Electrostatic Contributions to the Energetics of Dimer–Tetramer Assembly in Human Hemoglobin: pH Dependence and Effect of Specifically Bound Chloride Ions†

Margaret A. Flanagan, Gary K. Ackers, James B. Matthew,‡ George I. H. Hanania,§ and Frank R. N. Gurd*

ABSTRACT: The pH dependence and effects of specifically bound chloride ions on the electrostatic contribution to the energetics of human hemoglobin dimer–tetramer assembly were computed for deoxy- and liganded hemoglobin. In the absence of bound chloride, the electrostatic contribution models the observed contrasting pH dependence of dimer–tetramer assembly for deoxy- and oxyhemoglobin. The effect of specifically bound chloride on the computations depends on the number and placement of the anions. Deoxy assembly shows a greater sensitivity to anion binding, with effects propagating as far as 32 Å from the binding site. This sensitivity suggests a mechanism for electronic communication with the heme. At pH 7.4, 24–34% of the experimental value for deoxy and 73–85% for oxy dimer–tetramer assembly stabilization are predicted. Together with the findings of Chu and Ackers [Chu, A. H., & Ackers, G. K. (1981) *J. Biol. Chem. 256*, 1199] and other recent work, these results suggest that salt bridge formation is not the dominant energetic factor favoring deoxyhemoglobin dimer–tetramer assembly. Results of this work suggest that the marked electrostatic stabilization favoring oxy dimer–tetramer assembly may be a significant contributor to the quaternary enhancement observed in assembly reactions whereas the nonelectrostatic factors favoring deoxy dimer–tetramer assembly may be largely responsible for quaternary constraint.

Processes which contribute to protein subunit assembly are of interest from two standpoints: (1) self-assembly reactions produce the final products in biosynthetic pathways that result in functional oligomeric complexes; (2) regulation of biological function is frequently mediated through alterations in the intersubunit interactions during the functional cycle of a macromolecular assembly. In the case of human hemoglobin, subunit dissociation has been used as a sensitive probe of the changes in intersubunit interactions which accompany the binding of ligands, including oxygen, protons, and chloride ions (cf. Ackers, 1980). The resulting thermodynamic characterization provides a framework for considering the possible sources of energetic effects which contribute the driving forces in this regulatory system. This paper is a study of the electrostatic contributions to these processes.

The Tanford–Kirkwood discrete charge electrostatic theory (Tanford & Kirkwood, 1957), as modified by Shire et al. (1974a), has been used to calculate the additional electrostatic free energy gained when deoxyhemoglobin α and β monomers are assembled into $\alpha_2\beta_2$ tetramers (Friend et al., 1981). In efforts to compare these calculated electrostatic contributions to experimental results on the total free energy of tetramer assembly, an insurmountable limitation is imposed by the fact that free energies of $\alpha_1\beta_1$ dimer formation are immeasurably large. However, the assembly of $\alpha_1\beta_1$ dimers into tetramers proceeds with a simple stoichiometry (Ackers & Thompson, 1965; Kellett 1971) and is a well-characterized reaction (Ackers & Halvorson, 1974; Mills et al., 1976; Atha & Riggs, 1976; Ackers, 1980; Chu & Ackers, 1981). The Tanford–Kirkwood discrete charge electrostatic theory has therefore been extended to the treatment of deoxy- and oxyhemoglobin $\alpha\beta$ dimers in order to estimate the contribution made to the free energy of assembly of the respective tetramers from interactions involving charged groups.

In this paper, the pH dependence of the electrostatic contribution to dimer–tetramer assembly is presented as well as the effects of specifically bound chloride ion on its magnitude. An analysis of the individual contributions of some charged groups is described. The electrostatic interactions are found to be relatively more important for stabilizing the dimer–tetramer assembly for oxyhemoglobin than for deoxyhemoglobin. These results are rationalized with the recent experimental findings and with current concepts of protein association in general.

Electrostatic Calculations

The procedure for the iterative electrostatic interaction calculation as applied to tetrameric hemoglobin has been extensively described elsewhere (Matthew et al., 1979a, 1981a,b).[1] The electrostatic free energy of interaction, W_{ij},

† From the Department of Chemistry, Indiana University, Bloomington, Indiana 47405 (M.A.F., J.B.M., G.I.H.H., and F.R.N.G.), and the Department of Biology and McCollum–Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218 (G.K.A.). *Received May 5, 1981.* This is the 127th paper in a series dealing with coordination complexes and catalytic properties of proteins and related substances. For the preceding paper, see Gurd et al. (1981). This work was supported by U.S. Public Health Service Research Grant HL-05556 (M.A.F., J.B.M., G.I.H.H., and F.R.N.G.), National Science Foundation Grant PCM-8023321 (G.K.A.), and National Institutes of Health Grant GM-24486 (G.K.A.)

‡ Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06511.

§ Present address: Department of Chemistry, American University of Beirut, Beirut, Lebanon.

[1] Recently, Ohe & Kajita (1980) have published the pH dependence of the pseudo-first-order rate constants for the exchange reaction of the C-2 protons of the imidazole groups in deoxy- and carbonmonoxyhemoglobin. Under the conditions of their experiments, subunit dissociation may well have influenced the results along with variations in buffer composition. Another cause for concern is the incubation of the protein at 36 °C for 24 h with a 2-fold molar proportion of dithionite per heme (Antonini & Brunori, 1971). Their interpretations in terms of pK values are at odds with the elegant proton NMR work of Ho & Russu (1978; Russu et al., 1980), the potentiometric titration results for various hemoglobin species (deBruin & Janssen, 1973), the extents of exposure and burial of histidine residues described, for example, in terms of solvent accessibility (Lee & Richards, 1971), and our computed pK values (Matthew et al., 1979a). It is a strong possibility that the hydrogen exchange kinetics for all but the most exposed histidine rings are complicated by the same considerations which cloud the interpretation of amide proton exchange from proteins (Woodward & Hilton, 1979).

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is a function of several model parameters (Tanford & Kirkwood, 1957). The calculated value of W_{ij} depends on ionic strength, internal and external dielectric constants, and the distance of closest approach of a counterion to the protein surface. The magnitude of W_{ij} has been shown to have a stronger dependence on ionic strength than on sphere radius (Matthew et al., 1979a).

Proton Binding Calculations. So that the irregular protein–solvent interface can be accounted for, W_{ij} is modulated by the fractional accessibility of the charged atom to the solvent:

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

where SA_j is the static accessibility of the jth group (Lee & Richards, 1971; Matthew et al., 1978a).[2] The intrinsic equilibrium constant in the absence of effects from other charged sites, $(pK_{int})_i$, is modulated by the extent of electrostatic interactions of site i with other charged sites j:

$$pK_i = (pK_{int})_i - \frac{1}{(2.30kT)} \sum_{j \neq i} W'_{ij} z_i Z_j \quad (2)$$

where z_i is the charge on site i, ± 1, and Z_j assumes fractional values between ± 1 and 0. A consistent set of pK_i values is computed at each pH and ionic strength by using an iterative procedure [see, for example, Shire et al. (1974a,b); Matthew et al., 1978b; Botelho et al., 1978].[2] The parameter $pK_{1/2}$ is defined as the pH at which a particular residue is half-titrated and is the value used to compare computed with experimental pK values. Theoretical protein titration curves are generated by summing the fractional charge occupancy of each site, Z_i, over all charged sites on the protein as a function of pH.

Electrostatic Free-Energy Calculations. The overall electrostatic free-energy calculated for a protein summed over its n charged sites is (Friend & Gurd, 1979)

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

where W''_{ij} is defined as

$$W''_{ij} = W_{ij}(1 - SA_i)(1 - SA_j) \quad (4)$$

The electrostatic free energy gained upon dimer–tetramer assembly is obtained by subtracting the individual values of $\sum \Delta G''_{i,el}$ for two dimer units from $\sum \Delta G''_{i,el}$ for the tetramer. This additional electrostatic free energy gained upon dimer–tetramer assembly is referred to in the text as ΔG_{el}. Individual contributions to this sum are designated as $\delta G_{i,el}$.

