Energetics of Heme-Protein Interactions in Hemoglobin

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Abstract: Models for the energetics of cooperativity in hemoglobin are subjected to quantitative calculation. Force field calculations indicate that the strain mechanism is unlikely to account for the entire 3.6-kcal/mol heme-heme interaction energy, $\Delta\bar{G}$. Exploring the alternative of storing $\Delta\bar{G}$ electrostatically, we find that the electrostatic potential of the protein at the porphyrin changes drastically upon the t → r transition and that this change stabilizes the transfer of positive charge to certain regions of the heme expected to occur upon binding of an electronegative sixth ligand. The changes in the porphyrin charge distribution upon ligand binding are evaluated by correlating observed shifts of "oxidation state marker" resonance Raman lines with calculated shifts. The interaction between the charges on the porphyrin and the protein electrostatic potential is calculated to contribute about 1 kcal/mol to $\Delta\bar{G}$, of which a significant fraction is associated with the interaction between the vinyl side chain of ring III of the porphyrin and the protein dipoles, a finding supported by experiment. It is proposed that the r state of hemoglobin stabilizes the charges of the heme-ligand system more than the t state, while the t state stabilizes the intersubunit salt bridges more than the r. Qualitative comparison of strain and electrostatic contributions to $\Delta\bar{G}$ for binding O_2 and CO suggests that electrostatic effects are much larger for O_2.

(I) Introduction

Understanding the cooperativity exhibited by the binding of oxygen to hemoglobin is a fundamental problem in molecular biology. Many studies (for reviews see ref 1-3) have examined the heme-heme interaction, the increase in binding energy of the fourth oxygen relative to the first. Perutz and co-workers (see ref 1-3) compared the structures of the high and low oxygen affinity states of hemoglobin. They demonstrated that the low-affinity state is stabilized by salt bridges between subunits and suggested that in that state the protein pulls the iron away from the heme plane and thereby reduces the oxygen-binding energy. Experiments have not found evidence for significant strain in the heme group.[4,5] This has been rationalized by a qualitative argument that the strain energy should be distributed around the protein rather than localized on the heme.[6] A recent theoretical study[7] gave the reason for the heme geometry change upon ligand binding and showed quantitatively that little of the heme-heme interaction energy, $\Delta\bar{G}$, could be stored in heme distortions. This study also estimated the potential relating the protein energy in the r and t states to the distance from the proximal histidine to the heme plane, assuming some form of the strain model is correct.

Theoretical interpretation[7] of the available experimental evidence indicates that in the absence of a sixth ligand there is little or no strain in the low affinity form of hemoglobin; this has been confirmed by resonance Raman (RR) studies.[8] It is reasonable to assume that binding of a sixth ligand to the low-affinity form of hemoglobin is associated with storage of a major fraction of $\Delta\bar{G}$ as strain energy in the globin. However, there is no quantitative experimental estimate of this fraction, and recent RR experiments[9] which showed that the Fe-O_2 stretching frequency changes by less than 2 cm^{-1} upon transition of the globin from the low- to high-affinity form have indicated that the strain contribution to $\Delta\bar{G}$ might be smaller than previously assumed. Thus, it is important to estimate the strain contribution theoretically. This paper uses force field calculations to obtain the first quantitative estimate of an upper limit for the strain energy distributed around the protein.

There is a recent accumulation of cases of which energy is stored electrostatically in biological systems.[11-13] This form of energy has already been associated with the salt-bridge interactions in hemoglobin.[2,10] The calculations described in this work suggest that the change in the heme charges upon binding of a ligand is stabilized more by the high-affinity state of the protein than by the low-affinity state. Therefore, we propose that part of the cooperativity in hemoglobin is due to competition between electrostatic stabilization of the charge distribution of the hemes and electrostatic stabilization of the salt bridges.

(II) How the Heme-Heme Interaction Energy is Stored

The hemoglobin molecule has two quaternary structures, R (relaxed, high ligand affinity) and T (tense, low affinity). The tertiary structures of the subunits in these forms are designated here as r and t, with superscripts (d) and (o) to denote the unbound (deoxy) and bound (oxy) sixth ligand states.

The hemoglobin molecule is so designed that intersubunit interactions stabilize the t conformation and binding a ligand stabilizes the r. Figure 1 illustrates the energetics of two subunits of hemoglobin as functions of the least energy coordinates for changing the tertiary structures from t to r in each subunit. Competition between the energy of binding a ligand to a subunit and the energy holding the subunits together leads to cooperativity. The difference between the oxygen affinities of the last oxygen bound and the first is $\Delta\bar{G}$, which is also approximately the difference in the oxygen-binding energies of the r and t conformations. Figure 1 provides a phenomenological description of the energy balance that leads to cooperativity but does not tell us how $\Delta\bar{G}$ is related to the conformational change.

