Electrostatic Effects in Hemoglobin: Electrostatic Energy Associated with Allosteric Transition and Effector Binding†

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

ABSTRACT: The pH dependence of the summed electrostatic stabilization for deoxy- and liganded hemoglobin was computed for several ionic strength values. The computed contribution to the stabilization of deoxyhemoglobin by binding of 2,3-diphosphoglycerate in the β cleft compared well with experimental binding behavior for human hemoglobin A_0 and hemoglobin F. The contribution of diphosphoglycerate binding to the alkaline Bohr effect was computed correctly for both hemoglobins A_0 and F. The computed effects of simultaneous binding of diphosphoglycerate and formation of Val-1β carbamino adducts suggested a competition between these effectors. A direct competition was formulated between these two effectors, with extension to include a simple anion such as chloride or bicarbonate binding in competition with diphosphoglycerate but not with Val-1β carbamino formation. This model was found to hold at pH 7.3–7.4 over a range of concentrations of the effectors involved and to predict the pH dependence of Val-1β carbamino formation over the pH range 7.0–8.0. The pH dependence of the computed differential stability of liganded vs. unliganded hemoglobin A compared well with observation.

The successful applications of the modified Tanford–Kirkwood discrete charge electrostatic theory for globular proteins (Shire et al., 1974a,b, 1975; Botelho et al., 1978; Matthew et al., 1979a,b), incorporating a solvent accessiblity parameter (Lee & Richards, 1971; Matthew et al., 1978a) for each protein binding site, are underlined by current reports of electrostatic controls in biological systems (Wojtczak & Nalecz, 1979; Jähnig et al., 1979; Wagner et al., 1979). The pressing questions in the protein field of motion and mobility, conformational specificity and folding, specific and general ion effects, and ligand effects including transduction of ligand interactions and cross interactions between ligands, are all intimately associated with electrostatic interactions (Gurd & Rothgeb, 1979). It has recently been demonstrated that the static protein charge array in sperm whale myoglobin can account for several kilocalories of stabilizing energy in the native structure (Friend & Gurd, 1979a,b). Moreover, the variations of these interactions with pH have been shown to parallel the observed pH dependence of the overall stability.

The heterotropic effectors of hemoglobin, like the majority of known effectors, produce changes in the state and distribution of charges on the protein. The treatment of electrostatic interactions involving hydrogen ions and chloride ions with human deoxyhemoglobin (Matthew et al., 1979a,b) is now extended to include the carbamino adducts and organic phosphate polyanions.[1] A treatment of human fetal hemoglobin is included to exemplify the extension to protein variants (Matthew et al., 1981).

The present calculations follow the previous work (Matthew et al., 1979a,b) in the inclusion of two chloride ion binding sites per deoxyhemoglobin dimer at an ionic strength of 0.10 M. The Val-1α...Arg-141α site is firmly established experimentally (Arnone et al., 1976; O'Donnell et al., 1978). The location of the additional chloride site (His 117β...Arg 30β) used in this and the previous work is less well established, and similar results are obtained if alternative locations are assumed (Matthew et al., 1979a,b, 1981).[2] This finding means that the alternative of more than two chloride ion sites that are partially occupied is approximately satisfied by the treatment. The computed electrostatic contributions at I = 0.10 M and pH 7.60 were seen to total 0.97 Bohr protons without differential Cl^- binding to deoxyhemoglobin, representing 50% of the observed maximum Bohr effect, and 1.88 Bohr protons when bound Cl^- was included at these two sites per deoxyhemoglobin dimer (Matthew et al., 1979a,b). At I = 0.01 M, Cl^- binding is much reduced and is not included in the computation, yielding a value of 1.68 Bohr protons, which is not far from the experimental value (Rollema et al., 1975).

An extensive study of the effects of Cl^- and H^+ concentration on Bohr protons substantiates the assignment of 50% of the alkaline Bohr effect (I = 0.10, pH 7.6) to differential Cl^- binding to deoxyhemoglobin, the remainder being assigned to Cl^--independent proton binding sites (Van Beek et al., 1979). These chloride ion interaction studies, when taken with recent proton NMR studies (Ho & Russu, 1979; Russu et al., 1980) and discrete charge calculations, point to a more complete interpretation of the stereocheimcal model for the alkaline Bohr effect as well as the pH and ionic strength dependence of quaternary and tertiary rearrangements.

While crystallographic structural results have been invaluable in the elucidation of functional details for the solution state, contrasting results may be encountered. A case in point is His-146β, which is observed to be involved in a salt bridge in the deoxyhemoglobin crystal structure (Perutz, 1970; Fermi, 1975) and untethered in liganded crystal forms (Perutz, 1970; Baldwin & Chothia, 1979). Proton NMR studies of the ligation and ionic strength dependence of the $pK_{1/2}$ for His-146β show that, under conditions approaching the physiological

† From the Department of Chemistry, Indiana University, Bloomington, Indiana 47405. *Received May 5, 1980.* This is the 122nd paper in a series dealing with coordination complexes and catalytic properties of proteins and related substances. For the preceding paper, see Wittebort et al. (1980). This work was supported by U.S. Public Health Service Research Grants HL-05556, GM 22778, and T01 GM-1046.

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

[1] Abbreviation used: P_2-glycerate, 2,3-diphosphoglycerate.

[2] The alkaline Bohr effect is accurately predicted if any of the following second Cl^- sites are assumed to be fully occupied, expressed in terms of equidistant placement between the positive sites: His-20α... His-112α; His-45α...Lys-90α; His-89α...Arg-92α; Lys-82β...Val-1β; or Lys-82β...His-2β...His-143β (Matthew et al., 1981). A distribution of occupancy of such sites amounting to 1 equiv of bound Cl^- naturally also fulfills the observations.

0006-2960/81/0420-0571$01.00/0 © 1981 American Chemical Society

rather than the crystallization conditions, the His-146β salt bridge is intact in both liganded and unliganded hemoglobin (Ho & Russu, 1979; Russu et al., 1980).

The above assumptions and limitations have allowed calculation of changes in effective pK values and site occupancy for individual proton-binding groups over a wide range of pH, including consideration of P_2-glycerate (Matthew et al., 1979a,b; Matthew, 1978). In the present report they are employed to extend the treatment of the interactions with effectors, especially in terms of changes in summed electrostatic free energy. The calculation of electrostatic free energies is particularly important in light of recent studies which designate proton reactions as the major source of enthalpic effects in hemoglobin ligation and self-assembly (Mills et al., 1979; Imai, 1979; Mills & Ackers, 1979).

Electrostatic Computations

Free energies of interaction of the ionizable groups have been calculated (Matthew, 1978; Matthew et al., 1978a,b), according to the procedures described in a previous paper (Matthew et al., 1979a) from atomic coordinates obtained from the Brookhaven Protein Data Bank (Bernstein et al., 1977). Theoretical hydrogen ion titration curves for individual sites were generated as already described (Matthew et al., 1979a). The treatment adjusts the characteristic pK_{int} for the ionizable group in question to the effective pK_i at a given pH by the inclusion of an electrostatic ΔpK_i term reflecting the sum of electrostatic free energy contributions, W'_{ij}, between the particular group i and all other groups j. W'_{ij} is equal to W_{ij}, computed from the Tanford–Kirkwood treatment (Tanford & Roxby, 1972), multiplied by the factor $(1 - SA_j)$ where SA is the fractional static solvent accessibility.[3] Computed pK_i values are sometimes expressed as p$K_{1/2}$, those values applying at the pH of half-titration. The charge borne on a given site, Z_i or Z_j, varies between −1 and 0 or 0 and +1 to allow for fractional saturation. Net sums of charges at individual sites are expressed as $\bar{Z}$, for example, in titration curve plots.