Application to Dimeric Hemoglobin. It is difficult to assign a precisely equivalent radius to the hemoglobin dimer which does not conform to spherical geometry as closely as do the proteins previously treated (Shire et al., 1974a; Matthew et al., 1979a). The dimer is roughly elliptical with half-axes of 18 and 27 Å (Fermi, 1975). The larger sphere radius of 27 Å was selected to avoid artificial compression of the dimer into an 18-Å sphere. The difference between calculated values of $\Delta G''_{i,el}$ using W_{ij} values calculated from an 18-Å and a 27-Å sphere radius is 5–10%, depending on the distance between charged groups. This is the same magnitude of difference seen if the SA values are varied by 5% or if the r_{ij} value is changed by less than 0.2 Å. Thus errors which may be introduced by using an unrealistically large sphere radius are well within the error introduced by the other parameters defining $\Delta G''_{i,el}$. It is difficult to evaluate errors that may arise from the nonspherical geometry of the hemoglobin dimer within the terms of the simple extension of the Tanford–Kirkwood treatment (Matthew et al., 1981a,b). Recent application of the electrostatic theory to other nonspherical proteins, bovine pancreatic trypsin inhibitor (March et al., 1981), and ribonuclease A (Matthew & Richards, 1981) has resulted in good agreement with experimental data.

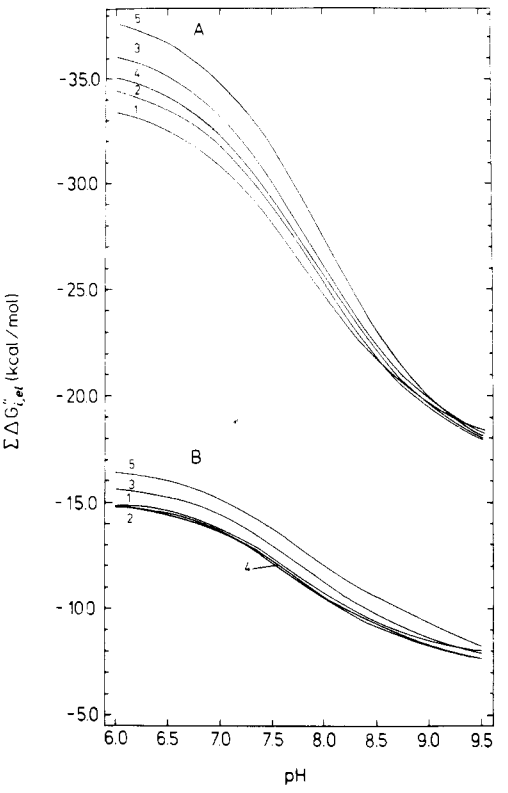


FIGURE 1: Computed effect of specific chloride ion binding to human deoxyhemoglobin dimer and tetramer on the magnitude and pH dependence of the summed electrostatic free energy of interaction between charged sites, $\sum \Delta G''_{i,el}$, in kcal/mol, at 25 °C, I = 0.1 M. For a full description of chloride ion locations, see Table II and text. (A) Deoxyhemoglobin tetramer: (1) no bound chloride; (2) chloride bound at CLR 142A; (3) chloride bound at CLR 142A and CLR 147B; (4) chloride bound at CLR 142A and CLR 148B; (5) chloride bound at CLR 142A and CLR 150B. (B) Deoxyhemoglobin dimer: (1) no bound chloride; (2) chloride bound at CLR 143A; (3) chloride bound at CLR 143A and CLR 147B; (4) chloride bound at CLR 143A and CLR 148B; (5) chloride bound at CLR 143A and CLR 150B.

The atomic coordinates for human deoxyhemoglobin were obtained from the Brookhaven Protein Data Bank (Bernstein et al., 1977). Atomic coordinates for human oxyhemoglobin were those generated by the rigid rotation of the deoxyhemoglobin α and β chains into the oxyhemoglobin quaternary structure (Matthew et al., 1979a,b, 1981a,b; Friend et al., 1981). Human carbonmonoxyhemoglobin coordinates were kindly provided by J. Baldwin previous to deposition in the Brookhaven Protein Data Bank (Baldwin & Chothia, 1979; Baldwin, 1980). The atomic coordinates for free dimers were assumed to be identical with those for the dimers in the tetrameric structure (Matthew et al., 1978b; Friend et al., 1981).

Results and Discussion

Electrostatic Stabilization of Dimers and Tetramers. The results for the individual dimers and the tetramers are first presented without consideration of bound chloride ions.

[2] In the computation of the change in pK, the SA_i value does not enter directly [see Tanford (1961); Matthew et al., 1979a]; however, SA_i and Z_i influence all sites j and hence their interaction with site i. For the discussion of an alternative formalism involving $\overline{SA}_{ij}$, see Matthew et al. (1981b).

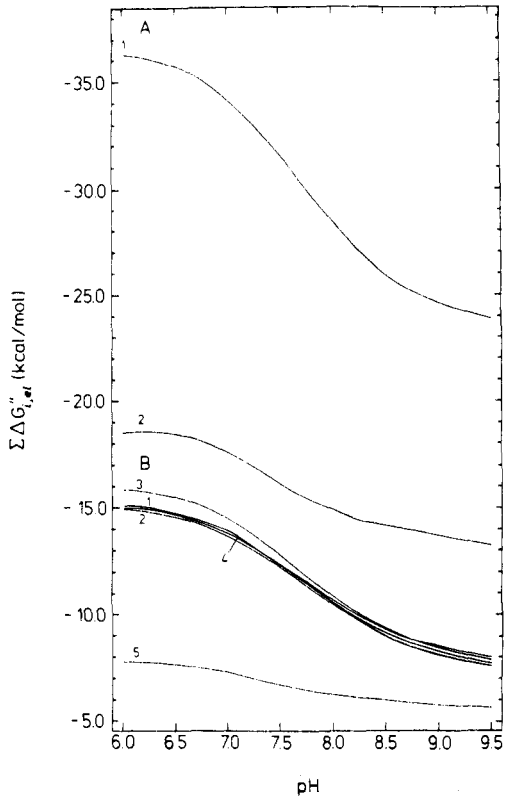


FIGURE 2: Computed effect of specific chloride binding to human oxyhemoglobin dimer and tetramer on the magnitude and pH dependence of $\sum\Delta G''_{i,el}$, in kcal/mol, at 25 °C, I = 0.1 M. For a full description of chloride ion locations, see Table II and text. (A) Oxyhemoglobin tetramer with no bound chloride. Curve 1 was computed by using the rigid rotation oxyhemoglobin structure and is taken from Matthew et al. (1981a). The computation using the carbonmonoxy structure is shown in curve 2. (B) Oxyhemoglobin dimer. Curves 1–4 were calculated by using the rigid rotation oxyhemoglobin structure. The placement of chloride ion is the same as that of curves 1–4 of Figure 1B. The carbonmonoxyhemoglobin dimer calculated with no bound chloride is shown in curve 5.

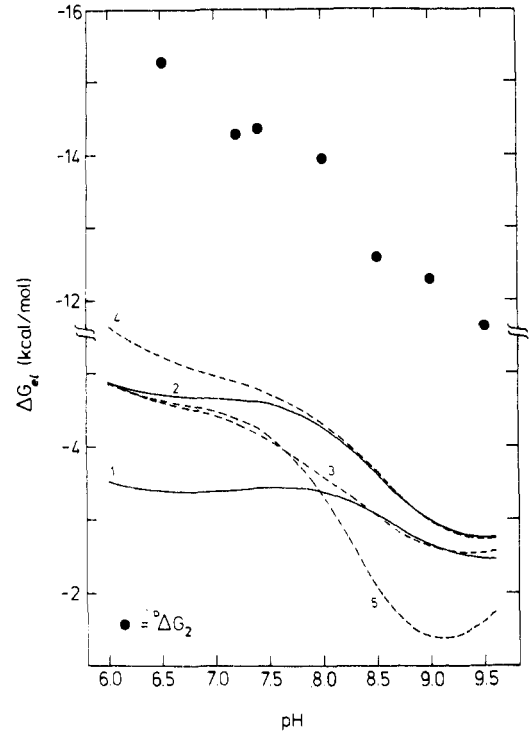


FIGURE 3: Computed pH dependence of the electrostatic contribution to the free energy of deoxyhemoglobin dimer–tetramer assembly, ΔG_{el}, in kcal/mol, at 25 °C, I = 0.1 M. Each curve, 1–5, is the difference between the corresponding curve in Figure 1A and twice the corresponding curve in Figure 1B (see also Table I). The experimental data, $^0\Delta G_2$ as a function of pH at 21.5 °C, are taken from Chu & Ackers (1981). The buffers used were 0.1 M BisTris-HCl (pH 6.5), 0.1 M Tris-HCl (pH 7.2–8.5), or 0.1 M glycine–HCl (pH 8.95 and 9.5) with 1 mM Na_2EDTA and 0.1 M NaCl.

