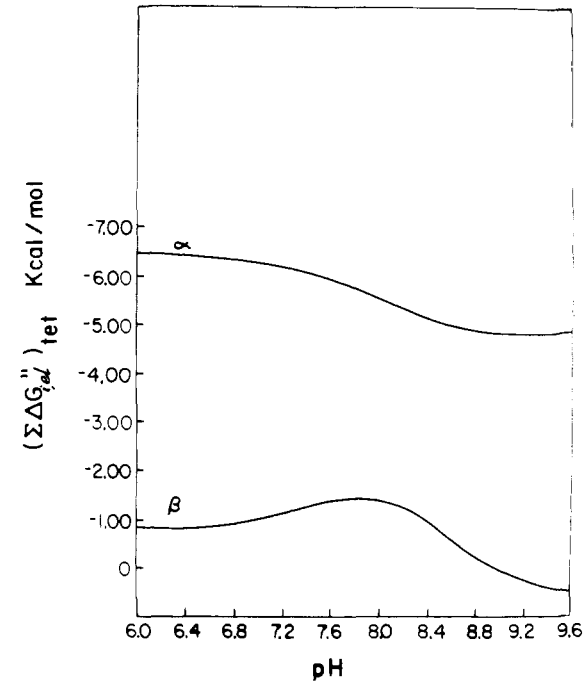

FIGURE 4: The electrostatic contribution to tetramer formation, $(\sum\Delta G''_{i,el})_{tet}$, in kcal/mol, plotted separately for the contributions from the two α and two β chains as a function of pH at 25 °C, I = 0.01 M.

Contributions of Individual Chains to Electrostatic Stabilization of Tetramer Formation. For a better display of the characteristics of the electrostatic changes on tetramer formation, the overall $(\sum\Delta G''_{i,el})_{tet}$ is broken down in Figure 4 into the contributions of the individual chains. The sum for the two α chains is significantly greater than that for the two β chains. The pH range is again pH 6.0 to 9.6 and I = 0.01 M. Important differences are apparent between the electro-