(A) Storage of $\Delta\bar{G}$ as Strain Energy. The strain hypothesis is described schematically in Figure 5 of ref 7 and in Figure 2 in this paper. According to this hypothesis, most of $\Delta\bar{G}$ is stored in the $t^{(0)}$ protein as a result of motion of the proximal histidine toward the heme upon binding of a ligand.[2,3,7,14,15] A proper calculation of the strain contribution to $\Delta\bar{G}$ requires starting from an energy-minimized $t_1^{(d)}$ reference subunit, in the presence of fixed interfaces with the other subunits and the solvent and then simulating the $t_1^{(0)}$ system by forcing the iron toward the heme plane and reminimizing the energy of the system with respect to all the coordinates of the reference subunit. The strain-energy contribution will be given by the difference between the minimum energies of the $t_1^{(0)}$ and $t_1^{(d)}$ systems. At present, unfortunately,

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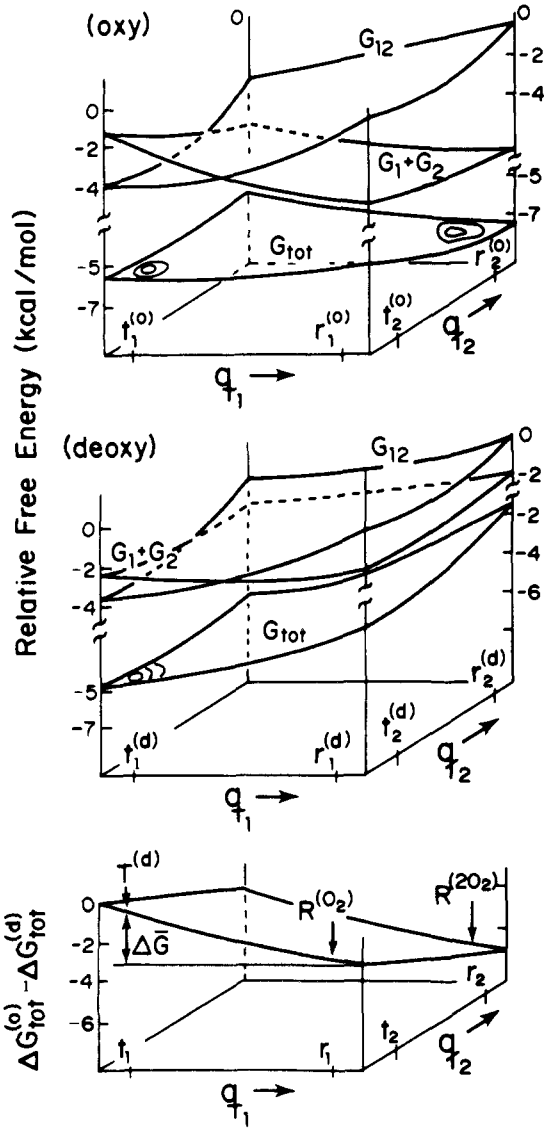

Figure 1. Schematic description of the energetics of cooperativity in a hypothetical two subunit system as a function of two effective coordinates q_i that describe the least energy paths for changes in tertiary structure from r to t in each subunit. G_{12}, G_i, and G_{tot} are respectively the subunit interaction energy, the internal energy of the ith subunit, and the sum of $G_{12} + G_1 + G_2$. The figure illustrates how a balance between the changes in G_{12} and $G_1 + G_{12}$ leads to conformational changes from t_1t_2 to r_1r_2 upon binding of two oxygen molecules. The cooperativity of the system can be understood by considering the oxygen binding energy $G_{tot}^{(o)} - G_{tot}^{(d)}$ (lowest figure). In the t_1t_2 configuration the binding energy is $\Delta\bar{G}$ less than in the r_1r_2 configuration.

such calculations are not expected to provide reliable results.

It is possible, however, to obtain an accurate estimate of the *upper limit* of the strain contribution. Instead of minimizing the energy of the reference subunit with respect to all its coordinates we fix most of this subunit, allowing only the proximal histidine and a few attached residues (Figure 3) to accommodate the motion of the iron toward the heme plane. Obviously the energy stored by these motions is larger than the energy stored when more coordinates are allowed to relax; the calculations thus give an upper limit for the strain-energy contribution. Since the calculations involve only a limited number of residues, they are expected to be more reliable than those involving the entire subunit. The present calculations evaluate the strain energy that is stored in the t state of the protein when only parts of residues F7 to FG3 are allowed to accommodate the changes observed in the N_ϵ-heme plane and C_ϵ–N_1 and C_δ–N_3 distances upon the transition from deoxy to aquomet-Hb in the α subunit of horse hemoglobin. The geometry change was evaluated by the procedure described in

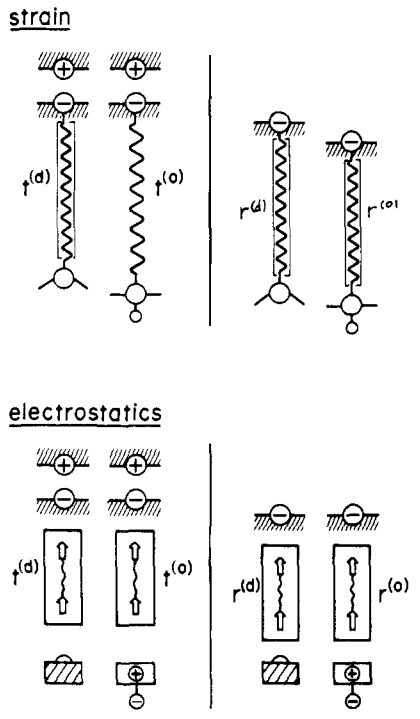

Figure 2. A schematic comparison of the strain and electrostatic mechanisms in storing $\Delta\bar{G}$. The salt bridges and the heme are represented respectively in the upper and lower part of each diagram. The upper diagrams depict storage of strain energy in the springlike bonds, bond angles, and van der Waals interactions of the protein (see also Figure 5 of ref 7). The lower diagrams represent storage of electrostatic energy in dipole–multipole interactions between the protein and liganded heme, where the stabilization of the heme$^{\delta+}$ charge by the protein dipoles competes with the stabilization of the salt bridges.