Following the treatment of the separate dimers and tetramers, the electrostatic contributions to dimer–tetramer assembly are computed by difference. Curve 1 in Figure 1A shows the computed value of $\sum\Delta G''_{i,el}$ for the deoxy tetramer at ionic strength 0.1 M as a function of pH in the absence of bound chloride, and the corresponding results for the deoxy dimer are shown in Figure 1B. The results for the tetramer correspond to those already reported (Friend et al., 1981).[1] In both cases, the electrostatic contribution to the stability of the structure is significant over the pH range shown and is greater at the lower pH values. The corresponding computed results for the oxyhemoglobin system are shown in Figure 2. The results for both the rigid rotation oxy- and carbonmonoxyhemoglobin structures are presented.

The much smaller values for $\sum\Delta G''_{i,el}$ calculated from the carbonmonoxyhemoglobin structure (Figure 2A, curve 2; Figure 2B, curve 5) indicate greatly reduced net stabilization within the charge array. A detailed study of the interactions of individual groups revealed that the source of this large difference is not limited to one or two charged sites (M. A. Flanagan, unpublished results). The carbonmonoxy structure imparts more positive environments to 8 of 11 histidine residues, depressing the computed $pK_{1/2}$ values below those observed by proton NMR (Ho & Russu, 1978). In contrast, the histidine pK_{eff} values are well modeled by the rigid rotation oxyhemoglobin structure (Matthew et al., 1979a). In particular, the $pK_{1/2}$ value predicted from the rigid rotation structure for His-146β (Matthew et al., 1979a) is in agreement with proton NMR studies conducted at low ionic strength (Ho & Russu, 1978; Russu et al., 1980), whereas that predicted from the carbonmonoxy structure is 6.0, more consistent with experimental pK_{eff} values determined at high ionic strength (Kilmartin et al., 1973; Ho & Russu, 1978; Russu et al., 1980). The net effect of the difference in the interactions of histidine and other residues is to depress the overall charge on the protein by two charges over the range pH 6–10 (M. A. Flanagan, unpublished results) relative to the experimental pH titration behavior at 25 °C, I = 0.1 M (Rollema et al., 1975). The overall depression of the predicted $pK_{1/2}$ values may be a reflection of an increased response of the liganded structure to the high salt concentration used for crystallization. In addition, at the pH used to grow the crystals, pH 6.8 (Perutz, 1968; Baldwin, 1980), tetramer to dimer dissociation is much more favored in liganded hemoglobin (Thomas & Edelstein, 1973; Atha & Riggs, 1976; Chu & Ackers, 1981).[3]

The computed pH dependence of the stabilization of the dimer forms is qualitatively similar to that of the tetramers and of the α and β monomers (Friend et al., 1981) or of myoglobin (Friend & Gurd, 1979), being greatest on the acid side of the isoelectric condition. As shown below, the same trend is found for the dimer–tetramer assembly step for the deoxy case, but for the rigid rotation oxyhemoglobin case, the pH dependence has the opposite trend.

Electrostatic Stabilization of Dimer–Tetramer Assembly. Curve 1 in Figures 3 and 4 shows the pH dependence at I = 0.1 M of the difference in summed electrostatic free energy,

[3] This subject will be dealt with in greater detail in a forthcoming review (F. R. N. Gurd, J. B. Matthew, S. J. Shire, and M. A. Flanagan, unpublished results).

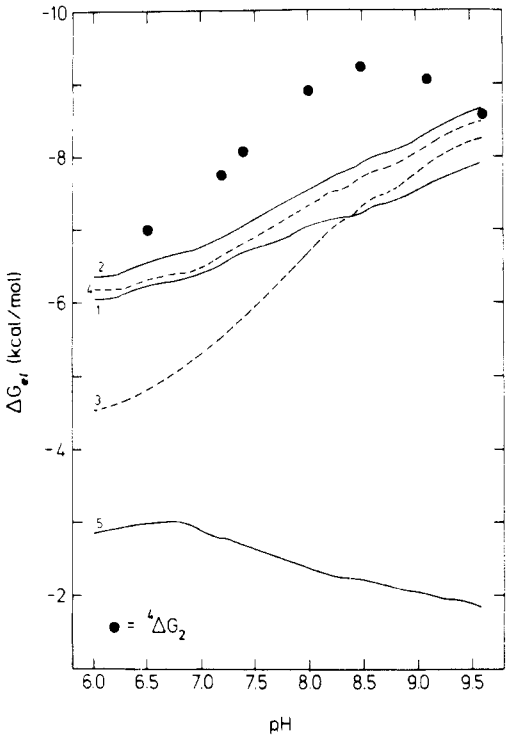


FIGURE 4: Computed pH dependence of the electrostatic contribution to the free energy of oxyhemoglobin dimer–tetramer assembly, ΔG_{el}, in kcal/mol, at 25 °C, $I = 0.1$ M. Each curve, 1–4, is the difference between curve 1, Figure 2A, and twice the corresponding curve, 1–4, in Figure 2B. These computations are based on the rigid rotation oxyhemoglobin structure. Curve 5 is calculated from the carbonmonoxy structure and is the result of the difference between curve 2, Figure 2A, and twice curve 5, Figure 2B (see also Table I). The experimental data, $^4\Delta G_2$ as a function of pH at 21.5 °C, are taken from Chu & Ackers (1981). The solution conditions are the same as those for Figure 3.

ΔG_{el}, between deoxyhemoglobin and rigid rotation oxyhemoglobin tetramers and dimers, respectively. The experimental data in Figures 3 and 4 were obtained by using 0.1 M buffers containing 0.1 M NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA) (Chu & Ackers, 1981).[4] At all pH values, it was computed that the tetramer has greater electrostatic stabilization than the dimers. The assembly of deoxy dimers into tetramers is known to be increasingly favorable as pH decreases whereas oxy assembly shows the opposite trend (Thomas & Edelstein, 1973; Atha & Riggs, 1976; Chu & Ackers, 1981). In each case, the pH dependence is predicted correctly (Figures 3 and 4). The pH dependence modeled for the electrostatic contribution to deoxy dimer–tetramer assembly is essentially the same as that calculated for the assembly of tetramers from monomers (Friend et al., 1981). From experimental results the assembly of dimers from monomers has been inferred to be independent of pH over the range between pH 7 and 9 (Chu & Ackers, 1981). Indeed, the calculated electrostatic contribution to the assembly of dimers from monomers has been found to be nearly independent of pH between pH 6.0 and 10.0 (Gurd et al., 1980).

The experimentally determined free-energy gain upon dimer–tetramer assembly is −14.3 and −8.0 kcal/mol for deoxy- and oxyhemoglobin, respectively, at pH 7.4 with a total chloride ion concentration of 0.18 M (Chu & Ackers, 1981).

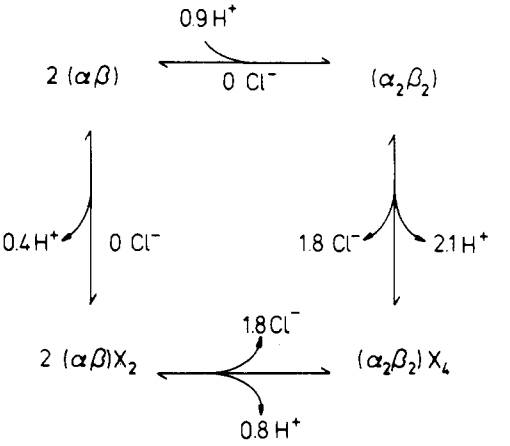
[4] Although it is difficult directly to compare the results obtained at two different ionic strengths, the trends computed at an ionic strength of 0.1 M are representative of those that would be seen at 0.18 M [see Friend et al. (1981)]. Direct calculations at 0.18 M ionic strength are prohibitive due to nonconvergence of the algorithm used for computation.