Extension to Human Deoxyhemoglobin F. For extension of the treatment to human deoxyhemoglobin F, the appropriate 39 amino acid substitutions into the human deoxyhemoglobin A_0 coordinate list were made by visual inspection of a structural model and by use of graphics projections. Estimated coordinates for substituted atoms were then checked for overlapping van der Waals radii and compatibility with the crystallographic results of Frier & Perutz (1977). While the differences involving only uncharged amino acid residues will affect the overall stability in several ways (Chothia et al., 1976; Friend, 1979), they only affect the electrostatic model if they alter the solvent accessibility of a charge site. In general, we neglect the possibility that the substitutions may induce more serious structural rearrangements (Frier & Perutz, 1977) and deal solely with the redistribution of the charge array. The β chain to γ chain proton binding site substitutions are: Val-1 → Gly-1; Pro-5 → Glu-5; Glu-7 → Asp-7; Asp-21 → Glu-21; Glu-22 → Asp-22; Glu-43 → Asp-43; Asp-47 → Asn-47; Asp-52 → Ser-52; Ala-76 → Lys-76; Asn-80 → Asp-80; Arg-104 → Lys-104; His-116 → Ile-116; Pro-125 → Glu-125; His-143 → Ser-143; Lys-144 → Arg-144. It should be pointed out that a substitution such as Glu to Asp, while conserving charge, usually entails changes in location and solvent accessibility involving the residue in question and often neighboring residues.

Table I: Estimated Coordinates for Charged Site Locations of Carbamino Adducts, Diphosphoglycerate, and Chloride Ions in the Deoxyhemoglobin β Cleft

	X	Y	Z	assigned pK_{int}
carbamino adducts	6.9	−20.0	1.2	5.0
	−6.9	−20.0	−1.2	5.0
diphosphoglycerate				
1-carboxyl	1.0	−20.0	0.0	3.00
2-phosphate	2.2	−20.0	−3.5	1.50
2-phosphate	3.5	−20.0	−2.2	4.50
3-phosphate	−2.2	−20.0	3.5	1.50
3-phosphate	−3.5	−20.0	2.2	4.50
chloride				
position 1	−4.7	−19.7	−2.8	1.0
	4.7	−19.7	2.8	1.0
position 2	−5.15	−20.1	6.65	1.0
	5.15	−20.1	−6.65	1.0

Following Friend & Gurd (1979a,b), the summation of all the computed electrostatic interactions felt by individual residues yields the overall electrostatic free energy contribution to the stability of the native structure, $\sum \Delta G''_{i,el}$, according to

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

where W''_{ij} is the electrostatic interaction energy for each pair of interacting sites, equivalent to $W_{ij}(1 - SA_j)(1 - SA_i)$.[4]

Simulation of Bound Carbamino and Diphosphoglycerate. The introduction of bound anions requires special consideration since the anions contribute to Coulombic influences and may represent in themselves sites from proton binding. Ambiguity can arise from uncertainty about the thermodynamics of anion binding and the pH dependence of the binding equilibrium, particularly so when the binding involves more than a single anchoring positive group.

P_2-glycerate is known to have one high affinity binding site in deoxyhemoglobin with an association constant of about 10^4 M^{-1} at pH 7.3 and 25 °C which is reduced 20- to 50-fold for oxygenated hemoglobin (Hedlund & Lovrien, 1974). The position of this high affinity site in deoxyhemoglobin has been verified on the dyad axis of symmetry between β chains by X-ray crystallography (Arnone, 1972). Placement of the five negative charges of P_2-glycerate in the coordinate list followed this crystallographic difference map (Table I). Since the binding of P_2-glycerate is dependent on the protonation state of its binding domain, it is possible to compare by calculation the proton binding behavior of deoxy tetramer with pH in the presence and absence of the P_2-glycerate. In essence this allows the pH dependence of the P_2-glycerate binding stability to be calculated. The pK_{int} values for free P_2-glycerate were assigned such that the calculations approximate its solution titration behavior.[5] An SA of 0.9 was used for each of the five charged groups. The sphere radius was varied from 14 to 27 Å with no appreciable effect upon the pK_i values. It was found that

[3] In computation of ΔpK_i 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, indirectly, the ΔpK_i term. For a discussion of an alternative formalism involving $\overline{SA}_{ij}$, see Matthew et al. (1981).

[4] The inherent symmetry of the relations is completely apparent for the pairwise value W''_{ij}. The factor $^1/_2$ in eq 1 corrects for double counting of the pairwise terms.

[5] P_2-glycerate has been determined by ^{31}P NMR to be essentially a pentavalent anion at physiological pH with p$K_{1/2}$ values of 1.2, 1.2, 6.45, and 6.85 for the four phosphate ionizations and 4.30 for the carboxyl moeity; epxerimental [P_2-glycerate] ≅ 0.10 M (J. B. Matthew, unpublished data). The calculated p$K_{1/2}$ values for free P_2-glycerate, I = 0.10 M, using the relative coordinates and pK_{int} values in Table I are 1.6, 1.6, 6.35, and 6.7 for the four phosphate ionizations and 3.9 for the carboxyl moiety.

Table II: Values of $pK_{1/2}$ for Various Sites in Deoxygenated Hemoglobin in the Presence and Absence of Carbamino Formation, Chloride Ion Binding, or Diphosphoglycerate Binding in the β Cleft

	adduct, I = 0.10 M					adduct, I = 0.01 M		
residue	Cl[a]	Cl[b]	Cl[c]	carbamino[d]	P_2-glycerate[d]	none	carbamino	P_2-glycerate
Val-1α	8.02	8.02	8.02	8.02	8.02	8.10	8.11	8.12
His-20α	6.76	6.76	6.76	6.76	6.77	6.87	6.88	6.88
His-45α	6.85	6.85	6.85	6.85	6.85	7.12	7.16	7.16
His-50α	7.45	7.45	7.45	7.48	7.48	7.84	7.87	7.88
His-72α	6.32	6.32	6.32	6.34	6.34	6.52	6.53	6.53
His-89α	7.21	7.21	7.21	7.21	7.21	7.48	7.51	7.49
His-112α	7.74	7.69	7.69	7.74	7.74	8.18	8.20	8.12
Val-1β	7.00	7.44	7.15		7.62	7.14		8.15
His-2β	6.53	6.60	6.90	6.84	6.84	6.53	7.09	7.08
His-77β	6.58	6.62	6.60	6.70	6.63	6.58	6.86	6.76
His-117β	8.20	7.75	7.75	7.75	7.75	8.08	8.11	8.11
His-143β	6.07	6.33	6.65	6.80	7.27	6.03	6.80	7.75
His-146β	8.48	8.48	8.52	8.52	8.57	9.00	9.15	9.26

[a] Chloride at Val-1α and His-117β sites. [b] Chloride at Val-1α and Lys-82β sites (position one). [c] Chloride at His-2β, Lys-82β, and His-143β sites (position two). [d] Chloride at Val-1α site only.

the calculated pK_i values respond to the protein charge array in the simulated binding.

The binding of both P_2-glycerate and CO_2 affects the computations through changes in solvent accessibility of protein sites as well as through the introduction of new members of the charge array. These two effectors are unique in that they mask their full effect on the hemoglobin proton binding sites by generating protons in their own binding reaction. The process of carbamino formation in the absence of organic phosphate has been shown to generate a substantial fraction of the protons, 25–50% depending on pH, for the alkaline Bohr proton absorbing groups (Matthew et al., 1977). The release of protons from P_2-glycerate on binding to deoxyhemoglobin is examined below.

The carbamino adduct, the covalent structure formed by the nucleophilic reaction of nonprotonated amine on free carbon dioxide, is observed to favor the deoxy quaternary state (Kilmartin & Rossi-Bernardi, 1973). Quantitative analysis of carbamino formation over the pH range 6.5–9.0 has shown that the effects of conversion from the deoxy to the liganded state occur predominately at the β-chain terminal amines (Morrow et al., 1976; Matthew et al., 1977; Matthew, 1978; Perrella et al., 1975). The computations were performed for both the presence and absence of the Val-1β carbamino adducts; a simple statistical distribution can be applied to conditions that produce partial occupancy (Matthew et al., 1977).[6] The coordinates chosen for the location of the negative carbamino sites (Table I) are in keeping with the crystallographic analyses of Arnone et al. (1980; Arnone, 1974) for the CO_2 adducts of hemoglobin A_0 and for the closely related, although uncharged, cyanate derivatives (O'Donnell et al., 1979). The experimental measurements of Val-1β carbamino formation were performed as previously described (Matthew et al., 1977; Matthew, 1978; Morrow et al., 1980).

