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Control of the Redox Potential of Cytochrome *c* and Microscopic Dielectric Effects in Proteins†

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ABSTRACT: X-ray structural information provides the opportunity to explore quantitatively the relation between the microenvironments of heme proteins and their redox potentials. This can be done by considering the protein as a "solvent" for its redox center and calculating the difference between the electrostatic energy of the reduced and oxidized heme. Such calculations are presented here, applying the protein dipoles–Langevin dipoles (PDLD) model to cytochrome *c*. The calculations focus on an evaluation of the difference between the redox potentials of cytochrome *c* and the octapeptide–methionine complex formed by hydrolysis of cytochrome *c*. The corresponding difference (~7 kcal/mol) is accounted for by the PDLD calculations. It is found that the protein provides basically a low dielectric environment for the heme, which destabilizes the oxidized heme (relative to its energy in water). The effect of the charged propionic acids on the heme is examined in a preliminary way. It is found that the negative charges of these groups are in a hydrophilic rather than a hydrophobic environment and that the protein–water system provides an effective high dielectric constant for their interaction with the heme. The dual nature of the dielectric effect of the cytochrome (a low dielectric constant for the self-energy of the heme and a high dielectric constant for charge–charge interactions) is discussed. The findings of this work are consistent with the difference between the folding energies of the reduced and oxidized cytochrome *c*.

The oxidation–reduction (redox) potentials of cytochromes span a range of nearly 800 mV [for example, see Xavier et al. (1981), Margoliash & Schejter (1966), and Meyer & Kamen (1982)]. In thermodynamic terms, the variation in cytochrome redox potentials corresponds to a *range* of 17 kcal/mol in the free energy of oxidation of the ferrous heme prosthetic group. Since 17 kcal/mol amounts for more than

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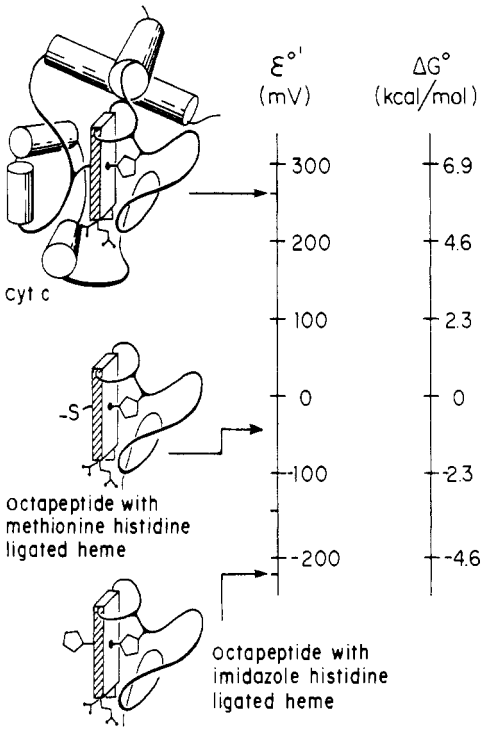

FIGURE 1: Redox potentials of cytochrome *c*, octapeptide of cytochrome *c* in 2 M *N*-acetyl-DL-methionine (Harbury et al., 1965), and octapeptide of cytochrome *c* in 0.05 M imidazole (Harbury et al., 1965).

half of the free energy required to split water into H_2 and O_2, the variability of cytochrome redox potentials is an important phenomenon in bioenergetics. Structural studies of cytochromes [see, for example, Salemme (1977), Adman (1979), and Takano & Dickerson (1981)] provide the opportunity to explore this key question on a detailed molecular level.