Figure 3. This change does not correspond to the least energy t → r transition and does not alter the position of any surface group that interacts with neighboring subunits. The calculated increase in protein energy as a result of the deformation is only 2 kcal/mol. If more groups, in particular the heme, were allowed to relax, the amount of strain would be smaller.

It is difficult to rationalize the storage of a large amount of strain energy in a protein as a result of atomic motions of less than 0.5 Å in a prosthetic group or substrate.[11] Only better knowledge of the heme geometry change upon ligation and convergent energy calculations[16] involving the complete protein can give a conclusive estimate of the contribution of strain to $\Delta\bar{G}$.

It is possible that the t state of the protein accommodates the heme–O_2 system without storing significant strain, but only at the expense of restricting the freedom of motion of the proximal histidine or some other part of the system. In this case, an entropic effect might contribute to $\Delta\bar{G}$.

(B) Storage of $\Delta\bar{G}$ as Electrostatic Energy. Recent studies have pointed out the importance of electrostatic interactions in controlling the energetics of biological processes.[11,13,17] Electrostatic forces vary much more slowly with distance than do steric forces and therefore cannot be relaxed by small atomic movements. Figure 2 gives a schematic description of an allosteric system controlled by changes in electrostatic energy. In order for such a mechanism to operate in hemoglobin the charge distribution at the active site must change significantly (a few hundredths of an electron on some atoms) upon ligand binding. Evidence for such changes is provided by the following experiments. (i) the RR spectra of porphyrins contain an "oxidation state marker" line[18] which is shifted from 1360 to ~1375 cm^{-1} upon oxidation.

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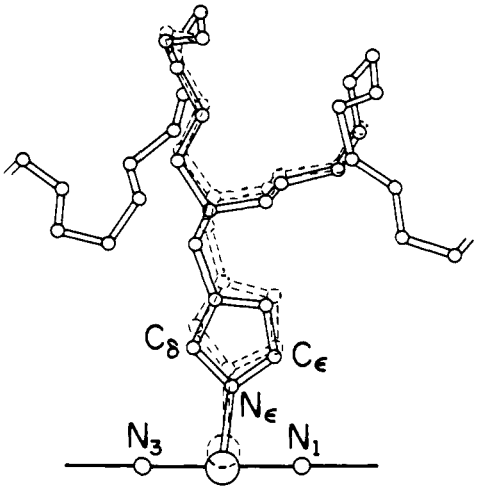

Figure 3. Estimating the *upper limit* for the contribution strain energy to $\Delta\bar{G}$. The figure describes the calculated geometry changes of the F7, F8, F9, and FG1 residues that accommodate the observed change in the distance between the N_ϵ, C_ϵ and C_δ of the histidine and the heme nitrogens upon transition from deoxy to met-Hb. These geometry changes were obtained as follows. (i) Starting from the X-ray coordinates[32] of the α chain of horse deoxy-Hb, we constrained the distance between N_ϵ and the plane of the heme nitrogen to 2.6 Å and minimized the energy of the protein with respect to the positions of the atoms of the F6-FG3 residues by using the protein energy functions of ref 11 and convergent minimization.[16] The results of these calculations are represented by the dashed lines. (ii) Starting from these energy-minimized deoxy coordinates, we constrained the position of N_ϵ to 2.2 Å from the plane of the heme nitrogens and the C_ϵ–N_1 and C_δ–N_3 distances to 3.18 and 3.39 Å, respectively, to reproduce the observed distances in the α chain of met-Hb. The total energy of the α subunit was minimized, under the above constraint, with respect to the positions of the F7, F8, F9, and FG3 side chains and the main chain atoms between F7 and FG3. All other atoms, including the heme, were kept fixed. The results of this calculation are described by the straight lines. The geometry change shown in the figure preserves the ligand–heme and intersubunit interactions, yet stores only 2-kcal/mol strain energy in the protein, which is far from completely relaxed.