Bound chloride ions are included in the computations for I = 0.10 but not below (Matthew et al., 1979b). In each such case the site near Val-1α is assumed to be occupied in each $\alpha\beta$ dimer. In some computations the site near His-117β in each $\alpha\beta$ dimer was assumed to be occupied also (Matthew et al., 1979b), whereas in other computations two alternative positions 1 and 2 were considered (Benesch et al., 1969; Arnone, 1972; Chiancone et al., 1975; Nigen & Manning, 1975; Wiechelman et al., 1978) in the β cleft midway between Lys-82β and Val-1β or midway between Lys-82β, His-2β and His-143β, respectively (Table I).[2] Each tetramer was thus usually assumed to bear four bound chloride ions.

Generation of Oxyhemoglobin Coordinates by Rigid Rotation. Following previous work (Matthew et al., 1979a,b), atomic coordinates for human oxyhemoglobin were generated by the rigid rotation of the deoxyhemoglobin α and β chains into the oxyhemoglobin quaternary structure. The inverse rotation matrix specified by the Eulerian angles and the inverse of the translation components for the oxy- to deoxyhemoglobin transformation were used (Muirhead et al., 1967; Cox, 1967; Matthew, 1978).

Results and Discussion

Electrostatic Effects of CO_2, Chloride, and P_2-Glycerate Binding on $pK_{1/2}$ Values in Deoxyhemoglobin. The sensitivity of the proton binding behavior of hemoglobin A_0 tetramer to pH, ionic strength, and quaternary state has been described (Matthew et al., 1979a,b). The effects of carbamino formation at Val-1β and P_2-glycerate binding in the deoxyhemoglobin β cleft are now explored. Table II illustrates the computed electrostatic effects on $pK_{1/2}$ values, for the 13 sites that titrate within the physiological pH range, caused by anion binding at several potential sites and the allosteric interactions of β chain carbamino formation and P_2-glycerate binding. The predicted $pK_{1/2}$ changes due to carbamino adduct formation should be viewed as partial influences because there exist the possibilities of synergistic effects and specific binding of bicarbonate or carbonate anions.

For I = 0.10 M the effects of including one or two fully occupied chloride ion sites are shown. The computed $pK_{1/2}$ values in the three columns in Table II dealing with different sets of chloride binding sites primarily differ for those groups within or neighboring the β-cleft region.

The results for carbamino and P_2-glycerate are striking in that computed perturbations induced by the negatively charged effectors are observed throughout the structure even at I = 0.10 M. The general increase in $pK_{1/2}$ values in the presence of carbamino formation, particularly for the β-chain residues illustrated, is generally amplified for the binding of the pentavalent anion, P_2-glycerate.[5] It is significant that the magnitudes of the calculated $pK_{1/2}$ changes are of the proper degree to account for an increased alkaline Bohr effect in the presence of P_2-glycerate (deBruin et al., 1971) and are intermediate

[6] Under conditions where the mole fraction of deoxy-β-chain carbamino derivative is 0.45, the probability of a tetramer having a single β-chain carbamino adduct in near 0.50 while the probability of binding two tetramer is 0.23.

Table III: Effect of Binding of Diphosphoglycerate to Deoxyhemoglobin A_o on Computed pK_i and Z_i Values of Various Proton Sites at pH 7.60[a]

	diphosphoglycerate, I = 0.10 M[b]					diphosphoglycerate, I = 0.01 M				
	absent		present			absent		present		
residue	pK_i	Z_i	pK_i	Z_i	ΔZ_i[c]	pK_i	Z_i	pK_i	Z_i	ΔZ_i[c]
Val-1α	8.01	0.72	8.01	0.72	0.00	8.02	0.73	8.04	0.73	0.00
His-20α	6.85	0.15	6.86	0.15	0.00	7.02	0.21	7.02	0.21	0.00
His-45α	6.87	0.16	6.89	0.16	0.00	7.19	0.28	7.23	0.30	0.02
His-50α	7.48	0.43	7.49	0.44	+0.01	7.80	0.61	7.84	0.63	0.02
His-72α	6.36	0.06	6.37	0.06	0.00	6.63	0.10	6.63	0.10	0.00
His-89α	7.18	0.30	7.23	0.30	0.00	7.51	0.45	7.52	0.46	0.01
His-112α	7.70	0.57	7.73	0.57	0.00	8.05	0.74	8.07	0.75	0.01
Val-1β	7.03	0.21	7.62	0.51	0.30	7.22	0.30	8.05	0.74	0.44
His-2β	6.68	0.11	6.96	0.19	0.08	6.82	0.14	7.22	0.29	0.15
His-77β	6.64	0.09	6.68	0.11	0.02	6.74	0.12	6.85	0.15	0.03
His-117β	8.19	0.80	8.20	0.80	0.00	8.03	0.73	8.06	0.74	0.01
His-143β	6.27	0.05	7.33	0.35	0.30	6.43	0.06	7.70	0.56	0.50
His-146β	8.43	0.88	8.52	0.89	0.01	8.86	0.95	9.01	0.96	0.01
Lys-82β	10.33	1.00	12.08	1.00	0.00	10.51	1.00	12.43	1.00	0.00
					0.72[a]					1.20

[a] At pH 7.6 and I = 0.10 M the proton release from P_2-glycerate on binding to hemoglobin is calculated to be only 0.06 per dimer (see Figure 1). [b] Chloride ions are assumed to occupy sites at Val-1α and His-117β. See text for effects of considering β cleft sites in place of the His-117β site. [c] Per $\alpha\beta$ dimer.

Table IV: Computed Protonation State of Diphosphoglycerate in the Free State and Bound to the Deoxyhemoglobin A_o Charge Array[a]

	pH 6.0				pH 7.0				pH 8.0			
	free		bound		free		bound		free		bound	
	pK_i	Z_i	pK_i	Z_i	pK_i	Z_i	pK_i	Z_i	pK_i	Z_i	pK_i	Z_i
1-carboxyl	4.13	−0.99	3.58	−0.99	4.58	−0.99	3.97	−1.00	4.81	−0.99	4.31	−1.00
2-phosphate	2.48	−1.00	2.05	−1.00	3.19	−1.00	2.64	−1.00	3.58	−1.00	3.05	−1.00
	6.62	−0.20	5.81	−0.61	6.69	−0.67	5.99	−0.91	6.72	−0.95	6.32	−0.98
3-phosphate	2.46	−1.00	2.05	−1.00	3.16	−1.00	2.52	−1.00	3.43	−1.00	2.89	−1.00
	6.32	−0.32	5.52	−0.75	6.39	−0.80	5.72	−0.95	6.44	−0.97	6.05	−0.99
		−3.50		−4.35		−4.47		−4.86		−4.91		−4.97
		(−3.36)[b]				(−4.36)[b]				(−4.91)[b]		

[a] Ionic strength = 0.10 M. [b] Values in parentheses are the net charge on P_2-glycerate at the respective pH as derived from the ^{31}P NMR $pK_{1/2}$ values (see footnote 5).

in magnitude to observed $pK_{1/2}$ changes in the presence of inositol hexaphosphate. In the presence of the polyanion inositol hexaphosphate the $pK_{1/2}$ value of His-2β in deoxyhemoglobin has been observed to increase by 0.80 pK unit while the His-146β residue was little affected (Ohe & Kajita, 1977). Table II indicates the calculated $\Delta pK_{1/2}$ values for P_2-glycerate binding as 0.31 for His-2β and 0.05–0.09 for His-146β at 0.10 M ionic strength. The calculated effect of P_2-glycerate on the $pK_{1/2}$ value of Val-1β at ionic strength 0.10 M would decrease the concentration of nonprotonated amine and reduce carbamino formation by half at pH 7.0 (Matthew et al., 1977) without invoking the additional considerations of steric crowding and electrostatic repulsion. Simultaneous binding (Perrella et al., 1975) and mutually exclusive binding (Benesch & Benesch, 1967) models have been suggested for the competition between carbamino formation and P_2-glycerate binding in the β cleft. Neither model has proved to be completely satisfactory. A scheme that includes elements of both models is described below.