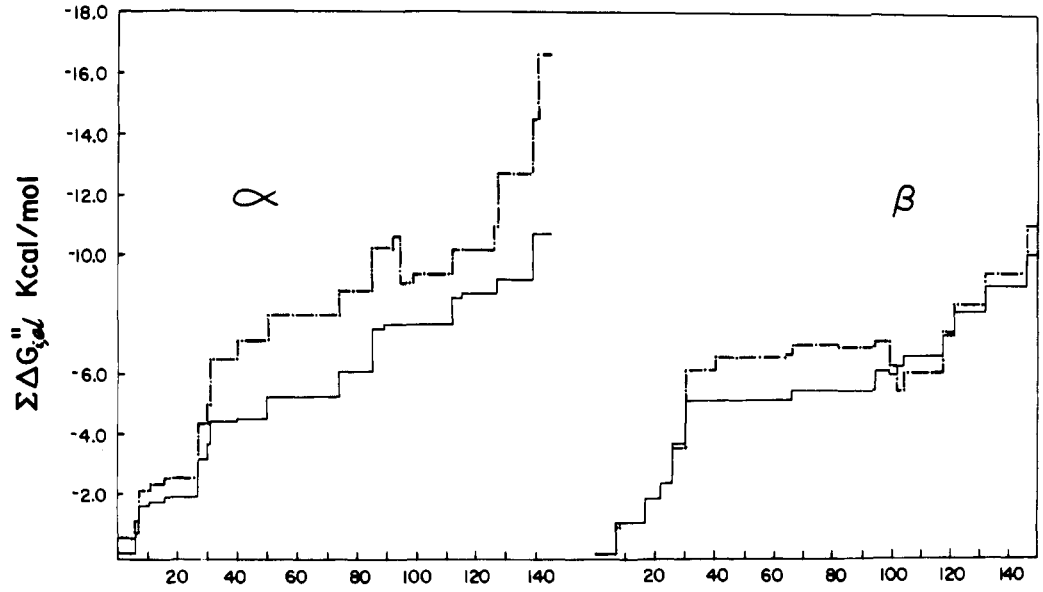

FIGURE 5: Summation plots of the electrostatic free energy contributions from individual sites, $\Delta G''_{i,el}$, in kcal/mol, in deoxyhemoglobin A_0 tetramer at 25 °C, $I = 0.01$ M, and pH 7.60. The solid lines sum $\Delta G''_{i,el}$ for two isolated α chain monomers and two β chain monomers. The broken lines sum $\Delta G''_{i,el}$ for two α chains and two β chains in the tetramer.

static contributions from the α and β chains. At pH 6.0 the α chains contribute almost 90% of the total electrostatic stabilization of tetramer formation. Above pH 9.0, charge-site interactions sensed by the β chains are actually more destabilizing upon tetramer formation, and at these high pH values the stabilization of tetramer formation results from the contribution of the α chains alone.

Because there is little contact between like subunits in the tetramer, it is interesting that the α and β chains are not reciprocally stabilized by each other, and that the pH dependence of $(\sum \Delta G''_{i,el})_{tet}$ is not similar for the α and β chains. In order to explain these phenomena it is necessary to break down the summed electrostatic free energy of formation into the contributions from individual groups.

Individual Group Contributions to Electrostatic Stabilization of Tetramer Formation. Figure 5 presents cumulatively the electrostatic contributions from individiaul sites to tetramer stability. $\sum_{i=1}^{n} \Delta G''_{i,el}$ is plotted as n increases integrally from 1 up to the residue positions given for both α chains and both β chains at pH 7.6 and $I = 0.10$ M. The solid lines represent the elctrostatic stabilization of the two α chain monomoers on the left and the two β chain monomers on the right. The dotted lines similarly show the contributions of the same residue sites in the deoxyhemoglobin tetramer.

Both the α-chain and β-chain monomers display a wide range of electrostatic interactions sensed by their individual charged sites. For each type of subunit certain sites sense a few particularly strong summed stabilizing interactions. Although no charged sites experience strong destabilizing interactions resulting from strong influences of like charged sites, many of the charged sites are located in environments which are not strongly stabilizing and some experience slightly destabilizing environments. The minimal stabilizations occur either because these sites are isolated from other charged sites by distance or solvent accessibility or because they are influenced rather equally by the positive and negative fields of other charges or because they are themselves not fully charged at pH 7.6. Both α and β monomers show similar overall behavior, with the pair of α chains showing about 1 kcal/mol more of stabilization than the pair of β chains at pH 7.6 (cf. Figure 1 for the singly counted chains). The difference in the electrostatic stabilization of tetramer formation between the α and β chains does not arise, therefore, from an inherent difference in electrostatic interactions within the two types of monomers considered in isolation.

Figure 5 shows that the increase in $(\sum \Delta G''_{i,el})_{tet}$, already attributed primarily to the α subunits in Figure 4, results from changes in electrostatic stabilization sensed by a small number of charge sites. Most of the electrostatic interactions are little altered on tetramer formation. Only eight sites in the α and β chains together undergo significant stabilizing alterations in electrostatic interactions. In addition to the increased electrostatic stabilization sensed at those eight sites, there are three groups, Asp-94α, Asp-99β, and Glu-101β, that actually experience significant electrostatic destabilization upon tetramer formation.

Table I lists the eight groups which provide more than 400 cal/mol of electrostatic stabilization to deoxytetramer formation at pH 7.6, $I = 0.10$ M, $T = 25$ °C. The charge and (1 − SA) values for each group are listed for both the monomer and tetramer along with the respective $(\Delta G''_{i,el})_{tet}$ value. Table I also lists all charge sites within 10 Å of each of these groups according to the respective subunits on which they occur, the distance r_{ij} to these groups, and both burial and charge for the monomer and tetramer. The last column lists the individual electrostatic contributions (eq 2) to stabilization of tetramer formation, $(\Delta G''_{el})_{tet}$.