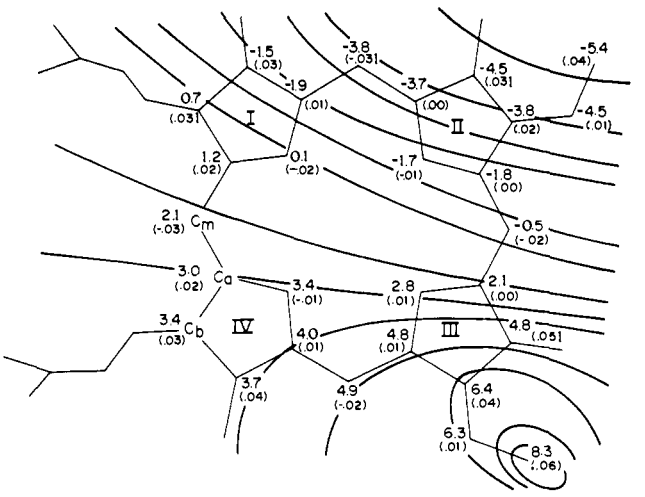

Figure 4. The changes in the electrostatic potential of the protein at the heme π-atom locations upon the r → t transition and the calculated changes in the heme charges upon binding of oxgyen (in brackets). The potential is defined as the energy in kcal/mol of a positive test charge. The contribution of a given set of atomic charges to $\Delta\bar{G}$ is given approximately by eq 2. Thus, for example, a unit positive charge on the terminal carbon of the rings on ring III will contribute 8.3 kcal/mol to $\Delta\bar{G}$. The calculation of the protein electrostatic potential is as described in the text and Table I. The changes in the porphyrin charges upon oxygen binding were evaluated assuming $\Delta n_{\pi^*} = -1$ and $\Delta n_\pi = 0.7$ (see text). The change in iron charge was considered negligible.[26] The calculation of the change in charges includes evaluation of the polarization of the heme electrons by the potential of the protein (which was included in the π-electron Hamiltonian by using eq 4 of ref 11). The deoxy porphyrin charges were evaluated by using the t state potential and the oxy charges by using the r state potential. The figure also presents a contour representation of the change in electrostatic potential at the atomic locations upon the r → t transition.

This shift results from significant loss of electron density from the π^* antibonding orbital of the porphyrin (almost a full electron as will be demonstrated below). The oxidation state marker line is in similar positions in oxy-Hb and the oxidized hemes of aquomethemoglobin and ferric cytochrome *c*.[18] This indicates that the porphyrin π^* orbital loses a significant amount of electron density in oxy-Hb relative to deoxy.[19] A similar conclusion also applies to carbonmonoxyhemoglobin.[20] (ii) The stretching frequency of the bound oxygen is 1107 cm^{-1},[21] much lower than that of O_2 (1566 cm^{-1}) and close to the stretching frequency of O_2^- (1145 cm^{-1}). This indicates significant charge transfer to the π^* orbital of the oxygen. However, the amount of negative charge on the O_2 ligand is not known since the bond to the iron might also involve electron transfer from the oxygen lone pair.

If the π^* orbital of the porphyrin loses significant electron density upon binding of oxygen to the heme, then electrostatic stabilization by the protein of positive charge on the porphyrin will increase the oxygen-binding energy. Such stabilization would contribute to cooperativity if it were stronger in the r state than in the t state.

In order to evaluate the contribution of electrostatic interactions to $\Delta\bar{G}$, we calculated the electrostatic stabilization of the heme–O_2 system by the protein charge distributions of the r and t states. The changes in the heme charges upon binding of oxygen were evaluated as follows. Using the QCFF/PI method[22,23] modified to include iron orbitals[7] and our method for RR calculations,[24] we calculated the dependence of the frequency of the oxidation state marker normal mode of the porphyrin on the occupancies of the $\pi^*(E_g)$ and $\pi(A_{2u})$ orbitals of the porphyrin. We obtained an approximately bilinear correlation

$$\Delta\nu = -14\Delta n_{\pi^*} + \Delta n_\pi \qquad (1)$$

where $\Delta\nu$ is the calculated frequency shift of the oxidation state marker (in cm^{-1}) and Δn_{π^*} and Δn_π are the changes in occupancies of the π^* and π orbitals.[25] Correcting the observed shift upon binding of oxygen, 19 cm^{-1}, for the ~−4-cm^{-1} shift due to contraction of the heme core upon the deoxy → oxy transition[24] gives $\Delta\nu \simeq 15$ cm^{-1}. This frequency shift, eq 1, and a reasonable estimate of a net transfer of 0.3 electron from the porphyrin to the oxygen[26] give $\Delta n_{\pi^*} = -1.00$ and $\Delta n_\pi = 0.70$. These Δn and the relation $\Delta\rho_i = \Delta n_{\pi^*}(c_{\pi^*,i})^2 + \Delta n_\pi(c_{\pi,i})^2$ (where c_{π^*} and c_π are the π^* and π molecular orbital vectors, respectively) give the changes in charges $\Delta\rho_i$ on the porphyrin atoms shown in Figure 4. Different assumptions give different charges but all involve very significant electron transfer from the π^* states and shift of positive charge to the C_b and the vinyl carbons.