Part of the observed variation in the redox potentials of cytochromes can be accounted for by differences in the atomic makeup of the heme–ligand complex. Empirically (Xavier et al., 1981 Margoliash & Schejter, 1966; Harbury et al., 1965; Marchon et al., 1982; Mashiko et al., 1981), substitutions of different ligands axial to the heme shift the redox potential by ~150 mV, and peripheral substituents on the porphyrin (associated with various heme skeletons such as *a*, *b*, and *c*) give rise to shifts of similar magnitude. On the other hand, hemes with the same skeleton and axial ligands exhibit a 300-mV range in redox potential, depending on the protein environment. Evidently, "solvation" of the heme complex by the protein–aqueous environment is important in shifting the redox potential, and one would like to correlate this solvation effect with the protein structure. In fact, significant progress has already been made in the identification of the possible effect of a low dielectric environment around the heme (Kassner, 1972) and in assessing the effect of charge–charge interactions [for example, see Rees (1980), Schejter & Eaton (1984), and Moore (1983)]. Nevertheless, the actual effect of the local protein environment could not be evaluated without taking into account the microscopic dielectric effect of the protein. In this work, we present a study of the effect of the microenvironment of proteins on their redox potential, taking cytochrome *c* as an initial test case. Cytochrome *c* is a good starting point because of the availability of a reference octapeptide system with a methionine–histidine ligated heme (Figure 1). This reference system, formed by hydrolysis of cytochrome *c* (Harbury et al., 1965), has the same heme–ligand complex as in cytochrome *c*. Thus, the 300 mV difference between the redox potentials of cytochrome *c* and the reference system (Figure 1) can be attributed to environmental effects. The effect of the ligands can be determined by changing the ligand in the octapeptide system (Figure 1) and in model compounds (Marchon et al., 1982). The primary objective of this work is in accounting for about a 300-mV (~7 kcal/mol) protein effect on the difference between the redox potential of the heme in cytochrome *c* and in the octapeptide (methionine) complex. We also consider the effect of ionizing the heme propionic group on the heme redox potential. These phenomena present a theoretical challenge since the protein–water system acts both as a low dielectric medium (with regard to the solvation energy of the oxidized heme) and as a high dielectric medium (with regard to the interaction between the heme and various ionized amino acids). These effects cannot be simulated simultaneously in a consistent way by available macroscopic models [e.g., Matthew et al. (1979)]. Here, as in previous studies of electrostatic energies in proteins (Warshel & Russell, 1984), we use a microscopic model that simulates the microscopic nature of the permanent and induced dipoles of the protein–water system. Our model reproduces the experimental trend and sheds some light on the global nature of the control of redox potential in proteins.

Our approach and method of calculation are outlined under Methods. The results of the calculations are described under Results. The implications of these results for redox potentials of cytochromes are discussed and related to the molecular meaning of dielectric effects in proteins under Discussion.

METHODS

Relation between Redox Potentials and Solvation Energetics. The standard free-energy change on adding one electron to an oxidized molecule, A^{ox}, to give the reduced form, A^{red}, is related to the redox potential by the well-known equation:

$$\Delta G^\circ_{red} = -\mathcal{F}\mathcal{E}^\circ + \text{constant} \qquad (1)$$

By convention, the potential $\mathcal{E}^\circ$ is given in volts, and the more favorable the reduction, the more positive is $\mathcal{E}^\circ$; the coefficient $\mathcal{F}$ is 23.06 kcal mol^{-1} V^{-1}. The "constant" in eq 1 depends on the chemical nature of the reference system (e.g., standard hydrogen electrode) as well as on temperature. Thus $\mathcal{E}^\circ$ determines the relative, but not absolute, free-energy changes for the redox process. Equation 1 applies to environmental effects of different solvents on the reduction of the same molecular entity. These effects are quantitatively described by using a Born cycle to decompose the overall free-energy change ΔG° into intramolecular and solvation energy components. The overall reduction process is carried out in three hypothetical stages:

$$\begin{array}{ccc} A_g^{ox} & \xrightarrow[II]{\Delta G^\circ_{II}} & A_g^{red} \\ {\scriptstyle \Delta G^\circ_I}\uparrow\ I & & III\ \downarrow{\scriptstyle \Delta G^\circ_{III}} \\ A_s^{ox} & \xrightarrow[\Delta G^\circ_{red}]{} & A_s^{red} \end{array} \qquad (2)$$

In stage I, A^{ox} is taken from the given solvent to the gas phase; $-\Delta G^\circ_I$ is the solvation energy of A^{ox} in solvent s. In stage II, A^{ox} is reduced in vacuum; $-\Delta G^\circ_{II}$ is the ionization potential of A^{red}. In stage III, A^{red} is taken from the gas phase to the solvent, and ΔG°_{III} is the solvation energy of the reduced species. The overall free-energy change is given as

$$\Delta G^\circ_{red} = \Delta G^\circ_I + \Delta G^\circ_{II} + \Delta G^\circ_{III} = -\Delta G^\circ_{sol}(A^{ox}) + \Delta G^\circ_{II} + \Delta G^\circ_{sol}(A^{red}) \qquad (3)$$

Combining eq 1 and 3, one obtains the relation:

$$-\mathcal{F}\Delta\mathcal{E}^\circ = \Delta[\Delta G^\circ_{sol}(A^{ox}) - \Delta G^\circ_{sol}(A^{red})] = \Delta\Delta G_{sol} = \Delta\Delta G^\circ_{red} \quad (4)$$

This equation provides a rigorous formal relation between *changes* in solvation energy and changes in redox potential. The next section will describe our approach for calculating solvation energies.