The stabilizing interactions sensed by each of these eight groups are not simply the result of contacting a neighboring opposite charge on another subunit. The stabilizations result from a diversity of interactions with various charged and uncharged groups on as many as all four subunits. The general concepts apparent from this detailed breakdown of some of the charged site interactions in tetramer formation are as follows:

(1) By comparison in Table I of the sum of all electrostatic stabilization provided by interactions within 10 Å of each of the eight groups with the overall stabilization listed below each group, it may be seen that as much as 25% of the overall effect is contributed by charged-site interactions spanning greater than 10 Å. This evidence for extensive domains of electrostatic interactions agrees well with what was found for azide binding to sperm whale myoglobin where several important heme-linked groups were found as much as 15 Å or more away from

Table I: Electrostatic Interactions Sensed within 10 Å of Charged Sites Providing Greater Than 400 cal/mol of Stabilization to Tetramer Formation at pH 7.6, $I = 0.10$ M, $T = 25$ °C

group	$1 - SA_M$[b]	$1 - SA_T$[b]	Z_M[c]	Z_T[c]	group	$1 - SA_M$[b]	$1 - SA_T$[b]	Z_M[c]	Z_T[c]	r_{ij}[d]	$(\Delta G''_{el})_{tet}$[a]
Arg-31α_1 [$(\Delta G''_{i,el})_{tet} = -840$][a]	0.46	0.93	1.00	1.00	Glu-27α_1	0.90	0.90	−1.00	−1.00	3.2	−750
					Glu-30α_1	0.73	0.73	−1.00	−1.00	8.9	−200
					His-112α_1	0.95	0.95			6.1	+186
Lys-40α_1 [$(\Delta G''_{i,el})_{tet} = -480$]	0.21	0.41	1.00	1.00	Cys-93β_2	0.90	0.90			7.9	
					Asp-94β_2	0.12	0.95	−1.00	−1.00	9.1	−110
					His-146(Im)β_2	0.73	0.73	0.72	0.88	6.8	+130
					His-146(OH)β_2	0.24	0.78	−1.00	−1.00	3.7	−410
Asp-126α_1 [$(\Delta G''_{i,el})_{tet} = -930$	0.21	0.95	−1.00	−1.00	Lys-127α_1	0.29	0.60	1.00	1.00	8.8	−160
					Cl-2α_1		0.50		1.00	6.7	+230
					Arg-141(N)α_2	0.05	0.66	1.00	1.00	2.7	−1240
					Tyr-35β_1	0.32	0.74			3.7	
Lys-127α_1 [$(\Delta G''_{i,el})_{tet} = -1290$]	0.29	0.60	1.00	1.00	Val-1(N)α_1	0.28	0.56	0.75	0.72	6.3	+110
					Asp-6α_1	0.98	0.98	−1.00	−1.00	3.1	−560
					Cl-2α_1		0.50		−1.00	5.7	−200
					Asp-126α_1	0.21	0.95	−1.00	−1.00	8.7	−110
					Arg-141(N)α_1	0.05	0.61	1.00	1.00	7.8	+160
					Arg-141(OH)α_1	0.31	0.59	−1.00	−1.00	3.9	−430
Arg-141(N)α_1 [$(\Delta G''_{i,el})_{tet} = -2200$]	0.05	0.66	1.00	1.00	Arg-141(OH)α_1	0.31	0.59	−1.00	−1.00	8.4	−140
					Val-1(N)α_2	0.28	0.56	0.75	0.72	8.8	−180
					Cl-2α_1		0.50		−1.00	4.4	−340
					Asp-6α_2	0.98	0.98	−1.00	−1.00	9.4	−180
					Asp-126α_2	0.21	0.95	−1.00	−1.00	2.7	−1240
					Lys-127α_2	0.29	0.60	1.00	1.00	7.8	+160
					Tyr-35β_2	0.32	0.74			5.5	
Arg-30β_1 [$(\Delta G''_{i,el})_{tet} = -1130$]	0.73	0.93	1.00	1.00	Glu-26β_1	0.86	0.86	−1.00	−1.00	2.6	−590
					His-117β_1	0.81	0.81	0.55	0.80	8.7	+110
					Cl-117β_1		0.50	−1.00	−1.00	4.6	−440
His-117β_1 [$(\Delta G''_{i,el})_{tet} = -750$]	0.81	0.81	0.55	0.80	Glu-22β_1	0.62	0.62	−1.00	−1.00	2.7	−290
					Glu-26β_1	0.86	0.86	−1.00	−1.00	7.8	−90
					Arg-30β_1	0.73	0.93	1.00	1.00	8.7	+110
					His-116β_1	0.05	0.95	0.07		8.0	
					Cl-117β_1		0.50	−1.00	−1.00	4.2	−370
His-146(N)β_1 [$(\Delta G''_{i,el})_{tet} = -600$	0.73	0.73	0.72	0.88	Lys-40α_2	0.21	0.41	1.00	1.00	7.2	+120
					Glu-90β_1	0.16	0.16	−1.00	−1.00	7.8	−10
					Cys-93β_1	0.90	0.90			4.5	
					Asp-94β_1	0.88	0.88	−1.00	−1.00	2.9	−240
					Lys-144β_1	0.46	0.46	1.00	1.00	9.3	+20
					Tyr-146β_1	0.95	0.95			9.9	
					His-146(OH)β_1	0.24	0.78	−1.00	−1.00	4.5	−400