Table I: Effect of Specifically Bound Chloride on the Electrostatic Contribution to the Free Energy of Dimer–Tetramer Assembly, pH 7.4, $I = 0.10$ M

	position of chloride[a]	ΔG_{el} (kcal/mol)
deoxyhemoglobin		
no bound chloride		−3.4
1 chloride per dimer	CLR 143A (dimer)	−4.6
	CLR 142A (tetramer)	
2 chlorides per dimer	CLR 143A + 147B (dimer)	−4.1
	CLR 142A + 147B (tetramer)	
	CLR 143A + 148B (dimer)	−4.8
	CLR 142A + 148B (tetramer)	
	CLR 143A + 150B (dimer)	−4.2
	CLR 142A + 150B (tetramer)	
rigid rotation oxyhemoglobin		
no bound chloride		−6.7
1 chloride per dimer	CLR 143A (dimer)	−7.1
2 chlorides per dimer	CLR 143A + 147B (dimer)	−5.8
	CLR 143A + 148B (dimer)	−6.8
carbonmonoxyhemoglobin		
no bound chloride		−2.7

[a] See Table II and text for a description of chloride ion binding sites.

Scheme I



The computed electrostatic contribution shows the oxy tetramer formation to be more stable than deoxy tetramer formation by 3.3 kcal/mol (Table I), yet the contrasting effects of pH are well modeled.

The pH dependence of the electrostatic contribution to the oxy dimer–tetramer assembly calculated by using the carbonmonoxy structure is shown in Figure 4 (curve 5). The net electrostatic contribution at pH 7.4 is 30% of that value calculated from the rigid rotation structure (Table I), and the pH dependence predicted is not in agreement with the experimental data (Chu & Ackers, 1981). The rigid rotation oxy structure provides a predictive model, whereas the carbonmonoxyhemoglobin structure in its present form does not.[3] Subsequently, all oxy dimer–tetramer assembly calculations referred to are based on the rigid rotation oxyhemoglobin structure.

Effects of Chloride Binding on Dimer–Tetramer Assembly. Recent studies of the chloride binding linkage in the dimer–

Table II: Coordinates and Static Accessibilities Used for Bound Chloride Ions in Oxy- and Deoxyhemoglobin[a]

	deoxy					oxy				
	SA		coordinates			SA		coordinates		
	dimer	tetramer	X	Y	Z	dimer	tetramer	X	Y	Z
CLR 142A[b]	0.39	0.22	7.4	12.5	4.2	0.38	0.32	6.2	11.5	4.2
CLR 143A[b]	0.31	0.31	8.0	19.6	0.3	0.31	0.31	6.3	19.0	1.0
CLR 147B[c]	0.35	0.35	4.7	−19.7	2.8	0.34	0.30	5.7	−20.1	−0.8
CLR 148B[c]	0.77	0.50	−5.2	−20.1	6.6	0.76	0.52	−4.4	−21.2	2.1
CLR 150B[b,d]	0.24	0.24	6.8	−18.5	2.1					

[a] The coordinates listed are for a single dimer. A simple reflection across the y axis will give the coordinates for the second dimer in the tetramer. [b] Anion binding sites identified by difference maps of bromide- and chloride-soaked deoxy-HbA crystals (A. Arnone and B. Foster, unpublished results). CLR 142A is between the α_1-chain NH_2-terminal and the guanidinium of Arg-141α_2. CLR 143A is between the hydroxyl of Ser-131α_1 and the α-chain NH_2-terminal. CLR 150B is between Val-1β_1 and Lys-82β_1. [c] Possible Cl^- binding sites in the β cleft used by Matthew et al. (1981a). CLR 147B is equidistant between Val-1β_1 and Lys-82β_1. CLR 148B is located between Lys-82β_1 and His-143β_1 in the dimer and interacts also with His-2β_2 in the tetramer. [d] This site was not used in oxyhemoglobin calculations.

tetramer assembly have been published (Chu & Ackers, 1981) and are summarized in Scheme I. Scheme I indicates the number of moles of hydrogen ion and chloride ion coupled to the reactions of dimer–tetramer assembly and oxygen binding at pH 7.4, 21.5 °C, with a total chloride ion concentration of 0.18 M. Reactions are taken from left to right and top to bottom; X represents ligand, CO, or O_2. In the assembly of tetramers from dimers of deoxyhemoglobin, there are no reaction-linked chloride ions. Oxyhemoglobin tetramer assembly shows a loss of approximately two chloride ions per tetramer formed that is subsequently offset upon deoxygenation in the chloride-linked part of the alkaline Bohr effect (Van Beek et al., 1979).[5] Thus it appears that at least one chloride ion is bound per dimer in the deoxy tetramer and in both the oxy and deoxy dimers. There may be, in addition, several other specific chloride ion binding sites that are not linked to oxygenation or association.

Location of Chloride Binding Sites. The locations of chloride binding sites on deoxy- and oxyhemoglobins are not fully known. However, it is known that an oxygen-labile chloride ion binds in the $\alpha_1\alpha_2$ tetramer contact between the α-amino-terminal of one α chain and the guanidinium group of Arg-141 on the other α chain (O'Donnell et al., 1979). This site will be referred to as CLR 142A (Table II). The apparent loss of this chloride ion on oxygenation is thought to contribute to the chloride-linked portion of the alkaline Bohr effect (Van Beek et al., 1979; Matthew et al., 1979b). Another anion binding site in this contact region has been identified in the X-ray diffraction difference map of tetrameric hemoglobin at high salt concentration (O'Donnell et al., 1979) between the α-amino-terminal and the hydroxyl group of Ser-131α on the same chain (Table II, CLR 143A).

Several lines of evidence suggest that Lys-82β binds some oxygen-linked chloride (Nigen & Manning, 1975; Nigen et al., 1980). An examination of the deoxy β cleft electrostatic potential surface suggests multiple isoenergetic chloride binding sites (Matthew et al., 1981a,b). One of these is between Lys-82β and the NH_2-terminal of the same chain (Table II, CLR 147B), and another is between Lys-82β, His-143β, and His-β_2 (Table II, CLR 148B).[6] An anion site has in fact been recognized between Lys-82β and Val-1β (Table II, CLR 150B) in a slightly different orientation (A. Arnone and B. Foster, unpublished results). It is known that oxyhemoglobin tetramers bind detectable chloride ion at acid pH or under conditions of high chloride ion concentration, but no binding sites have been identified (Van Beek & deBruin, 1980).

The analysis of electrostatic stabilization of the dimer–tetramer assembly of deoxy- and oxyhemoglobin was extended to conditions in which various potential chloride binding sites are taken to be fully occupied, although at 0.1 M ionic strength, fully occupancy of any given chloride site would probably not occur (Matthew et al., 1981b). If flexible side chains are allowed to rearrange or chelate in response to the binding of an anion, greater differential proton binding will occur. In this treatment, local rearrangements due to charge binding are not allowed; thus the binding constants for protons and anions will, in most cases, be an underestimate (Matthew et al., 1981a). The pH dependence of chloride binding was previously found to have a negligible effect on the calculations below pH 9 and thus was not taken into account in these or previous calculations [see Matthew et al. (1979b)]. Recent experiments have shown that chloride binding to deoxyhemoglobin tetramers persists at pH 9.0, although less marked than at pH 7.0 (Van Beek & deBruin, 1980). A thermodynamic analysis of oxygen-linked chloride binding suggests the involvement of either a single site per dimer or of multiple independent, partially occupied sites (Haire & Hedlund, 1977). Therefore, a linear combination of results for several different chloride binding sites may yield a reasonable estimate of the true molecular situation (Matthew et al., 1979b, 1981b). The coordinates used and SA values calculated for these various sites are compiled in Table II.