Microscopic Electrostatic Model for G°_{sol}. On oxidation of the ferrous heme, the net charge of the heme complex changes by 1. Accordingly, the difference in solvation energy of the oxidized and reduced heme depends mainly on the electrostatic interaction of the heme charges, Q, with charges and dipoles in the surrounding protein and water. That is, the solvation energy ΔG_{sol} includes the following contributions: (i) the interaction $\Delta V_{Q\mu}$ between the heme charges and the protein permanent dipoles (e.g., the N–H and C=O dipoles of the peptide bonds); (ii) the interaction $\Delta V_{Q\alpha}$ between the heme charges and the induced dipoles associated with the polarizability of individual atoms; (iii) the solvation energy ΔG_{Qw} of the heme and the protein by the surrounding water molecules; (iv) the interaction energy $V_{QQ'}$ between the heme and other ionized groups (where Q′ are the charges of the ionized groups). The overall electrostatic free energy associated with the change of the heme charges can be approximated by

$$\Delta\Delta G^p_{sol}(Q^{red}\rightarrow Q^{ox}) \simeq \Delta\Delta V_{Q\mu} + \Delta\Delta V_{Q\alpha} + \Delta\Delta V_{\mu\mu} + \Delta\Delta G_{Qw} + \Delta V_{QQ'} \quad (5)$$

where $\Delta\Delta V_{\mu\mu}$ is the change in the protein conformational energy associated with the stabilization of the oxidized heme.

The energy components of eq 5 are evaluated by the protein dipoles–Langevin dipoles (PDLD) model, which is described in detail in Warshel and Russell (1984) and Russell and Warshel (1985) and presented schematically in Figure 2. We outline here only key points that are relevant to the redox problem. The term $\Delta V_{Q\mu}$ is calculated by considering the interaction between the heme charges, Q, and the residual charges of the protein atoms, q, in vacuum:

$$\Delta V_{Q\mu} = 332\sum_{ij} Q_i q_j / r_{ij} \quad (6)$$

where the energy is given in kilocalories per mole, the distances are in angstroms, and the charges are in units of an electron charge. The delocalized heme charges can be evaluated by the QCFF/PI method [see the treatment of Warshel & Weiss (1981)]. However, if the four pyrrole nitrogens are assigned a charge of 0.25 in the ferric state and 0.0 in the ferrous state, one obtains almost the same solvation energy as with the QCFF/PI charges. Thus, we used the simplified heme charge distribution for the present calculations. The residual atomic charges of the protein are assigned as in Russell and Warshel (1985). The interaction between the heme charges and the protein-induced dipoles is given (in kcal/mol) by

$$\Delta V_{Q\alpha} = -166\sum_i \mathbf{E}^\circ_i \mu_i \quad (7)$$

$$\mu_i = \alpha \mathbf{E}_i$$

where $\mathbf{E}^\circ_i$ is the local field on the ith protein atom due to the charges Q and Q' and the protein permanent dipoles. $\mathbf{E}_i$ is the local field on the ith protein dipole due to $\mathbf{E}^\circ$ and the field from all other protein-induced dipoles. The fields $\mathbf{E}_i$ and the induced dipoles are evaluated by a self-consistent iterative approach (Warshel & Levitt, 1976; Warshel & Russell, 1984). The atomic polarizabilities are taken as 0.5 Å^3 for H and 1.0 Å^3 for other atoms.

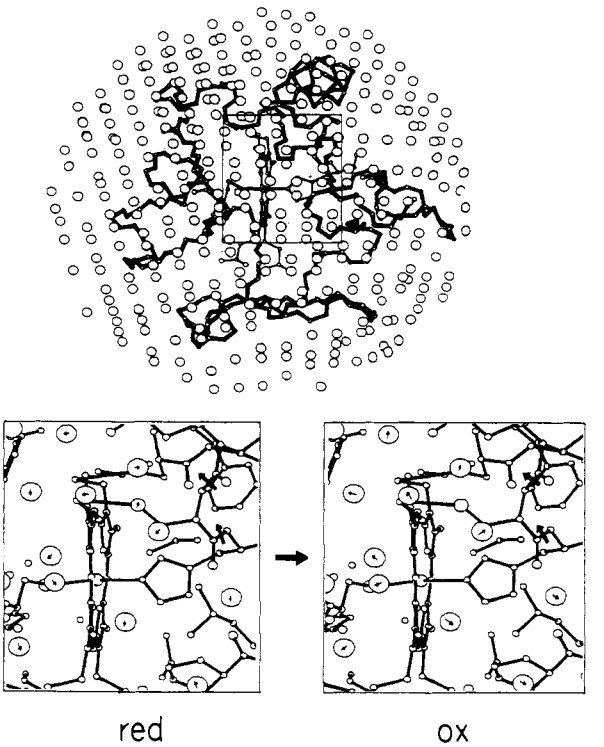

FIGURE 2: PDLD model for cytochrome *c*–water system. The figure shows the protein surrounded by a grid of Langevin dipoles (which represent the water molecules). The lower part of the figure describes schematically the reorientation of the Langevin dipoles upon oxidation of the heme.