The increased alkaline Bohr effect attributable to P_2-glycerate binding can be computed at a given pH by using the increased hemoglobin pK_i values calculated in the presence of bound P_2-glycerate and the calculated proton release by P_2-glycerate sites. Table III shows the appropriate effective pK_i values at pH 7.6, I = 0.10 and 0.01 M, and the theoretical changes in bound protons, ΔZ_i, by deoxyhemoglobin. The calculated proton uptake by the protein residues requires correction for P_2-glycerate proton release. Table IV tabulates the computed effective pK_i values and proton occupancy at three pH values for the five ionizable sites of P_2-glycerate for the bound and free conditions. It is clear that the last two phosphate ionizations are most relevant in the 6.0–8.0 pH range. The computed net charge for unbound P_2-glycerate compares well with the values derived from experimental $pK_{1/2}$ values, shown in parentheses. These computations predict that the correction for proton release from P_2-glycerate sites ranges from 0.85 proton at pH 6.0 to 0.06 proton at pH 8.0.

The experimental values of deBruin et al. (1971, 1973) for the P_2-glycerate-induced hydrogen ion uptake by deoxyhemoglobin as a function of pH are compared in Figure 1 with the computed values. Curve 1 represents the computed values of proton uptake per deoxyhemoglobin tetramer when P_2-glycerate is placed in an empty β cleft, while curve 2 is calculated assuming P_2-glycerate displaces two chloride ions (Table I, position 2) from the deoxyhemoglobin β cleft. When a β-cleft chloride site is considered to be occupied prior to P_2-glycerate binding, the calculated effect is to reduce the computed proton uptake, as several cleft proton sites have elevated proton affinities due to the chloride which are amplified by the pentavalent anion (see Table II).

The experimental points in Figure 1 are bracketed by the calculations for empty and fully occupied β-cleft chloride sites, which leads to the conclusion that a 70–80% occupancy of these chloride sites satisfies the observed proton uptake as well

Table V: Effect of Binding of Diphosphoglycerate to Deoxyhemoglobin F on Computed pK_i and Z_i Values of Various Groups at pH 7.60

	diphosphoglycerate, I = 0.10 M[a]					diphosphoglycerate, I = 0.01 M				
	absent		present			absent		present		
residue	pK_i	Z_i	pK_i	Z_i	ΔZ_i[b]	pK_i	Z_i	pK_i	Z_i	ΔZ_i[b]
Val-1α	8.01	0.72	8.01	0.72	0.00	8.01	0.72	8.04	0.73	0.01
His-20α	6.86	0.15	6.86	0.15	0.00	7.02	0.21	7.03	0.21	0.00
His-45α	6.88	0.16	6.88	0.16	0.00	7.20	0.28	7.23	0.30	0.02
His-50α	7.54	0.46	7.54	0.47	0.01	7.88	0.66	7.92	0.68	0.02
His-72α	6.46	0.07	6.46	0.07	0.00	6.73	0.12	6.73	0.02	0.00
His-89α	7.22	0.29	7.22	0.29	0.00	7.48	0.43	7.50	0.44	0.01
His-112α	7.74	0.58	7.74	0.58	0.00	8.06	0.74	8.09	0.76	0.02
Gly-1γ	7.08	0.23	7.68	0.54	0.31	7.29	0.33	8.17	0.79	0.46
His-2γ	6.71	0.11	7.01	0.20	0.09	6.86	0.15	7.31	0.34	0.19
His-77γ	6.62	0.09	6.62	0.10	0.01	6.71	0.11	6.84	0.15	0.04
His-117γ	8.18	0.79	8.19	0.79	0.00	8.00	0.72	8.03	0.73	0.01
Ser-143γ		0.00		0.00	0.00		0.00		0.00	0.00
His-146γ	8.47	0.88	8.54	0.90	0.02	8.88	0.95	9.06	0.97	0.02
Lys-82γ	10.37	1.00	12.20	1.00	0.00	10.57	1.00	12.66	1.00	0.00
					0.44					0.80

[a] Chloride ions are assumed to occupy sites at Val-1α and His-117γ. [b] Per αγ dimer.

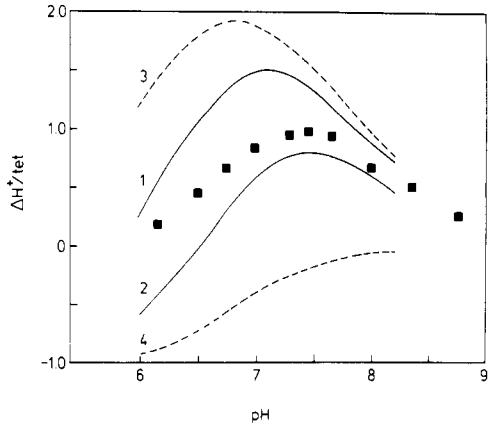


FIGURE 1: The proton uptake per deoxyhemoglobin tetramer induced by P_2-glycerate binding as a function of pH. The experimental points (■) are those of deBruin et al. (1973) with 0.3 mM deoxyhemoglobin tetramer and 0.4 mM P_2-glycerate in 0.10 M KCl at 25 °C. The solid curves are the computed proton uptake with P_2-glycerate binding when a pair of β-cleft chloride binding sites (Table I, position 2) are assumed to be unoccupied (curve 1) or fully occupied (curve 2). The overall computed hydrogen ion uptake in the absence of specifically bound β-cleft chloride (curve 1) is further resolved into the dashed curves: proton uptake (curve 3) and P_2-glycerate hydrogen ion release (curve 4).

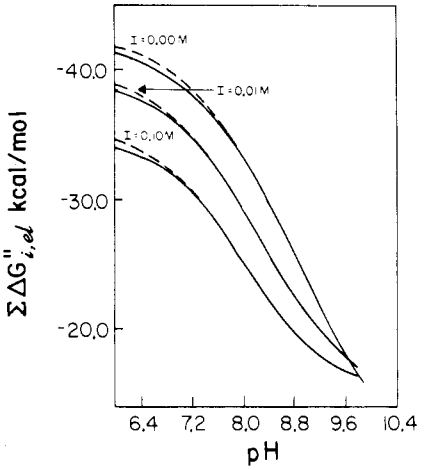


FIGURE 2: Summed electrostatic free energy of interaction between charged sites, $\sum\Delta G''_{i,el}$, expressed in kcal/mol as a function of pH for human hemoglobin A_0 as solid curves, and human hemoglobin F as broken curves, at the indicated ionic strength values.

as fitting the existing estimates of β-cleft chloride association constants [see Tables V and VI and Figure 4, as well as Van Beek et al. (1979) and Guesnon et al. (1979)].

Experiments on $^{35}Cl^-$ binding in the presence and absence of inositol hexaphosphate support the idea of partial occupancy of multiple Cl^- binding sites (Chiancone et al., 1975). Figure 1 also shows the resolution of proton uptake by deoxyhemoglobin in the presence of P_2-glycerate (curve 1) into two contributions: the deoxyhemoglobin proton uptake (curve 3) due to increased side-chain pK_i values (Table III) and the release of protons from P_2-glycerate on binding (curve 4) due to suppression of the phosphate pK_i values (Table IV).

Table V shows the corresponding computations for the effects of P_2-glycerate on pK_i and Z_i values and the resulting ΔZ_i values for tetrameric human deoxyhemoglobin F, summed to the α,γ dimer, at I = 0.10 and 0.01 M. The most striking difference from the results in Table III for deoxyhemoglobin A_0 reflects the substitution of Ser-143γ for His-143β.

Electrostatic Free Energy of Deoxyhemoglobin and the Effect of Diphosphoglycerate Binding. The solid curves in Figure 2 show the summed electrostatic free energy of stabilization of deoxyhemoglobin A_0 at ionic strength values of 0, 0.01, and 0.10 M. Here $\sum\Delta G''_{i,el}$ (expressed in kilocalories/mole) is plotted as ordinate against pH. Over the range of pH the free energy resulting from the full constellation of charge point interactions is negative, indicating substantial stabilization, as is found from similar computations applied to myoglobin (Friend & Gurd, 1979a). The dependence on ionic strength is marked, showing that the native state is destabilized as the electrostatic interactions are lessened with increasing ionic strength. The greatest electrostatic stabilization is computed to occur well below the isoionic point, as is true with myoglobin (Friend & Gurd, 1979a). The basis for this effect is explored in detail in the following paper (Friend et al., 1981). The decrease in stabilization with increasing pH reflects especially the loss of charge pairs between carboxyl groups and histidine residues that become discharged.