Calculated Chloride Binding Effects on Dimer–Tetramer Assembly. For studies on the effect of specific chloride binding on the free energy of deoxyhemoglobin dimer–tetramer assembly, the NH_2-terminal α chain was assumed to be present in both the dimer and tetramer. The site that bridges the $\alpha_1\alpha_2$ contact region was used in the tetramer, CLR 142A, and the intrasubunit site, CLR 143A, was used in the dimer. In addition, a second chloride binding site was introduced in both dimer and tetramer at one of the three β chain sites: CLR 147B, CLR 148B, or CLR 150B. For oxyhemoglobin dimer–tetramer assembly, the NH_2-terminal α-chain site was assumed to be present only in the dimers. The intrasubunit site, CLR 143A, was used. In subsequent calculations, an additional chloride ion was placed near Lys-82β, either CLR

[5] Matthew et al. (1979a, 1981b) computed that four chloride ions differentially bound to deoxyhemoglobin, at an ionic strength of 0.1 M, were able to account for the alkaline Bohr effect, although the number of differentially bound chloride is probably less (Matthew et al., 1981a). In addition, at chloride ion concentrations greater than 0.1 M, specific chloride binding to oxyhemoglobin increases, decreasing the amount of differentially bound chloride (Van Beek & deBruin, 1980) but not substantially decreasing the alkaline Bohr effect (Rollema et al., 1975; Van Beek et al., 1979).

[6] These sites were previously referred to as Lys-82β, positions 1 and 2, respectively (Matthew et al., 1981a).

147B or CLR 148B, in the oxyhemoglobin dimer. No bound chloride was included in the oxy tetramer under any conditions. The results for the individual dimers and tetramers are shown in Figures 1 and 2. As before, ΔG_{el} is computed by difference.

Curve 2 in Figures 3 and 4 shows the pH dependence of ΔG_{el} when the α-chain NH_2-terminal chlorides are included. The difference between curves 1 and 2 is due only to the difference in the interactions of charged groups with the chloride site in the tetramer and dimer. These differences arise from greater burial of groups near the chloride binding site as well as from direct contributions to the charge array. The chloride binding makes tetramer formation more stable over the entire pH range studied and contributes significantly between pH 6 and 8 to deoxy assembly. At pH 7.4, an additional −1.2 kcal/mol of electrostatic stabilization is contributed to deoxy assembly by specific chloride binding at the α-chain sites (Table I). The increase for oxy assembly is less, −0.4 kcal/mol (Table I).

Curves 3–5 in Figure 3 and curves 3 and 4 in Figure 4 demonstrate the effect of an additional chloride ion bound near Lys-82β in the β cleft. Sensitivity to the exact placement of the anion is readily apparent. Chloride ion at either position between Val-1β and Lys-82β, CLR 147B (Figures 3 and 4, curve 3) or CLR 150B (Figure 3, curve 5), is destabilizing, relative to curve 2. It lowers the electrostatic contribution to association and also brings the slope of the pH dependence closer to that seen experimentally. Above pH 7.8, a relatively significant destabilization occurs in deoxy assembly when chloride is placed at the anion binding site identified by X-ray crystallography, CLR 150B (Figure 3, curve 5). A slight shift in the position of the anion makes a difference in the overall stability of association and its pH dependence that is comparable to the stability differences for the presence and absence of chloride ion. Inclusion of the other possible anion binding site involving Lys-82β, CLR 148B (Figure 3 and 4, curve 4), provides a significant increase in the stability of the deoxy tetramer at acid pH but makes little change in oxy assembly. The calculations entailing the presence of two chlorides per dimer (Figure 3, curves 3–5) result in a pH dependence for deoxy assembly that more closely resembles the experimental data than do the calculations done in the absence of chloride (Figure 3, curve 1) or with only one chloride bound per dimer (Figure 3, curve 2). Table I summarizes the electrostatic contribution to the free energy of dimer–tetramer assembly, pH 7.4, with occupancy of various chloride binding sites.

At pH 8.95, there is a net loss of bound chloride ion during deoxyhemoglobin dimer–tetramer assembly, in contrast with pH 7.4 where there are no assembly linked chloride ions observed (Chu & Ackers, 1981). The observed change in chloride ion linkage as pH increases represents an increasing destabilization of bound chloride in the deoxy tetramer relative to the dimer and may involve sites similar to those modeled in Figure 3, curve 3–5.

It has been shown that the binding of chloride ions can have a large effect on the stability of the hemoglobin tetramer relative to the dimer. A definitive understanding of the contribution that charge interactions make to the overall free energy of dimer–tetramer assembly will require a careful identification of anion binding sites and their occupancies under various conditions. However, the calculated electrostatic contributions to deoxy and oxy dimer–tetramer assembly are sufficiently distinct, irrespective of chloride ion placement, to warrant a closer examination of the interactions responsible for the calculated stability differences.

Contributions of Individual Charged Groups. The contributions of selected individual groups to the free energy of dimer–tetramer assembly are summarized in Tables III and IV. The groups are arranged into four main categories: $\alpha_1\alpha_2$ contact, $\alpha_1\beta_2$ contact, $\beta_1\beta_2$ contact (β cleft), and bound chloride ions. The contribution from each category is summed, and then the total contribution from all four categories is determined and compared to the electrostatic free energy summed over the entire charge array. The stabilizations and destabilizations result from a diversity of interactions (Friend et al., 1981).

Table III: Contribution of Selected Individual Groups to the Electrostatic Free Energy of Dimer–Tetramer Assembly of Deoxyhemoglobin, pH 7.4, I = 0.10 M

contact region between dimers	$\delta G_{i,el}$ (cal/mol)	$[\delta G_{i,el}(Cl^-) - \delta G_{i,el}]$ (cal/mol) 2Cl⁻	4Cl⁻ CLR 147B	4Cl⁻ CLR 148B	4Cl⁻ CLR 150B
$\alpha_1\alpha_2$					
Val-1α	−170	−408	−408	−408	−408
Asp-6α	−418	+36	+38	+38	+38
Asp-126α	−1076	+282	+284	+284	+282
Lys-127α	−952	−182	−184	−184	−184
Arg-141α	−1808	−820	−824	−822	−824
Arg-141α COOH	−234	+182	+176	+174	+176
	−4658	−910	−918	−918	−920
$\alpha_1\beta_2$					
Lys-40α	−490	−4	−8	−16	−6
Arg-92α	−144	−20	−20	−20	−20
Asp-94α	+1336	+106	+114	+112	+116
Arg-40β	−380	−14	−16	−16	−16
Glu-43β	−18	+0	+0	+0	+0
Asp-94β	+172	−6	−10	−24	−8
His-97β	+6	+0	+0	+0	+0
Asp-99β	+1138	+50	+62	+58	+60
Glu-101β	+982	+62	+76	+76	+72
His-146β	−432	−14	−12	−46	−6
His-146β	−402	+6	+16	+34	+14
	+1768	+166	+202	+158	+206
$\beta_1\beta_2$					
Val-1β	+0	+12	+210	+48	+154
His-2β	+0	+0	−4	−30	−6
Lys-82β	−10	−16	−54	−22	−10
His-143β	+0	+0	−2	−54	+0
	−10	−4	+150	−58	+138
chloride					
CLR 142A		−296	−296	−296	−294
CLR 147B			+258		
CLR 148B				−104	
CLR 150B					+206
	+0	−296	−38	−400	−88
Σ (kcal/mol)	−2.9	−1.0	−0.6	−1.2	−0.7
total (kcal/mol)	−3.4	−1.2	−0.7	−1.4	−0.8

In the second column of Tables III and IV, individual contributions to the free energy of association, $\delta G_{i,el}$, calculated in the absence of bound chloride are listed. A striking difference is seen to exist in the contributions from the $\alpha_1\alpha_2$ and the $\alpha_1\beta_2$ contacts. The $\alpha_1\alpha_2$ contact contributes −4.7 kcal/mol to the free energy of deoxy assembly (Table III). The large contribution from this region was first seen in the assembly of the deoxy tetramer from monomers (Friend et al., 1981). This is in sharp contrast with the contribution from the deoxy $\alpha_1\beta_2$ contact, +1.6 kcal/mol, due largely to three significant destabilizations: Asp-94α, Asp-99β, and Glu-101β. The β-cleft region makes no significant contribution to deoxy dimer–tetramer assembly. Taken together, the three contact regions contribute −3.0 kcal/mol. For oxyhemoglobin dimer–tetramer assembly, the charged groups in the $\alpha_1\alpha_2$ contribute −7.8 kcal/mol to the dimer–tetramer assembly (Table IV). This is −3.2 kcal/mol more than that contributed from the deoxy $\alpha_1\alpha_2$ contact (Table III). The $\alpha_1\beta_2$ contact has a