[a] Values expressed in cal/mol. [b] Burial values in the monomer and tetramer, respectively. [c] Charge at each site under the conditions stated in the text for the monomer and tetramer. [d] Distance in angstroms between the interacting charged sites.

the azide binding site at the heme (Friend et al., 1980). Such long-range interactions are also in accord with studies on the electrostatic domains of myoglobin denaturation (Friend, 1979; Gurd et al., 1980). These extensive domains of electrostatic interactions, furthermore, may provide a rationale by which to explain the importance of the numerous widespread mutations which affect tetramer formation (Bunn et al., 1977).

(2) Each of the eight groups has interactions with nearby similarly charged groups that are destabilizing even though all eight groups provide significant overall net stabilizations. For this reason a complete consideration of the charge-site contributions to tetramer formation in terms of all their individual site interactions is desirable even for the groups providing the most stabilization of tetramer formation. This approach parallels that required to explain the electrostatic stabilization of myoglobin (Friend & Gurd, 1979b).

(3) Three of the eight groups, Arg-31α_1, Arg-30β_1, and His-117β_1, provide preferential stabilization to the tetramer not because they interact strongly with any oppositely charged sites on other subunits but because nonpolar intersubunit contacts increase the burial of charged sites on the given subunit. The accompanying increase in the factor $(1 - SA)$ provides that the stabilizing interactions already present in the given monomer can be sensed more intensely. For these three sets of charged-site interactions, the stabilization of tetramer formation does not result from new intersubunit charged-site interactions but from new intersubunit interactions involving noncharged residues. Noncharged contact regions are therefore able to create significant long-range effects through their modulation of intrasubunit charged-site interactions. Such a mechanism can be postulated to be important for a variety of protein–protein interactions such as antibody function or protein–membrane interactions. One of the major functions of noncharged groups may be such a regulation of charged-site interactions.

(4) Four factors determine the magnitude of the electrostatic contributions to tetramer formation. First, the change in radius of the monomer (18 Å) to the tetramer (27 Å) only slightly increases all interactions and so represents a small factor in tetramer formation. Second, the site occupancies of all but the histidine residues and the NH_2-terminal residue remain constant during tetramer association at pH 7.6, and so the association process can be seen to depend to a limited extent on changes in site occupancy. As shown in Table I, the two other factors, distance and site burial, most significantly alter the electrostatic stabilization of tetramer formation. This point again stresses the importance of both charged and noncharged sites as modulators of the electrostatic contribution to tetramer formation.