(18) Spiro, T. G.; Strekas, T. C. *J. Am. Chem. Soc.* **1974**, *96*, 338–345.
(19) According to the detailed calculations of ref 24, the effect of motion of the iron toward the heme plane upon binding of oxygen to deoxy heme contributes only 4 cm^{-1} to the 19-cm^{-1} shift of the 1360-cm^{-1} oxidation state markers. The rest of the shift is due to change in the heme π-electron bond orders.
(20) Kitagawa, T.; Ozaki, Y.; Kyogoku, Y. *Adv. Biophys.* **1978**, *11*, 153–196.
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(24) Warshel, A. *Annu. Rev. Biophys. Bioeng.* **1977**, *6*, 273–300.
(25) This relation can be verified by using the rough approximation $\Delta\nu_s/\nu_s \simeq [\sum_l \Delta P_l L_l^s / \sum_l P_l L_l^s]^{1/2}$ where l runs over the porphyrin bonds, L_l^s is the lth bond stretching component of the sth normal mode, and P_l is the bond order of the lth bond, given by $P_l = \sum_m n_m c_{m,i} c_{m,j}$, where n_m is the number of electrons in the mth molecular orbital and the lth bond connects the i and j atoms.
(26) Zerner, M.; Gouterman, M.; Kobayashi, H. *Theor. Chim. Acta* **1966**, *6*, 363–400.

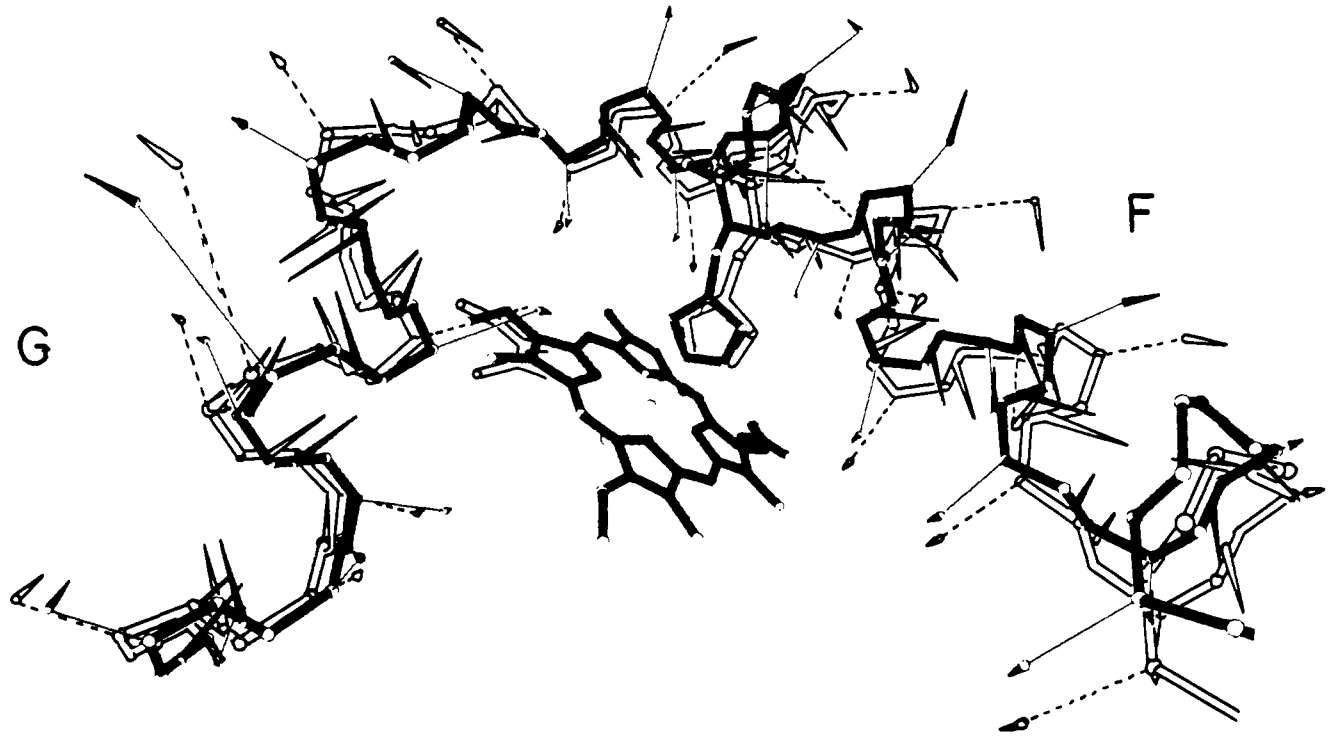

Figure 5. The change in relationship of some dipoles with the heme upon the t → r transition. The chains of the r and t states in the F-G region are drawn as ━ and ═, respectively. The ring III vinyl is on the left.

The electric potential from the protein at the porphyrin atoms was evaluated by using the atomic point charges of ref 11. The change in electrostatic potential between the r and t states of the protein is given in Figure 4. An estimate of the electrostatic contribution to $\Delta\bar{G}$ is given by eq 2, where ΔV_i and Δq_i are

$$\Delta\bar{G}_e = \sum_i \Delta V_i \Delta q_i \qquad (2)$$

respectively the change in potential on the ith atom upon the r → t transition and the change in charge of the ith atom upon ligand binding. This relation and the charges of Figure 4 give $\Delta\bar{G}_e$ = 1.1 kcal/mol. The conribution of the oxygen to $\Delta\bar{G}_e$ was not included since it is subject to a large error due to the uncertainty in the charge on the oxygen and the position of the distal histidine (see below). Taking the dielectric effect of the protein into account by the method of ref 11 increases the calculated $\Delta\bar{G}_e$ to 1.13; a larger increase is obtained when more positive charge is transferred to the porphyrin. The largest contributions of the protein groups to $\Delta\bar{G}_e$ are summarized in Table I. The table shows that most of the contributions come from the main-chain dipoles, whose positions are probably more accurate than those of the side chains. Taking only the main chain into account gives a larger $\Delta\bar{G}$.