The broken curves in Figure 2 show the corresponding computations for the deoxyhemoglobin F. As described under Electrostatic Computations, the γ chains of hemoglobin F differ in 39 positions from β chains of hemoglobin α_0, of which 15 involve charge site substitutions. Eight of these amino acid substitutions conserve the charge nature of the specific site, while the others effect a new placement of two negative sites

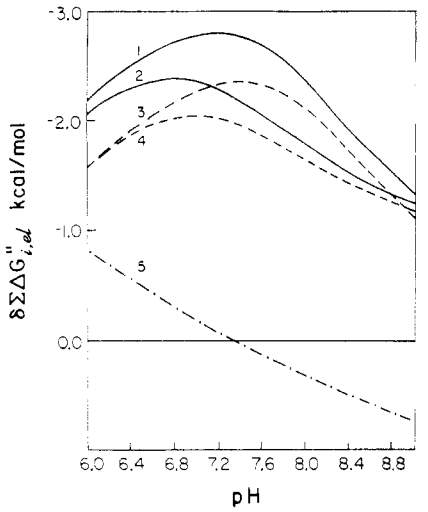


FIGURE 3: Additional electrostatic stabilization, $\delta\sum\Delta G''_{i,el}$, in kcal/mol, produced by the binding of P_2-glycerate to deoxyhemoglobin A_0, curves 1 and 2 at $I = 0.01$ and 0.10 M, respectively, and to deoxyhemoglobin F, curves 3 and 4 at $I = 0.01$ and 0.10 M, respectively, at 25 °C. The corresponding stabilization, passing to destabilization at higher pH, with P_2-glycerate binding in the presence of carbamino adducts to both Val-1β sites is shown in curve 5 for $I = 0.10$ M.

and the addition of one positively charged site and one negatively charged site, net charge being conserved. As reflected in Figure 2, the substitution of Ser-143γ for His-143β produces a net difference in charge-site occupancy below the region of pH 7. The computed overall electrostatic stabilization of the fetal protein is essentially identical above pH 7.0 with that of the adult form. The close but coincidental match between the curves for deoxyhemoglobin A_0 and deoxyhemoglobin F in Figure 2 reflects only the common form of the pH dependence of the electrostatic interactions and does not reflect the different stabilizing and destabilizing interactions resulting from the numerous substitutions between these protein, nor are any differences in effects on dissociation of the tetrameric species and in the nature of denaturated stated taken into account (Friend et al., 1981).

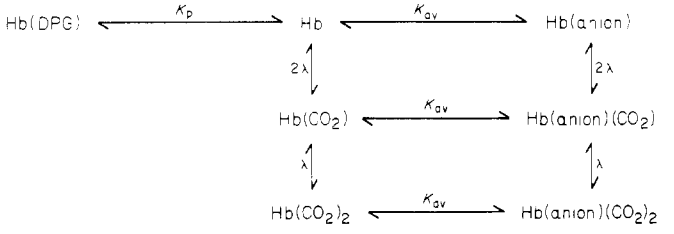
Figure 3 shows the additional electrostatic stabilization produced by binding of P_2-glycerate to deoxyhemoglobin A_0 (curves 1 and 2) and deoxyhemoglobin F (curves 3 and 4), (in kilocalories per mole) as a function of pH, at $I = 0.01$ and 0.10 M, respectively, at 25 °C. Curves 1 and 3, at $I = 0.01$ M, indicate stabilization from P_2-glycerate binding at pH 6 to be 2.2 and 1.6 kcal/mol, respectively, and at pH 9 to be 1.4 and 1.1 kcal/mol. The maxima, which occur close to pH 7.3 ± 0.1, are 2.8 and 2.3 kcal/mol, respectively, for hemoglobins A_0 and F. The experimentally determined free energy of interaction between oxygen binding sites is increased by 5.1 kcal/mol on P_2-glycerate binding at pH 7.4 and 25 °C to deoxyhemoglobin A_0 and by 2.7 kcal/mol on binding to deoxyhemoglobin F (Tyuma et al., 1973; Hedlund & Lovrien, 1974). While the experimental values fall somewhat above those presented in curves 1 and 3, the trends are comparable. Stabilizing charge pairs are brought together by conformational changes attending binding of P_2-glycerate to the deoxyhemoglobin A_0 structure (Arnone, 1972). Since such structural alterations are not included in the computations, it is not surprising that the stabilizaton with respect to deoxyhemoglobin A_0 is underestimated. Futhermore, other effects apart from electrostatics may contribute to the stabilization.

Effects of β-Chain Carbamino Formation on Diphosphoglycerate Binding Energy. Since P_2-glycerate and CO_2 both preferentially bind to the β chain NH_2-terminal site in the deoxy tetramer, it is to be expected that they will experience competitive binding at that locus. At pH 7.4 the extent of carbamino adduct formation at the β-chain terminus is significantly reduced in the presence of P_2-glycerate (Morrow et al., 1976; Matthew et al., 1977; Perrella et al., 1975).

Calculation of the electrostatic free energy of stabilization from P_2-glycerate binding in the presence of β-chain carbamino adducts allows an estimation of the implications of simultaneous binding, as shown in Figure 3, curve 5. The placement of the negative charge borne by the carbamate derivative was made as described in the section on simulation of bound carbamino and P_2-glycerate.

It is clear that perturbation of the P_2-glycerate binding domain by the introduction of the negatively charged carbamino adducts in place of the protonated NH_2-terminal valine virtually eliminates the stabilization of P_2-glycerate. Conversely, the presence of P_2-glycerate will decrease the concentration of the nonprotonated amine form of the β-chain terminal with which CO_2 reacts and may also decrease the CO_2–amine association constant by steric crowding or by electrostatically enhancing the carbamino off-rate (Gurd et al., 1980).

A Scheme for P_2-Glycerate–CO_2 Competition at the β Cleft. Several other lines of evidence point to a likely scheme for the interaction of deoxyhemoglobin A_0 with CO_2 and P_2-glycerate, as outlined in Figure 4. These include (1) the absence of perturbation of the β-chain carbamino ^{13}C nuclear magnetic resonance position in the presence of P_2-glycerate (Matthew et al., 1977); (2) the effect of NaCl at 0.1 M in mimicking



$$Z_\beta = \frac{\lambda[CO_2] + 2\lambda^2[CO_2]^2 + K_{av}\lambda[CO_2][anion] + 2K_{av}\lambda^2[anion][CO_2]^2}{1 + K_p[DPG] + K_{av}[anion] + 2\lambda[CO_2] + 2\lambda^2[CO_2]^2 + 2K_{av}\lambda[CO_2][anion] + 2K_{av}\lambda^2[anion][CO_2]^2}$$

$$[anion] = [Cl^-] + [HCO_3^-]\,;\lambda = [RNHCO_2^-]/[RNH_2][CO_2]\,;K_{av} = [Hb\text{-}anion]/[Hb][anion]$$

FIGURE 4: Scheme for mutually exclusive binding of P_2-glycerate and CO_2 to the β-cleft region of the deoxyhemoglobin tetramer. The vertical arrows represent the formation and decomposition of carbamino derivatives written as $Hb(CO_2)$ and $Hb(CO_2)_2$ to denote occupancy of one and both Val-1β sites. The combined anion population, e.g., HCO_3^- and Cl^-, is assumed to compete with P_2-glycerate for the β cleft site but much less with the carbamino formation. Indeed, the effect of anion binding on the pH-dependent carbamino formation constants λ is not distinguished because λ is determined in the presence of the anion. The anion binding is treated in terms of one anion site per P_2-glycerate binding site.