A similar analysis of the componenets of the three major destabilizations involving residues Asp-94α, Asp-99β, and Glu-101β reveals the same general trends and so need not be

discussed in detail. In these cases the net destabilization is brought about primarily by interactions with like charged groups on the other chains.

Conclusions

The isolated α and β monomers are both stabilized by charged-site interactions to a similar degree as the myoglobins in which such stabilizations have been shown to be conserved among numerous mammalian species (Friend & Gurd, 1979a,b). These results, taken along with experimental observations on electrostatic contributions to the stabilization of cytochrome *c* (Schejter et al., 1979), suggest that the heme proteins may be commonly stabilized by charged-site interactions.

In deoxyhemoglobin the net stabilization does not arise equally from the four chains but instead arises almost entirely from the interactions sensed by the two α chains on association into the tetramer. Much of this effect comes from increased interaction between oppositely charged sites within each α chain, reflecting the reduction in accessibility to the aqueous solvent caused by apposition of neighboring subunits. The effect is maximal in the physiologic pH range because of the substantial interactions between many of the titratable histidine residues and nearby acidic residues which poise the occupancies of histidine sites so that they can contribute to $(\sum \Delta G''_{i,el})_{tet}$ in the pH range between 7.2 and 7.8. The same characteristics provide for heightened buffer capacity in this pH range and contribute to the mechanism of the Bohr effect (Matthew et al., 1979b; Ho & Russu, 1979; Russu et al., 1980). The change in site occupancy is illustrated for pH 7.6 for His-117β and His-146β and applies significantly to other histidine residues as the pH is lowered through the normal range. Table I is rich in examples of the effect of changes in solvent accessibility of sites that contribute to $(\Delta G''_{el})_{tet}$ terms.

In many of the examples in Table I $(1 - SA)_M$ is a relatively small number, meaning that the site in question in the monomer can contribute relatively little toward stabilizing that form and its major stabilizing role is reserved for the tetramer form.

The entries in Table I for Arg-30β_1 and His-117β_1 both involve the inclusion of a Cl^- site located between these groups. Following the preceding treatment (Matthew et al., 1979a,b), the site is assumed to be fuly occupied by the Cl^-. It can be seen that in such a case the contribution of $(\Delta G''_{el})_{tet}$ toward the overall stabilization is substantial. Essentially identical results were obtained when alternative sites were taken to be occupied by chloride ions. It is more probable that a number of sites of anion binding will be partially occupied under a given set of conditions so that overall contributions of this magnitude may be made up of a larger number of individual contributions distributed over the tetrameric structure (Matthew et al., 1981). Since the identification of simple anion binding sites in the crystalline state of proteins usually is made under conditions rather far removed from the physiological or experimental conditions applying in solution, direct evidence of such sites is difficult to obtain.

The electrostatic site interactions are computed to stabilize tetramer formation by as much as 8 kcal/mol and to vary with ionic strength and pH. As Figure 2 shows most clearly, the electrostatic interaction pattern works to stabilize the native tetramer structure well below the physiological pH range. As would be expected on general grounds (Tanford, 1970) and as found for sperm whale myoglobin (Friend & Gurd, 1979a,b; Breslow & Gurd, 1962), the disordered state obtained at low pH is probably less sensitive to variation in ionic strength and less directly stabilized by charge-site interactions. Again by analogy with the myoglobin studies, it is reasonable to ascribe the stabilization of the denatured state to protonation of newly exposed histidine residues in the hemoglobin chains (Breslow & Gurd, 1962; Acampora & Hermans, 1967; Steinhardt & Beychok, 1964; Friend & Gurd, 1979a,b).