As illustrated in Table I and Figure 5, the change in folding of the protein upon the t → r transition pushes the heme closer to protein dipoles that stabilize positive charge on the porphyrin ring (see also Figure 6). The largest contribution to this stabilization effect comes from the interactions between the vinyl side chain of ring III and the neighboring protein dipoles, particularly those of the peptide bond connecting residues 93 and 94. The vinyl gains a significant amount of positive charge in the heme$^{\delta+}$ system, as can be rationalized by considering the C_b=C—CH_2^+ valence bond configuration. This charge interacts much more strongly with the protein electrostatic potential (Figure 4) in the r state than in the t state and contributes significantly to $\Delta\bar{G}$. Experimental support for this will be given in the next section.

The significance of the calculations depends on the sensitivity of the results to the atomic charges and coordinates used to represent the r and t subunits. We tested the former in two ways. (i) Using the atomic charges of ref 33 decreased the calculated $\Delta\bar{G}_e$ by 15%. (ii) The surface ionizable groups, which were treated as neutral in the above calculations, were given unit charges. This had a large effect on the potentials in the r and t states but surprisingly little effect on ΔV_i and $\Delta\bar{G}_e$. The validity of using the structures of horse deoxy- and aquomethemoglobin to represent the r and t state was examined by also using the structures of human deoxy- and carbonmonoxyhemoglobin. Although much of the potential difference on the heme periphery disappeared, the positive peak on the ring III vinyl of the heme remained. This peak also persists in calculations on the β subunits. A possible deficiency in the use of the observed structures is that they represent the $t^{(d)}$ and $r^{(o)}$ structures but not the $r^{(d)}$ and $t^{(o)}$ structures.[7] We particularly expect errors resulting from this approximation

Table I. Some Contributions to $\Delta\bar{G}$ from the Electrostatic Interaction Energies[a] (kcal/mol) between the Heme and the Protein groups[b]

residue	heme[c]–residue[d]	heme–main chain[e]	vinyl III[f]–residue
Leu F4	−0.45	−0.41	−0.01
Val G3	0.37	0.40	0.09
Asn G4	1.07	0.94	0.29
Ser G9	−0.40	−0.10	−0.04
Val H15	0.41	0.41	0.02
Leu H19	0.67	0.65	0.06

[a] The calculated electrostatic portion of $(E_t(o) - E_t(d)) - (E_r(o) - E_r(d))$: thus an entry is the contribution of the specified group to $\Delta\bar{G}_e$. [b] The coordinates of the r and t states are taken as those of the α chains of horse aquomet- and deoxyhemoglobin in the Brookhaven protein data file.[32] The protein charges are those of ref 11 except that the imidazole ring of residue F8 (the proximal histidine), which is connected electronically to the heme system, and residue E7 (the distal histidine), which probably moves in the $t^{(o)}$ structure relative to the $t^{(d)}$ structure (a bound water molecule in $t^{(d)}$ is displaced by the bound ligand in $t^{(o)}$) were left uncharged. The changes in the heme charges are given in Figure 4. [c] Includes charges of the π system of the porphyrin. The oxygen charge was included only in the calculation of the interaction with the induced dipoles of the protein since it interacts primarily with the distal histidine (see above). [d] Includes all atoms of the specified residue. [e] Includes $NHC_\alpha HCO$ of the specified residue. [f] Includes the two carbon atoms of the vinyl moiety on ring III of the heme.