Table VI: Model Assuming Mutual Exclusion of Diphosphoglycerate Binding and Val-1β Carbamino Formation, with Small Anion Competition with Diphosphoglycerate Only[a]

	case					
	1	2	3	4	5	6
P_2-glycerate (mM)	5.0	5.0	5.0	7.0	9.0–10.0	9.0–10.0
hemoglobin (mM)	2.5	2.5	2.5	3.5	3.1	3.1
pCO_2 (torr)	45	72	100	140	45	72
bicarbonate (mM)	32	50	70	100	32	50
pH	7.40	7.35	7.38	7.35	7.35	7.40
Z_β	0.19	0.28	0.40	0.50	0.10	0.16
K_{av}	35	25	25	25	30	25

[a] The fraction, Z_β, of Val-1β as carbamino derivative was measured in the presence of various concentrations of P_2-glycerate with varying levels of competing anion (Figure 4) made up in all cases with 50 mM NaCl in addition to the bicarbonate concentrations shown. Chloride and bicarbonate were treated interchangeably in computing the association constant with a single site, K_{av}. Values of λ were taken from Matthew et al. (1977) and of K_p by interpolation and extrapolation of the results of Hedlund & Lovrien (1974).

Table VII: Parameters Employed to Compute pH Dependence of Z_β According to the Scheme of Figure 4

	concentrations				
pH	CO_2 (mM)	anion (mM)	λ ($M^{-1} \times 10^{-3}$)	K_p ($M^{-1} \times 10^{-3}$)	K_{av} (M^{-1})
7.0	5.90[a]	97.5[b]	0.126[c]	15.0*[d]	30
7.1	4.70	98.7	0.176	12.4	30
7.2	3.73	99.9	0.241	10.5	30
7.3	2.96	101	0.327	7.5*	30
7.4	2.34	102	0.435	5.0	30
7.5	1.86	102	0.579	4.0	30
7.6	1.48	103	0.761	2.9*	30
7.7	1.17	103	0.993	1.9	30
7.8	0.93	104	1.28	1.2	30
7.9	0.74	104	1.68	0.8	30
8.0	0.58	104	2.13	0.5	30

[a] Concentration of free CO_2 required to achieve the pH value at total carbonates of 55 mM. [b] Total anion concentration made up of 50 mM NaCl and the bicarbonate concentration at the pH. [c] $\lambda = (K_z Kc)/[K_z(H^+) + (H^+)^2]$, where $K_z = 1.23 \times 10^{-7}$ and $K_c = 2.3 \times 10^{-5}$ (Matthew et al., 1977). [d] Starred K_p values are those of Hedlund & Lovrien (1974). The remaining values are extrapolated and correlate with the calculated pH-dependent binding energy for deoxyhemoglobin and P_2-glycerate (Figure 3).

the P_2-glyerate action on deoxyhemoglobin A_0 and reducing the effect of added P_2-glycerate (Benesch et al., 1969; Tyuma et al., 1973); (3) the electrostatically unfavorable interactions produced by simultaneous occupancy of the β cleft by P_2-glycerate and carbamino adducts (Figure 3). Accordingly Figure 4 describes the competition in terms of the mutually exclusive binding of P_2-glycerate and carbamino formation in the β cleft of deoxyhemoglobin, with interference from competing anions such as Cl^- and HCO_3^- with respect to P_2-glycerate binding. This description is particularly attractive in view of the crystallographic evidence for a small anion binding site in the β cleft near the dyad axis (Fermi, 1975; Arnone et al., 1980). The formulation in Figure 4 is made in terms of stepwise formation of the carbamino derivatives, $Hb(CO_2)$ and $Hb(CO_2)_2$, the pH-dependent formation constant, λ, a formation constant for anion binding, K_{av}, and the formation constant for binding of P_2-glycerate, K_p.

Table V presents the results of several determinations by ^{13}C NMR of carbamino adduct formation at Val-1β at relatable conditions of pH and P_2-glycerate concentration (Matthew et al., 1977; Matthew, 1978). The mole fraction of Val-1β site converted to the carbamino derivative is expressed as Z_β. K_{av}, Figure 4, expresses the association constant for small anion binding in the β cleft which is taken to be compatible with carbamino derivative formation but not with the binding of P_2-glycerate. The values of K_{av} in each case represent those that predict the observed values of Z_β when entered with the variables and constants in the long expression in Figure 4. Comparisons of cases 1, 2, 3, and 4 or 5 and 6 demonstrate the enhancement of Z_β with increasing pCO_2 and total carbonates in the presence of P_2-glycerate, while a comparison of cases 1 and 5 or 2 and 6 shows the effect of increased P_2-glycerate concentration on Z_β. In all cases, the data are consistent with an anion site association constant, K_{av}, of about 30. Deoxyhemoglobin chloride binding constants have been estimated (Van Beek et al., 1979) as varying from 10 to 650 with decreasing pH, with a value of 40 at pH 7.4, agreeing remarkably well with our analysis of K_{av} (Table VI). For comparison, estimates of the oxyhemoglobin Cl^- binding constant at pH 7.4 range from 1 to 10 depending on the number of assumed sites (Van Beek et al., 1979) and increases to 30 by pH 6.0. Besides fitting with the Cl^- association constant values of Chiancone et al. (1975) and Haire & Hedlund (1975) and other evidence for binding in this region of the molecule (Nigen & Manning, 1975), these results correspond in magnitude with careful measurements reported recently by Guesnon et al. (1979) on the binding of Cl^- and lactate.

The low value of K_{av} (Table VI) corresponds to less than stoichiometric occupancy of the assumed chloride binding site (Figure 4). To reach a total of approximately 4 bound chloride ions per deoxyhemoglobin tetramer, partial occupancy of more than two sites per dimer is therefore required. As was shown in Table II, similar electrostatic consequences can follow from various alternative placements of chloride ions.[2] As will be shown in the following paper (Friend et al., 1981), the summed electrostatic free-energy contributions from chloride ion binding are comparable for occupancy of the different sites considered here. Such similarities in interactions among charge sites suggest similar free energies of binding which in turn go with low and comparable values of the binding constant for the broader set of partially occupied sites. Because of the partial occupancy reflecting the weakness of the binding, the simple choice of a single small anion binding site was made for the computation of K_{av} (Figure 4). If a pair of symmetrically placed chloride sites are considered in the β cleft (a pair for position 1 or a pair for position 2; Table I), the value of K_{av} would be approximately one-half that shown in Table VI. The foregoing considerations underscore the difficulty of recognizing the precise distributions of site locations and occupancies either from solution or from crystallographic studies.

The parameters required to compute the dependence of fractional β-chain carbamino formation, Z_β, as a function of pH according to the scheme of Figure 4 are listed in Table VII for conditions encompassing those for which experimental values of Z_β have been measured (Matthew et al., 1977; Matthew, 1978). The experimental results for Z_β as a function of pH between 7.0 and 8.0 are compared in Figure 5 with computed values. The curves represent computed values allowing for different concentrations of free P_2-glycerate to correspond roughly with the three sets of conditions in which 2.5 mM deoxyhemoglobin was equilibrated with the following concentrations of P_2-glycerate: 0.0, 5.0, and 9.0 mM. The computed and observed values correspond well within experimental error. Note that for the computations in Tables VI and VII and Figure 5 the experimental pH dependence of λ and of K_p were taken into account and the electrostatic charge site interaction treatment was not applied, in contrast with

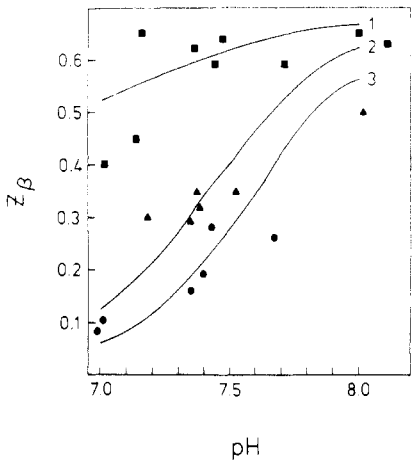


FIGURE 5: The mole fraction converted to the Val-1β carbamino derivative, Z_β, as a function of pH. Experimental Z_β values for deoxyhemoglobin in the absence and the presence of 5 and 9 mM of P_2-glycerate are represented as ■, ▲, and ●, respectively. Computed curves for Z_β under comparable conditions with free P_2-glycerate concentrations of 0.3 and 7 mM are marked 1, 2, and 3, respectively.