Kirshner & Tanford (1964) have shown tetramer dissociation to dimer to be favored at increased ionic strengths. They ascribed approximately 10 kcal/mol per mole of NaCl of destabilization for dilute aqueous solutions and found the experimental value to be less positive at higher salt concentrations, as would be expected for an electrostatic effect.[2] The present treatment does not deal with the electrostatic stabilization of the deoxyhemoglobin dimer or any specific ion binding effects involving that intermediate structure. However, the computations in Figure 3 correlate well with the experimental values of Kirshner & Tanford (1964).

The steep decrease in $(\sum \Delta G''_{i,el})_{tet}$ above pH 7.6 quite closely parallels the constant increase in the log of the association constant for dimers to tetramers, observed experimentally by Atha & Riggs (1976) to be 7.0 at pH 9.6 and 10.4 at pH 7.6. Below pH 7.6, where the computed values become only slightly more stabilizing, Atha & Riggs (1976) observed a more pronounced increase in the (dimer $\rightarrow$ tetramer) association constant. This slight discrepancy below pH 7.6 could be due to a pH dependence of dimer–monomer dissociation although this equilibrium has been shown to be relatively pH independent (Guidotti et al., 1963). It also might be due to pH-dependent conformational changes or increased anion binding at low pH values. Further crystallographic data would be most helpful concerning these last two points.

The results in this report lead to several generalizations about electrostatic contributions to protein–protein interactions. First, without alteration of their original charge-site geometries, the association of the protein molecules may be favored by stabilizing electrostatic interactions. Second, the stabilizing effects may result from direct interactions involving oppositely charged sites on the individual interacting molecules, or they may result from changes in solvent accessibility of given sites. Contributions of both types are found in Table I. The role of intermolecular contacts that exclude solvent in the region of charged sites will be particularly important if a protein or peptide becomes imbedded in a membrane or other nonpolar environment. Note that the presence of an extrinsic protein at such an interface and its orientation with respect to the interface can be controlled largely by electrostatic interactions within the protein in question. Conversely, changes in the external electrostatic field, controlled, for example, by membrane polarization, will influence the charge configuration and orientation of the protein. Third, an allosteric effect mediated by electrostatic communication need not require a measurable conformational change in the protein or subunit involved, and may be expected to range over distances of 10 Å or more.

Fourth, among the roles of any component, such as the hemoglobin α chain, that combines simultaneously in several different tetramer forms such as hemoglobins A_0, A_2, or F, may be the ability to confer stability on the aggregates by virtue of its own electrostatic charge configuration. Other normal and abnormal hemoglobin forms (Bunn et al., 1977; Bank et al., 1980) must be examined to establish whether this role for α chains represents a generally meaningful adaptation in the hemoglobin system. Such a specialized stabilization role

[2] At very low ionic strength values, Kirshner & Tanford (1964) found an increased contrary tendency to dissociate that is probably due to the loss of stabilization from the chloride ions preferentially bound to the tetramer (Matthew et al., 1979a,b).

may be found in other systems where common subunits are encountered (Pierce, 1971).

Finally, by defining the electrostatic free energy change attendant on tetramer formation in which no tertiary structural changes are permitted, the treatment indicates the maximum electrostatic free energy potentially available for driving changes in the tertiary structure between the monomer and tetramer states. Use of identical structures for the subunits in the monomeric and tetrameric states is artificial to the extent that small conformational rearrangements probably do occur, yet it is just this restriction which allows calculation of electrostatic free energy available for structural rearrangements or other interconversions of free energy (see eq 1). The site by site analysis by the present approach allows assessment of possible conformational changes by indicating where changes in charge-site interaction could provide driving force or require compensation.

Acknowledgments

Helpful discussion with Dr. C. F. Poyart and Miss M. A. Flanagan are gratefully acknowledged, as is the help of Mrs. D. Embry in preparing the manuscript.

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