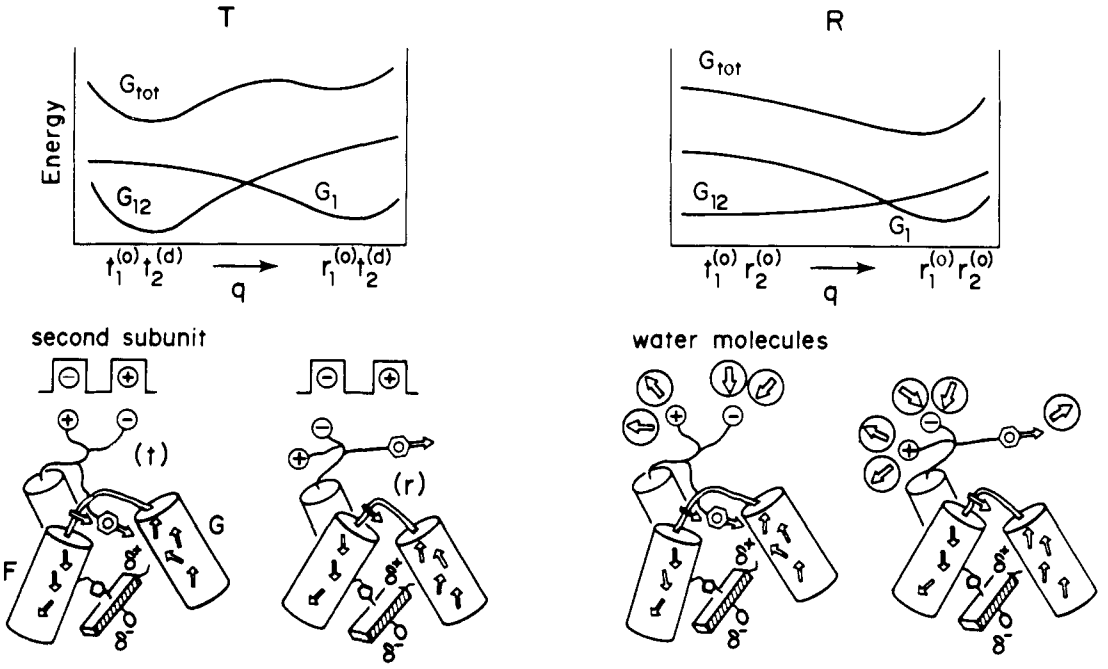

Figure 6. A schematic description of the energetics of cooperativity of a two-subunit system. q is the least energy coordinate for the $t_1^{(o)}t_2^{(d)} \rightarrow r_1^{(o)}t_2^{(d)}$ conformational change. G_1, G_{12}, and G_{tot} are respectively the internal energy of the first subunit, the subunit interaction energy, and the sum $G_1 + G_{12}$. In the T state the heme$^{\delta+}$–$O_2^{\delta-}$ system could be stabilized only at the expense of intersubunit interaction. In the R state, on the other hand, the $r^{(o)}$ configuration with low G_1 can be formed without changing G_{12}. The lower part of the figure describes schematically how the stabilization of the heme charge by the dipoles of the G helix (see also Figure 5) competes with the electrostatic interaction with the neighboring subunit. The HC2 tyrosine is drawn as a dipole attached to a benzene ring. In the R state the salt-bridge groups are shown stabilized by water molecules represented as dipoles embedded in spheres.

to be significant around the proximal and distal histidines, which should show the greatest conformational changes on ligand binding (see caption to Table I).

(III) Relation to Experimental Information

All available experimental information about hemoglobin (with the possible exception of the observation that the Fe–O_2 stretching frequency does not change upon the r → t transition[9]) is consistent with storage of significant amounts of $\Delta\bar{G}$ as strain. Explanations of various experiments in terms of the strain model are given in ref 1–3 and references cited therein.

Storage of $\Delta\bar{G}$ as electrostatic energy is also consistent with all available experimental information. Here we consider several important experiments. (i) The change in charge distribution of the heme upon binding of oxygen is supported by the change in the oxidation state marker as discussed in detail in section II. (ii) $\Delta\bar{G}$ of (cobalto)hemoglobin (CoHb) is about one-third that of hemoglobin.[27] This is consistent with our model since the change in the porphyrin charges upon binding of oxygen to CoHb is about one-third of the corresponding change in hemoglobin as indicated by the shifts in the oxidation state marker frequencies (7 and 19 cm^{-1}, respectively). (iii) Asakura and Sono[28] have demonstrated that substitution of the ring III vinyl by a formyl group greatly reduces the cooperativity, while the corresponding substitution on ring II has no effect. This is consistent with the present calculations, which predict that the largest contribution to $\Delta\bar{G}_e$ comes from the interaction between the positive charges on the vinyl of ring III and the protein electrostatic potential (see Figure 4). Substitution studies[29] have shown that the cooperativity decreases when the vinyls are substituted by Br or by CH_3 and is reduced drastically when the vinyls are replaced by hydrogens. Substitution by hydrogens leads to almost complete loss of the vinyl contribution to $\Delta\bar{G}_e$. Substitutions by Br and CH_3 are intermediate cases since both stabilize positive charge on the C_b pyrrole atoms (e.g., $=C_b^+—Br$). (iv) The oxygen affinity of ferrous hemes in solution increases by up to a factor of 30 upon increase in the solvent polarity.[30] This indicates that the interaction of the solvent dipoles with the heme$^{\delta+}$–$O^{\delta-}$ system helps determine the oxygen affinity. (v) The absorption maxima of the Soret and the Q bands of met-Hb are red shifted by 0.5–2 nm upon the r → t transition.[31] This is consistent with heme–protein electrostatic interactions since these transitions involve excitation of an electron to the π^* orbital where negative charge is more stable in the t state than in the r state.[34] We examined this point quantitatively by incorporating the protein electrostatic potential in the π-electron Hamiltonian of the heme by using the relation (3);[11] where μ designates orbitals, i designates atoms,

$$F_{\mu\mu}^{ii} = (F_{\mu\mu}^{ii})^0 + V^{(i)} \quad (3)$$

$(F_{\mu\mu}^{ii})^0$ is the diagonal SCF matrix element in vacuo, and $V^{(i)}$ is the electrostatic potential from the protein at the ith heme atom. The calculated effects of the r → t transition are red shifts of 1.5 and 2 nm for the Soret and the Q bands, respectively.

As was stated at the beginning of this section, virtually all the experimental information considered here is also consistent with the strain model. This might indicate that both steric and electrostatic interactions are important to cooperativity.