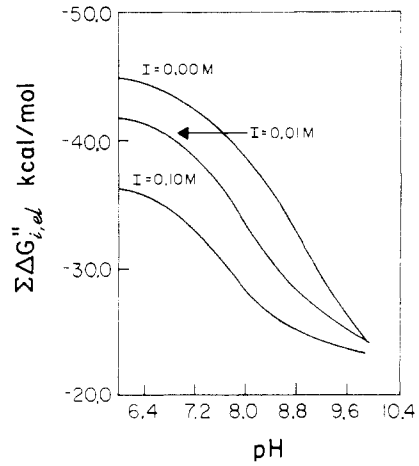


FIGURE 6: Summed electrostatic free energy of interaction between charged sites, $\sum\Delta G''_{i,el}$, expressed in kcal/mol as a function of pH for the oxyhemoglobin structure derived by rigid rotation from the deoxyhemoglobin A_0 structure, for ionic strengths as shown.

other computations reported here.

Electrostatic Free Energy of Oxyhemoglobin Charge Configuration. Figure 6 shows the results of the electrostatic free energy calculation for the rigid rotation structure of oxyhemoglobin A_0 at ionic strengths of 0, 0.01, and 0.10 M over the pH range 6–10. Here, as in deoxyhemoglobin (Figure 2), the free energy resulting from the full constellation of charge-point interactions indicates substantial stabilization over the pH range of interest. Over the pH range shown computations have been made as if tetramer to dimer dissociation were negligible, and pH-dependent conformational transitions were not taken into account, although such assumptions become critical at the extremes of pH. For this reason comparisons are limited to the pH range between 7.0 and 9.0.

The additional electrostatic free energy of stabilization of oxy- over deoxyhemoglobin, $\delta\sum\Delta G''_{i,el}$, as a function of pH is shown in Figure 7 for I = 0.10 M in the presence (curve 1) and absence (curve 2) of P_2-glycerate bound to deoxyhemoglobin. The summed electrostatic free energy terms for the two tetrameric configurations are large (see Figures 2 and 6), so that their differences carry considerable uncertainty. The introduction of P_2-glycerate (Figure 7, curve 1) substantially decreases the computed electrostatic free energy difference. Furthermore, as would be expected, the computed

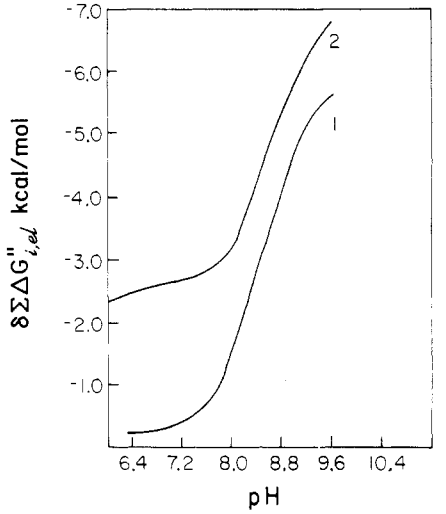


FIGURE 7: Additional electrostatic free energy of stabilization of oxy- over deoxyhemoglobin as a function of pH at I = 0.10 M in the presence (curve 1) and absence (curve 2) or P_2-glycerate. The left-hand ordinate is $\delta\sum\Delta G''_{i,el}$, obtained by subtracting from $\sum\Delta G''_{i,el}$ for oxyhemoglobin either $\sum\Delta G''_{i,el}$ for deoxyhemoglobin alone (curve 2) or $\sum\Delta G''_{i,el}$ for deoxyhemoglobin bearing the bound P_2-glycerate (curve 1).

differences between the deoxy- and oxyhemoglobin charge configurations increase with pH. The pH dependence shown in Figure 7, curve 2, reflects that of the interconversion parameter, L, and follows roughly the observed pH dependence of $-\log P_m$, where P_m is the median oxygen equilibrium pressure (Imai & Yonetani, 1975).

The electrostatic contribution to the relative stabilization of the liganded form would be lessened somewhat on the basis of a preliminary analysis of the electrostatic relationships in the carbomonoxyhemoglobin structure of Baldwin (J. M. Baldwin, Brookhaven Protein Data Bank; Baldwin & Chothia, 1979; M. A. Flanagan, personal communication), although qualitatively the pattern is not substantially altered. By comparison with the results obtained through the rigid rotation and translation operation used here, the new coordinates also reflect several tertiary rearrangements that have the effect of relieving unfavorable electrostatic interactions introduced in our generated oxyhemoglobin structure. The relationship between these rearrangements and incipient transitions of dimer dissociation which are driven by acid (Atha & Riggs, 1976) and by high salt concentration (Kirshner & Tanford, 1964; Ackers et al., 1975; Farmer, 1979) is unknown. A fuller comparison of the experimental carbonmonoxyhemoglobin structure with the rigid rotation structure is in preparation.

Conclusion

The results presented here illustrate the value of considering the role of effectors in terms of their influence on overall electrostatic stability and how they are sensed throughout the molecule. These differences in free energy can be directly related to individual site contributions. The trends of the results in Figure 3 for stabilization of deoxyhemoglobins A_0 and F by the binding of P_2-glycerate fit with observations of the free energy of linkage of O_2 binding (Tyuma et al., 1973; Hedlund & Lovrien, 1974), except that for the reasons noted above the stabilization of deoxyhemoglobin A_0 is understated. Likewise, the increment in proton binding to deoxyhemoglobin resulting from shifts in pK_i values on binding of P_2-glycerate is correctly computed (Tables IV and V, Figure 1) in terms of effects at a number of sites. Both the computed pH and ionic strength dependence of the effector interactions corre-

spond with experimental observations in several instances presented here and earlier (Matthew et al., 1979a,b).

The most complex interplay analyzed is the self-consistent treatment of interactions in the β-cleft in terms of the binding of protons and of P_2-glycerate, carbamino formation at Val-1β, and the binding of small anions such as chloride and bicarbonate. The results are foreshadowed in Figure 2 and Tables II–V and are shown in Figure 5 and Tables VI and VII. Because of the size of its binding site, P_2-glycerate finds itself in competition with both carbamino formation and the binding of small anions. As illustrated by Figure 3, curve 5, the binding of P_2-glycerate and the formation of the Val-1β carbamino adduct compete for different protonation states of the NH_2-terminal sites, and so the competition between them is strongly dependent on pH.

The treatment of the electrostatic interactions, with or without considering effectors, takes into account partial occupancy of binding sites (e.g., Tables III–V). The situations dealt with in Tables II–V and Figure 3, curves 1–4, and Figure 7, curve 2, assume complete occupancy of the β-cleft P_2-glycerate site, and that in Figure 3, curve 5, assumed full occupancy with respect to both P_2-gylcerate and Val-1β carbamino adducts. In any actual equilibrium situation with respect to deoxyhemoglobin, partial occupancy of sites will be the rule, and it is gradations in these several site occupancies with variations in concentrations of the effectors that will determine their influence on the properties of the protein. Such influences are expressed partly through the changes in proton distribution among sites (Tables III–V), as described also for the interaction of azide ion with ferrimyoglobin (Friend et al., 1980).

The present treatment deals with certain examples of effector interactions, omitting the established Val-1α carbamino site whose occupancy appears to vary little with ligand state (Matthew el al., 1977). The following paper (Friend et al., 1981) deals with the electrostatic consequences of bringing together α and β subunits to form the deoxyhemoglobin tetramer and complements the present analysis of effector interactions. An extension of these methods to various hemoglobin mutants, as well as ferrihemoglobin, is reported elsewhere (Matthew et al., 1981).

Acknowledgments

The kind advice and assistance of M. A. Flanagan is acknowledged with thanks. Drs. A. Arnone, J. M. Baldwin, M. Farmer, and B. A. Foster very kindly provided information in advance of publication. The advice and support of Professor F. M. Richards are gratefully acknowledged. Thanks are due to Mrs. D. Embry for help in the preparation of the manuscript.

References

Ackers, G. K., Johnson, M. L., Mills, F. C., Halvarson, H. R., & Shapiro, S. (1975) *Biochemistry 14*, 5128.

Arnone, A. (1972) *Nature (London) 237*, 146.

Arnone, A. (1974) *Nature (London) 247*, 143.

Arnone, A., O'Donnell, S., & Schuster, T. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 3*, 1604.