Table IV: Contribution of Selected Individual Groups to the Electrostatic Free Energy of Dimer–Tetramer Assembly of Oxyhemoglobin, pH 7.4, $I = 0.10$ M

contact region between dimers	$\delta G_{i,el}$ (cal/mol)	$[\delta G_{i,el}(Cl^-) - \delta G_{i,el}]$ (cal/mol) 2Cl⁻	4Cl⁻ CLR 147B	4Cl⁻ CLR 148B
$\alpha_1\alpha_2$				
Val-1α	−30	+164	+164	+164
Asp-6α	−758	−208	−208	−208
Asp-126α	−1660	−54	−56	−54
Lys-127α	−2348	+40	+40	+40
Arg-141α	−2090	+0	+0	+0
Arg-141α COOH	−960	−8	−10	−10
	−7846	−66	−70	−68
$\alpha_1\beta_2$				
Lys-40α	−30	+0	+0	+0
Arg-92α	−24	+0	+0	+0
Asp-94α	+400	−2	−2	−2
Lys-99α	−82	+4	+10	+6
Arg-40β	−706	+0	+0	+0
Glu-43β	−8	+0	+0	+0
Asp-94β	+104	+2	−6	−4
His-97β	+20	+0	+0	+0
Asp-99β	+384	−2	−4	−2
Glu-101β	−246	−6	−18	−12
Arg-104β	−116	0	+10	+4
His-146β	+4	+2	+18	+12
His-146β COOH	−146	+0	−2	+0
	−416	−2	+6	+2
$\beta_1\beta_2$				
Val-1β	+48	+0	+296	+32
His-2β	+4	+0	+0	+0
Lys-82β	+1550	+0	+200	+72
His-143β	−10	+0	+0	−12
	+1592	0	+496	+92
chloride				
CLR 143A		−256	−256	−256
CLR 147B			+640	
CLR 148B				+144
	+0	−256	+384	−112
Σ (kcal/mol)	−6.7	−0.3	+0.8	−0.1
total (kcal/mol)	−6.7	−0.4	+0.9	−0.1

net contribution of −0.5 kcal/mol, −2.1 kcal/mol more stabilizing than the deoxy $\alpha_1\beta_2$ contact. The large destabilizations provided by Asp-94α and Asp-99β in deoxy tetramer assembly are partially relieved in the oxy tetramer. Glu-101β actually becomes stabilizing. Several groups that provide stabilization in the deoxy tetramer are significantly less stabilizing in the oxy molecule: Lys-40α and His-146β are the most striking examples. The compensating destabilization in oxy dimer–tetramer assembly is due to the closing of the β cleft; +1.6 kcal/mol is contributed from this region.[7] The overall stabilization is −6.8 kcal/mol, −3.8 kcal/mol more than that calculated for deoxy dimer–tetramer assembly.

The next four columns in Table III and three columns in Table IV list the additional contribution to the electrostatic free energy of assembly, $[\delta G_{i,el}(Cl^-) - \delta G_{i,el}]$, that results from the inclusion of bound chloride in the charge array. When bound chloride is included in the deoxy $\alpha_1\alpha_2$ contact, its interactions with the groups in this region provide −0.9 kcal/mol additional free energy (Table III). In contrast, the $\alpha_1\beta_2$ contact is actually destabilized by almost 0.2 kcal/mol, although the closest group, Asp-94α, is 12.6 Å from the chloride ion. The other groups in the $\alpha_1\beta_2$ contact are 16.3–32.9 Å from the anion. The three strongest additional destabilizations are felt at Asp-94α, Asp-99β, and Glu-101β, the same three groups which provide the bulk of the $\alpha_1\beta_2$ instability. The total for the four categories is −1.0 kcal/mol additional stabilization of deoxy dimer–tetramer assembly.

When chloride is bound at the NH_2-terminal of the α chains in oxy dimers but is absent from the oxy tetramer, the net result in the $\alpha_1\alpha_2$ contact is −0.07 kcal/mol of additional stabilization of oxy tetramer formation (Table IV). This is a small difference in comparison with the magnitude of the interactions seen in deoxy dimer–tetramer assembly, but it suggests a rationale for the lower occupancy of bound chloride in the $\alpha_1\alpha_2$ contact in oxyhemoglobin tetramers. The $\alpha_1\beta_2$ contact is not as sensitive to bound chloride as it is in deoxy dimer–tetramer assembly. In contrast to the assembly of deoxy tetramers, bound chloride provides additional stabilization to Asp-94α, Asp-99β, and Glu-101β in oxy dimer–tetramer assembly.

When an additional chloride ion is included in the β-cleft region, the major changes are in the $\alpha_1\beta_2$ contact and in the β cleft. A chloride ion between Val-1β and Lys-82β preferentially stabilizes the dimer (Tables III and IV) because Val-1β interacts more strongly with the anion in the dimer. The deoxy $\alpha_1\beta_2$ contact is suprisingly sensitive to changes in the β cleft (Table III). The 10–40 cal/mol of additional destabilization of the tetramer is fairly uniformly distributed over seven of the nine $\alpha_1\beta_2$ groups, indicating that most of this region of the deoxy tetramer senses the addition of chloride ion in the β cleft.

It is noteworthy that a consistent 12–17% of the electrostatic interaction energy affecting deoxy dimer–tetramer assembly is attributable to sites outside of the contact regions. In the assembly of deoxy tetramers from monomers, a full 25% of the overall stabilization is contributed by charge site interactions spanning a distance greater than 10 Å (Friend et al., 1981). Widespread effects of changes in the charge array of a protein have previously been seen in hemoglobin oxygenation (Matthew et al., 1979b) and azide binding to ferrimyoglobin (Friend et al., 1980). The substitution of a histidine at Arg-141α results in effects sensed a full 55 Å away (Poyart et al., 1980).

The analysis of individual contributions to ΔG_{el} shows a sensitivity of the deoxy $\alpha_1\beta_2$ contact to chloride ion binding. The two α-chain heme iron atoms are 19.3 and 20.2 Å from the chloride ion bound between the α chains, the same as the average distance of the chloride ion from the $\alpha_1\beta_2$ charged groups. The coordinated heme iron does not carry a formal charge in the ferro form (Shire, 1974) so that the extent of electrostatic perturbation at the heme cannot be calculated by using the present treatment. However, electronic stabilization between heme and protein has been investigated by Raman difference spectroscopy (Shelnutt et al., 1979a,b). These interactions change in ferrihemoglobins when inositol hexaphosphate is bound (Rousseau et al., 1980b) and the observed electronic interaction has been implicated in hemoglobin cooperativity (Rousseau et al., 1980a). The far-reaching effects of perturbations of the charge array, of which chloride binding is an example, suggest a mechanism for communication between the heme iron and surface groups. Because of the interconvertibility of energy forms, changes in electronic interactions due to proton or anion binding may contribute to the free energy required for heme-linked conformational change.

[7] The destabilization of the β cleft calculated for the oxy tetramer is probably an overestimate because of the close placement of Lys-82β and His-143β in the rigid rotation structure. The calculated energy of interaction represents the maximum energy available for conformational rearrangement due to the repulsion between these groups (Matthew et al., 1981b).

Table V: Comparison of Experimentally Determined and Predicted Parameters for the Dimer–Tetramer Assembly of Oxy- and Deoxyhemoglobin, pH 7.4, $I = 0.10$ M

	exptl values[a]				predicted values[b]	
	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (eu)	n_{H^+} (H^+/tetramer)[c]	ΔG_{el} (kcal/mol)	n_{H^+} (H^+/tetramer)
deoxy	−14.3 ± 0.2	−28.9 ± 0.5	−49.8 ± 1.7	−0.9 ± 0.1	−3.4 to −4.8	−1.2 to +1.2
oxy	−8.0 ± 0.1	+3.9 ± 1.6	+40.4 ± 5.7	+0.8 ± 0.1	−5.8 to −6.8	+0.8 to +2.0

[a] Experimental values are taken from Ackers (1980); the reaction conditions were pH 7.4, 0.1 M Tris-HCl buffer, 0.1 M NaCl, and 1 mM EDTA. The total chloride ion concentration was 0.18 M. [b] The ranges shown are from different cases of chloride binding (see Table I and text). [c] n_{H^+} refers to the number of protons released per tetramer formed.