(IV) Concluding Remarks

We have argued that although strain energy probably contributes a large fraction of $\Delta\bar{G}$, it is unlikely to account for the entire amount. It is difficult to store energy as strain in a protein

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(34) The spectral shifts upon the r → t transition in hemoglobin are caused by two opposing contributions: the interactions with the protein is expected to lead to a red shift and the displacment of the iron from the heme plane is expected to cause a blue shift. Spectral shifts in five ligand systems are affected by the motion of the iron and only in ligated systems, where the iron position is relatively fixed,[7] do we expect the observed shift to reflect the change in interaction with the protein upon the r → t transition.

since small converted atomic motions can relax this energy without changing the interaction between subunits. It has been suggested before that the subunit interaction involves electrostatic energy in the salt bridges;[2] here we make a logical extension of this point and suggest that part of the energy between the subunit and its ligated heme can also be considered to be electrostatic. It is shown that binding of oxygen to the iron leads to significant transfer to positive charge to the π^* orbital of the porphyrin. The calculated stabilization of this positive charge by the permanent and induced protein dipoles increases by 1.1 kcal/mol upon the t → r transition. In particular, the r state stabilizes additional positive charge on the vinyl group and the C_b carbons of ring III, in agreement with substitution experiments. Thus we suggest that the electrostatic contribution to cooperativity in hemoglobin can be viewed as described schematically in Figure 6; the protein subunits are designed to have multiple folding conformations r and t, where the r state interacts more strongly than the t state with the charges of the heme-O_2 system while the t state interacts more strongly than the r state with the charges and dipoles of the neighboring subunits. The competition between intrasubunit electrostatic stabilization of the heme$^{\delta+}$-$O_2^{\delta-}$ system and intersubunit salt-bridge interactions is an important factor in cooperativity in hemoglobin.

Because of the appoximate nature of the electrostatics calculations, we cannot provide a quantitative estimate of the electrostatic contribution to $\Delta\bar{G}$ but wish rather to point out the feasibility of such an effect. A possibly interesting clue to the magnitude of this contribution might be provided by comparing the heme-heme interaction in bindings of CO and O_2 to hemoglobin; the cooperativity in CO binding is attributed to two factors: a steric barrier on the distal side[35] and protein strain at the proximal side. Since the strain contribution from the proximal side should be similar for CO and O_2, the additional cooperativity due to the distal effect should make $\Delta\bar{G}$ for CO binding larger[36] than for O_2 binding. Yet, $\Delta\bar{G}$ for CO is ~3.0 kcal/mol. We suggest that this is due to a smaller electrostatic contribution to cooperativity in HbCO than in HbO_2.

The present work might have general implications for other allosteric systems in showing that the allosteric effect may be associated with a balance of opposing electrostatic contributions. This is consistent with the idea that transfer of energy in biological systems is often associated with changes in electrostatic energy.[13]

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(36) The distal effect in CO is manifested in the "on rate", k_{on}, which is about 40 times larger in the R state than in the T state.[35] Assuming that the distal penetration barrier is determined by the steric resistance to a linear binding of CO and that the same steric strain still exists in the protein when the Fe-CO bond is completely formed, we would expect an extra distal contribution to $\Delta\bar{G}$.

A Reinvestigation of the Mechanism of *Pseudomonas testosteroni* Δ^5-3-Ketosteroid Isomerase

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Abstract: The mechanism of the isomerization of Δ^5-3-keto steroid by the enzyme of *P. testosteroni* has been reinvestigated with androst-5-ene-3,17-dione **1**. The formerly proposed 4β → 6β intramolecular transfer does not account for all the reaction. It has been demonstrated by using 4α- and 4β-deuterated substrates that this reaction is not stereospecific and involves a competitive abstraction of the 4α- and 4β-protons. The relative contribution of the two processes is temperature dependent. This may be attributed to some conformational change of the enzyme resulting in small modifications of the spatial relationships within the active site. The course of the reaction with other substrates was also examined. The 19-methyl group, very important for the binding, does not influence the proton transfer since the results are identical with **1** and **5**. Some 4β → 10 intramolecular transfer has also been found with a $\Delta^{5(10)}$-3-keto steroid, **4**, showing the flexibility of the active site. Another Δ^5-3-keto steroid, **3**, shows a very different behavior. In this case, the 4β-proton is selectively removed. An enzymic exchange of the 6β-proton is also taking place. Thus the examination of the protons involved in the isomerization and of the intramolecular character of the reaction offers a sensitive method for the study of the interaction in the enzyme-substrate complex.

Isomerases constitute a very important class of enzymes which catalyze either a ketol isomerization (phosphoglucose isomerases,[1] triosephosphate isomerases,[2,3] glyoxalases[4]) or a double bond migration (Δ^5-3-ketosteroid isomerases,[5] prostaglandin isomerases[6]). They represent a rather simple case of acid-base catalysis since they require no cofactor and generally no metal ion and some of them have been extensively studied. In most cases, an at least partially intramolecular stereospecific proton transfer has been demonstrated by using either a labeled substrate in H_2O or a nonlabeled one in D_2O (or ^3H-H_2O).

With the Δ^5-3-ketosteroid isomerase of *P. testosteroni* (EC 5.3.3.1) in which we are interested, this intramolecular transfer first discovered by Talalay et al.[5] was reinvestigated by Malhotra and Ringold,[7] who have shown that the 4β-proton was stereospecifically removed and transferred on carbon 6. They have

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