Arnone, A., Rogers, P. H., & Briley, P. D. (1980) in *Biophysics and Physiology of Carbon Dioxide* (Bauer, C., Gros, G., & Bartels, H., Eds.) Springer, Berlin, Heidelberg, and New York.

Atha, D. H., & Riggs, A. (1976) *J. Biol. Chem. 251*, 5537.

Baldwin, J. M., & Chothia, C. (1979) *J. Mol. Biol. 129*, 175.

Benesch, R., & Benesch, R. E. (1967) *Biochem. Biophys. Res. Commun. 26*, 162.

Benesch, R. E., Benesch, R., & Yu, C. I. (1969) *Biochemistry 8*, 2567.

Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Jr., Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T., & Tasumi, M. (1977) *J. Mol. Biol. 112*, 535.

Botelho, L. H., Friend, S. H., Matthew, J. B., Lehman, L. D., Hanania, G. I. H., & Gurd, F. R. N. (1978) *Biochemistry 17*, 5197.

Chiancone, E., Norne, J. E., Forsen, S., Bonaventura, J., Brunori, M., Antonini, E., & Wyman, J. (1975) *Eur. J. Biochem. 55*, 385.

Chothia, C., Wodak, S., & Janin, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3793.

Cox, J. (1967) *J. Mol. Biol. 28*, 151.

deBruin, S. H., Janssen, L. H. M., & Van Os, G. A. J. (1971) *Biochem. Biophys. Res. Commun. 45*, 544.

deBruin, S. H., Janssen, L. H. M., and Van Os, G. A. J. (1973) *Biochem. Biophys. Res. Commun. 55*, 197.

Farmer, M. (1979) Ph.D. Thesis, Duke University.

Fermi, G. (1975) *J. Mol. Biol. 97*, 237.

Foster, B. A. (1978) Ph.D. Thesis, University of Iowa.

Friend, S. H. (1979) Ph.D. Thesis, Indiana University.

Friend, S. H., & Gurd, F. R. N. (1979a) *Biochemistry 18*, 2612.

Friend, S. H., & Gurd, F. R. N. (1979b) *Biochemistry 18*, 2620.

Friend, S. H., March, K. L., Hanania, G. I. H., & Gurd, F. R. N. (1980) *Biochemistry 19*, 3039.

Friend, S. H., Matthew, J. B., & Gurd, F. R. N. (1981) *Biochemistry* (following paper in this issue).

Frier, J. A., & Perutz, M. F. (1977) *J. Mol. Biol. 112*, 97.

Guesnon, P., Poyart, C., Bursaux, E., & Bohn, B. (1979) *Respir. Physiol. 38*, 115.

Gurd, F. R. N., & Rothgeb, T. M. (1979) *Adv. Protein Chem. 33*, 73.

Gurd, F. R. N., Matthew, J. B., Wittebort, R. J., Morrow, J. S., & Friend, S. H. (1980) in *Biophysics and Physiology of Carbon Dioxide* (Bauer, C., Gros, G., & Bartels, H., Eds.) p 89, Springer, Berlin, Heidelberg, and New York.

Haire, R. N., & Hedlund, B. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4135.

Hedlund, B. E., & Lovrien, R. (1975) *Biochem. Biophys. Res. Commun. 61*, 859.

Ho, C., & Russu, I. M. (1979) in *Clinical and Biochemical Aspects of Hemoglobin Abnormalities* (Caughey, W. S., Ed.) p 179, Academic Press, New York.

Imai, K. (1979) *J. Mol. Biol. 133*, 233.

Imai, K., & Yonetani, T. (1975) *J. Biol. Chem. 250*, 2227.

Jahnig, F., Harlos, K., Vogel, H., & Eibl, H. (1979) *Biochemistry 18*, 1459.

Kilmartin, J. V., & Rossi-Bernardi, L. (1973) *Physiol. Rev. 53*, 836.

Kirschner, A. G., & Tanford, C. (1964) *Biochemistry 3*, 291.

Lee, B., & Richards, F. M. (1971) *J. Mol. Biol. 55*, 379.

Matthew, J. B. (1978) Ph.D. Thesis, Indiana University.

Matthew, J. B., Morrow, J. S., Wittebort, R. J., & Gurd, F. R. N. (1977) *J. Biol. Chem. 252*, 2234.

Matthew, J. B., Hanania, G. I. H., & Gurd, F. R. N. (1978a) *Biochem. Biophys. Res. Commun. 81*, 410.

Matthew, J. B., Friend, S. H., Botelho, L. H., Lehman, L. D., Hanania, G. I. H., & Gurd, F. R. N. (1978b) *Biochem. Biophys. Res. Commun. 81*, 416.

Matthew, J. B., Hanania, G. I. H., & Gurd, F. R. N. (1979a) *Biochemistry 18*, 1919.

Matthew, J. B., Hanania, G. I. H., & Gurd, F. R. N. (1979b) *Biochemistry 18*, 1928.
Matthew, J. B., Friend, S. H., & Gurd, F. R. N. (1981) in *Interaction Between Iron and Proteins in Oxygen and Electron Transport* (Ho, C., Eaton, W. A., Collman, J. P., Gibson, Q. H., Leigh, J. S., Jr., Margoliash, E., Moffat, J. K., & Scheidt, W. R., Eds.) Elsevier–North Holland, New York (in press).
Mills, F. C., & Ackers, G. K. (1979) *J. Biol. Chem. 254*, 2281.
Mills, F. C., Ackers, G. K., Gaud, H. T., & Gill, S. J. (1979) *J. Biol. Chem. 254*, 2875.
Morrow, J. S., Matthew, J. B., Wittebort, R. J., & Gurd, F. R. N. (1976) *J. Biol. Chem. 251*, 477.
Morrow, J. S., Matthew, J. B., & Gurd, F. R. N. (1980) *Methods Enzymol.* (in press).
Muirhead, H., Cox, J. M., Mazzarella, L., & Perutz, M. F. (1967) *J. Mol. Biol. 28*, 117.
Nigen, A. M., & Manning, J. M. (1975) *J. Biol. Chem. 250*, 8248.
O'Donnell, S., Mandaro, R., & Schuster, T. (1978) *Biophys. J. 21*, 201a.
O'Donnell, S., Mandaro, R., Schuster, T. M., & Arnone, A. (1979) *J. Biol. Chem. 254*, 12204.
Ohe, M., & Kajita, A. (1977) *J. Biochem. (Tokyo) 82*, 839.
Perrella, M., Kilmartin, J. V., Fogg, J., & Rossi-Bernardi, L. (1975) *Nature (London) 256*, 759.
Perutz, M. F. (1970) *Nature (London) 228*, 726.
Rollema, H. S., deBruin, S. H., Janssen, L. H. M., & Van Os, G. H. J. (1975) *J. Biol. Chem. 250*, 1333.
Russu, I. M., Ho, N. T., & Ho, C. (1980) *Biochemistry 19*, 1043.
Shire, S. J., Hanania, G. I. H., & Gurd, F. R. N. (1974a) *Biochemistry 13*, 2967.
Shire, S. J., Hanania, G. I. H., & Gurd, F. R. N. (1974b) *Biochemistry 13*, 2974.
Shire, S. J., Hanania, G. I. H., & Gurd, F. R. N. (1975) *Biochemistry 14*, 1352.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, Chapter 8, Wiley, New York.
Tanford, C., & Roxby, R. (1972) *Biochemistry 11*, 2192.
Tyuma, I., Imai, K., & Shimizu, K. (1973) *Biochemistry 12*, 1491.
Van Beek, G. G. M., Zuiderweg, E. R. P., & deBruin, S. H. (1979) *Eur. J. Biochem. 99*, 379.
Wagner, G. A., Kalb, J., & Wüthrich, K. (1979) *Eur. J. Biochem. 95*, 249.
Wiechelman, K. J., Fox, J., McCurdy, P. R., & Ho, C. (1978) *Biochemistry 17*, 791.
Wittebort, R. J., Szabo, A., & Gurd, F. R. N. (1980) *J. Am. Chem. Soc. 102*, 5723.
Wojtczak, L., & Nalecz, M. J. (1979) *Eur. J. Biochem. 94*, 99.

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 © 1981 American Chemical Society