Sources of Energetic Effects. Results of this study provide an estimate for the electrostatic contribution to the free energy of the hemoglobin dimer–tetramer assembly reaction. Those processes that depend on charge interactions in assembly reactions include the formation of salt bridges or ion pairs and changes in the protonation state of groups that titrate in the pH range of interest. The latter changes will of course affect the more general electronic interactions with other groups that do not carry formal charges. Such interactions can affect the energetics of hydrogen bonds and the energy of interaction of the heme with the protein. These interactions are not modeled by this method.

Ackers (1980) has analyzed the measured thermodynamic parameters for hemoglobin assembly against theories based on the predominance in the energetics of the reaction of each of the several types of noncovalent interaction. Results of this analysis indicated that the deoxy dimer–tetramer assembly may be favored over the oxy assembly reaction because of more favorable hydrogen bonds formed in the deoxy tetramer. The pattern of thermodynamic parameters was also found consistent with fewer vibrational degrees of freedom in the deoxy tetramer.

In this paper, an attempt is made to reconcile the predicted electrostatic contribution with the known thermodynamic parameters. The experimental parameters are reproduced in Table V along with the calculated electrostatic contribution, ΔG_{el}. The experimental free energies show deoxy assembly to be more favorable than oxy assembly by −6.3 kcal/mol. The electrostatic contribution, on the other hand, is computed to be 2.4–3.4 kcal/mol more stabilizing for oxy assembly than for deoxy assembly, implying that electrostatic interactions are more favorable in oxy than deoxy assembly. It is widely held, on the contrary, that salt bridge formation is responsible for the preferential stability of deoxy tetramers (Perutz, 1978). However, recent experimental evidence suggests that at pH 7.4, salt bridges do not play a dominant role in stabilizing the deoxy tetramer relative to the oxy molecule (Ackers, 1980; Chu & Ackers, 1981).

The signs of ΔH and ΔS are opposite for deoxy and oxy assembly (Table V). Clearly, oxy dimer–tetramer assembly is stabilized by different predominant factors from those important in the deoxy dimer–tetramer assembly. The substantial difference in n_{H^+}, the change in overall protonation state, between the two reactions also suggests that different interactions contribute to their relative stability. The predicted values of n_{H^+} are appreciably dependent on the location and quantity of bound chloride yet show strong general correlation with the experimental values.

The predominant forces stabilizing the deoxy dimer–tetramer assembly produce net negative entropy and enthalpy changes (Table V). The factors that would meet this requirement are an increase in van der Waals contacts (Ross & Subramanian, 1980), an increase in the number or energy of hydrogen bonds (Ackers, 1980), or the loss of vibrational degrees of freedom (Sturtevant, 1977). A small amount of net energy contributed from other factors can be accommodated so long as the sign of the entropy or enthalpy change is not altered. For example, ion pair formation results in a release of electrostricted water and characteristically results in small negative or positive enthalpies and large, positive entropies (Kauzmann, 1959; Fersht, 1971).[8] Protonation reactions are characterized by negative enthapies and positive entropies (Sober & Harte, 1968). An increase in hydrophobic interactions would give positive enthalpies and entropies (Tanford, 1980).

Conversely, the oxy dimer–tetramer assembly is stabilized by factors giving a net result of positive entropy and enthalpy. Hydrogen bonds and van der Waals forces cannot provide a substantial portion of the stabilization unless ion pair formation, deprotonation, or hydrophobic interactions contribute significantly also. It has been recently postulated that hydrophobic interactions constitute the driving force for association reactions but compensate only marginally over rotational and translational entropy losses (Ross & Subramanian, 1980). The net stabilization of association would then be due to more specific forces such as salt pairs, hydrogen bonds, and van der Waals contacts. In this view, hydrophobic interactions may contribute to the sum of energy terms in the assembly process (Chothia & Janin, 1975; Herskovits & Ibanez, 1976) but provide significant net contributions to neither deoxy nor oxy dimer–tetramer assembly.

The two factors left in consideration as major net contributors of oxy dimer–tetramer assembly are deprotonation and ion pair formation, or, more generally, alterations in the interacting charge array. The net loss of protons for this reaction (Table V) would contribute a positive enthalpy change and a negative entropy change. A major consequence of ion pair formation, the release of electrostricted water, contributes a large, positive entropy change.[8] This effect would be greater for oxy assembly than for deoxy assembly because the hemoglobin charge groups are more dehydrated in oxy tetramers as computed from solvent accessibility data (Matthew et al., 1979a). In the right proportions, a combination of the two factors could provide the appropriate result of positive enthalpy and entropy change.

The fraction of the overall free energy of dimer–tetramer assembly accounted for by ΔG_{el} is consistent with this analysis. The extent of ion pair formation and protonation or deprotonation will depend on the changes in electrostatic interactions as well as noncharged, electronic interactions. ΔG_{el} for deoxy assembly accounts for 24–34% of the observed free-energy change. The thermodynamic parameters require that major contributions come from noncharge-related forces. ΔG_{el} for oxy assembly accounts for 73–85% of the observed free energy. This is consistent with a large fraction of the assembly reaction

[8] Ion pair formation between closely neighboring sites, involving displacement of bound water molecules, will have greater consequences for entropy than when charged groups are at a sufficient distance that one hydration sphere is relatively unaffected by the other.

being contributed from charge-related forces, of which deprotonation and ion pair formation are examples.

Charge Interactions as Quaternary Enhancement. Model-independent thermodynamic results have established the existence of *quaternary enhancement* in human hemoglobins (Valdes & Ackers, 1978; Mills & Ackers, 1979; Chu et al., 1981); i.e., subunit assembly can lead to quaternary structures with enhanced affinity for oxygen. An alternative manifestation of quaternary enhancement is an energetically less-stabilized assembly reaction for the unliganded subunits as compared to the oxygenated ones. This phenomenon is observed in hemoglobin A at the last step of oxygenation; i.e. assembly of a triliganded tetramer from a doubly liganded dimer and a singly liganded dimer is less favorable by 0.8 kcal than the assembly of an oxy tetramer from two oxy dimers (Mills & Ackers, 1979). In the case of the β-chain self-assembly, the deoxy reaction is less favorable than the oxy reaction by 3.2 kcal, or again 0.8 kcal/heme. These observations reinforce the concept that the net energetic effects in hemoglobin are the resultant of oppositely directed effects. The 3.2-kcal electrostatic destabilization found in this study for assembly of deoxyhemoglobin relative to oxyhemoglobin may be a significant contributor to quaternary enhancement. This destabilization may be masked in deoxyhemoglobin by the larger, favorable interactions. As these constraints are progressively released upon oxygenation, a part of the destabilizing interactions may become dominant (e.g., at the last step of oxygenation). In suggesting that the electrostatic effects may contribute significantly to quaternary enhancement, we do not imply that these interactions are necessarily the sole source of quaternary enhancement. Since less than one quarter of the triliganded tetramers are in the deoxy quaternary state, the experimentally observed 0.8 kcal of quarternary enhancement seems unlikely to be completely attributable to the electrostatic effect of quaternary structure change.

Hydrogen exchange data are consistent with greater restraints in the $\alpha_1\beta_2$ contact of the deoxy tetramer, perhaps due to higher energy hydrogen bonds or other constraining interactions (Englander et al., 1980, 1981). The large electrostatic destabilization calculated for the $\alpha_1\beta_2$ region in deoxyhemoglobin may be necessary for the optimum arrangement of atoms for these interactions. The free-energy change associated with hydrogen-bond formation is of an adequate magnitude, −0.5 to −5.0 kcal/mol (Finney et al., 1980), to compensate for the free-energy losses due to unfavorable electrostatic interactions.

The role of factors other than electrostatic in stabilizing the deoxy structure is demonstrated for Asp-99β, one of the groups that is calculated to contribute a significant destabilization (Tables III and IV). An aspartic acid in this position allows for interactions with Tyr-42α in the deoxy form that are not available when an asparagine or histidine is substituted (Perutz & TenEyck, 1971; Matsukawa et al., 1979; Chu et al., 1981). In addition, at least two water molecules are seen bridging from Asp-99β to the α chain across the $\alpha_1\beta_2$ contact (Perutz, 1977). The interaction between Asp-99β and Tyr-42α is diminished in the quaternary structural change from T to R as seen in crystal structures (Perutz, 1970; Anderson, 1973) and in proton NMR (Viggiano & Ho, 1979). In oxyhemoglobin, this destabilization is calculated to decrease by 0.8 kcal/mol (Tables III and IV), consistent with diminution of interactions compensating for the large electrostatic destabilization of Asp-99β in deoxyhemoglobin.

Thus the present analysis suggests that electrostatic interactions are responsible in part for quarternary enhancement of oxygen affinity while the hydrogen bonds and van der Waals forces postulated to be responsible for the stability of deoxyhemoglobin tetramers (Ackers, 1980) are responsible for quarternary constraint. This is in direct opposition to the current belief that salt bridges provide quaternary constraint in tetrameric hemoglobin (Perutz, 1978).

The evidence in favor of the salt bridges as the dominant source of cooperative energy is the following: (1) Crystallographic structure work showed that in deoxyhemoglobin the C-terminal residues of the four chains form salt bridges with complementary charges of neighboring chains, while in liganded hemoglobins the C-terminal residues are free and delocalized. These crystallographic findings led Perutz to suggest that a major portion of the free energy of heme–heme interaction is stored in these salt bridges. (2) Kilmartin, Perutz, and their colleagues tested this hypothesis by preparing modified hemoglobins in which the salt bridges were selectively altered. These were found to have raised oxygen affinity, loss of cooperativity, and a deoxy quaternary structure similar to that of oxyhemoglobin. (3) Abnormal hemoglobins with substitutions that alter the C-terminal salt bridges have raised oxygen affinities. Much of this evidence has been reviewed by Baldwin (1975).

The current evidence against the salt bridges as the dominant source of cooperative energy may be summarized as follows: (1) The model-independent thermodynamic data for normal human hemoglobin are inconsistent with a dominant role of salt bridges in stabilizing the deoxy quaternary structure (Ackers, 1980), as discussed above. (2) Insensitivity of the dimer–tetramer association constant of deoxyhemoglobin to salt (Chu & Ackers, 1981; Thomas & Edelstein, 1973) argue against significant salt bridge stabilization of the deoxy structure. (3) In a study of dimer–tetramer assembly in 22 mutant and chemically modified hemoglobins, it was found that all alterations of amino acid residues in the $\alpha_1\beta_2$ intersubunit contact region lead to large alterations in energies of stability at the $\alpha_1\beta_2$ interface and large losses in energies of cooperativity (Pettigrew et al., 1981). In light of these findings, the results of Kilmartin and Perutz are understandable as arbitrary perturbations at the $\alpha_1\beta_2$ interface. Since other types of perturbation at this interface also lead to loss of cooperativity, the argument favoring salt bridges does not appear compelling. (4) The evidence from electrostatic calculations described in this paper argues against a dominant role of salt bridges as a major source of the cooperative energy.

The 12 groups identified as important for preferentially stabilizing the deoxy molecule (Perutz, 1978) are italicized in Tables III and IV. These groups provide a net stabilization of oxy tetramers relative to deoxy tetramers of 1–2 kcal/mol. The variety of changes occurring in the values of $\delta G_{i,el}$ on going from deoxy to oxy tetramers supports the original observation that the interactions of these groups in deoxyhemoglobin are appreciably altered in the liganded form (Perutz, 1970; Perutz & TenEyck, 1971), but the interactions unique to the liganded charge array in fact provide overall greater electrostatic stabilization.

The definition of the electrostatic free-energy contributions to assembly of hemoglobin tetramers from dimers is intended to shed light on the complex interplay of stabilizing forces of which electrostatic interactions account for only a part. The present results correctly model the pH dependence of the free energy of deoxy- and oxyhemoglobin dimer–tetramer assembly and rationalize their relative magnitudes with observed thermodynamic parameters of the assembly process. The value of the electrostatic model thus extends beyond its previous

applications to the analysis of the alkaline Bohr effect (Matthew et al., 1979b, 1981b), the prediction of histidine pK values which are independently verified by NMR measurement (Ho & Russu, 1978; Matthew et al., 1979a), and the analysis of the interactions of allosteric effectors (Matthew et al., 1981a,b).

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Structural Studies of Tobacco Mosaic Virus and Its Components by Laser Raman Spectroscopy†

S. R. Fish, K. A. Hartman,* G. J. Stubbs, and G. J. Thomas, Jr.*

ABSTRACT: Raman spectra have been recorded for oriented gels of tobacco mosaic virus, aqueous solutions of TMV protein in the form of capsid and disk, and protein-free TMV RNA. The protein-free RNA molecule contains about 80% ordered type-A structure, which is similar to other RNA molecules in solution. RNA in the virus particle does not exhibit the base stacking and pairing normally found in protein-free RNA but instead shows at least two distinct structures characterized by different phosphodiester group frequencies in the Raman spectrum. Approximately one out of three such linkages retains the type-A geometry characteristic of aqueous RNA, while two out of three appear to have a different geometry not encountered previously for RNA. The secondary structure of the TMV coat protein molecule is similar for the three aggregation states studied (virus, capsid, and disk) and consists of 40–50% α helix, 40–50% irregular structure, and 0–20% β sheet. Two of the three tryptophan residues per protein molecule reside in hydrophobic regions, and the third is in contact with water. The distribution of the four tyrosine residues per protein molecule among hydrogen-bonding states is the same for capsid and virus but different for the disk. The latter could be a consequence of the presence of Cl^- ions which may form hydrogen bonds with one or more *p*-hydroxyl groups of tyrosine residues.

The tobacco mosaic virus (TMV) particle, or virion, is a relatively simple system in which many copies of one kind of protein molecule are combined in a helical array with one strand of RNA to form a rod-shaped structure. TMV protein, which can be separated from the RNA, forms many different aggregates including a disk which is composed of 34 protein molecules and a rod-shaped capsid which has the same helical arrangement of protein subunits found in the virion. The disk and protein-free RNA interact to form the virion in vitro and also, it is believed, in vivo. The RNA, disk, capsid, and virion compose a system in which details of protein–protein and protein–RNA interactions may be investigated (Caspar, 1963; Holmes, 1980).

The TMV system has received considerable study by numerous techniques of biochemistry and biophysics, and recent reviews are available (Kaper, 1975; Holmes, 1980). In particular, X-ray diffraction studies have produced electron density maps at 4-Å resolution for the virion (Stubbs et al., 1977) and at 2.8-Å resolution for the disk (Bloomer et al., 1978). Molecular models derived from these maps show many details of the molecular structures and interactions.

Raman spectroscopy measures the scattering of light as modulated by the normal modes of vibration in molecules of the sample. The intensities and frequencies of the Raman lines can be related to the interactions and structures of the molecular subgroups involved and in favorable cases have been used to measure protein and nucleic acid secondary structures. These results have permitted the use of Raman spectroscopy for the study of structure of both RNA and DNA viruses and nucleoproteins (Thomas, 1978).

It was the goal of this work to obtain Raman spectra of TMV and its disk, capsid, and RNA and to interpret these in terms of protein and RNA structures and interactions. Two papers have recently appeared in which Raman spectra of TMV (Shie et al., 1978) and additionally some of its com-

† From the Department of Biochemistry and Biophysics, University of Rhode Island, Kingston, Rhode Island 02881 (S.R.F. and K.A.H.), Rosenstiel Basic Sciences Center, Brandeis University, Waltham, Massachusetts 02254 (G.J.S.), and Department of Chemistry, Southeastern Massachusetts University, North Dartmouth, Massachusetts 02747 (G.J.T.). *Received July 22, 1981.* Paper IX in the series Studies of Virus Structure by Laser Raman Spectroscopy. For paper VIII in this series, see Thomas & Day (1981). Supported in part by U.S. Public Health Service Grant AI 11855 (G.J.T.) and by the Department of Biophysics, University of Rhode Island (K.A.H.).

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