The total energy change associated with the change in the cytochrome geometry upon change in the heme oxidation state has been found to be smaller than 3 kcal/mol (Churg et al., 1983). If the protein reorganization energy can be described within the harmonic approximation, then $\Delta\Delta V_{\mu\mu}$ is smaller than 1.5 kcal/mol [see Churg et al. (1983)]. Thus, we neglect $\Delta\Delta V_{\mu\mu}$ in this work.

The energy contribution from the water around the protein is evaluated by

$$\Delta G_{Qw} = \Delta G_{QL} + \Delta G_{bulk} \quad (8)$$

where ΔG_{QL} represents the interaction between the protein and heme charges and the water molecules near the protein (up to a distance of $\bar{b}$ = 18 Å from the heme center). This energy is evaluated here by representing the water molecules as a grid of polarizable Langevin-type dipoles. ΔG_{bulk} is the interaction between the protein–heme charges and the bulk water molecules in the region from $\bar{b}$ to infinity. This contribution is evaluated by a continuum model (see below). The energy of polarization of the Langevin dipoles is evaluated by

$$\Delta G_{QL} = -166\sum_i \mu^L_i \mathbf{E}^\circ_i \quad (9)$$

where ΔG is given in kilocalories per mole and the polarization of the Langevin dipoles is evaluated by

$$\mu^L_i = \mathbf{e}_i \mu_0 (\coth X_i - 1/X_i)$$
$$X_i = C'\mu_0(\mathbf{E}_i - \mathbf{E}^c_i)\mathbf{e}_i/(k_b T) \quad (10)$$

where $\mathbf{e}_i$ is a unit vector in the direction of $\mathbf{E}^\circ_i$, $\mathbf{E}_i$ is the total local field on the ith dipole, and $\mathbf{E}^c$ is the field on the ith dipole from its nearest neighbors. The fields $\mathbf{E}_i$ and the dipoles μ^L_i are evaluated by an iterative self-consistent approach. The detailed implementation of the Langevin dipole moment and its relation to the actual polarization of real water molecules are considered in Warshel and Russell (1984) and Russell and Warshel (1985).

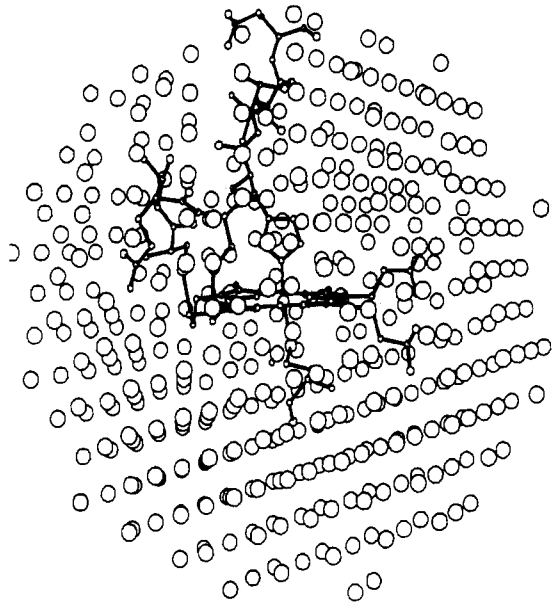

FIGURE 3: PDLD model for octapeptide–water system. The figure describes the octapeptide surrounded by a grid of Langevin dipoles. The permanent and induced dipoles of the octapeptide are not drawn in the figure.

The bulk energy is evaluated by a standard continuum approximation. For a given total charge Z of the heme–protein complex, it is approximated (in kilocalories per mole) by

$$\Delta G_{\text{bulk}} \simeq -166Z^2/\bar{b} \qquad (11)$$

where $\bar{b}$ is the radius (in angstroms) of the sphere included in the Langevin dipole calculations. This Born's approximation can be replaced by the more accurate Kirkwood's formula (Kirkwood, 1934), where the ionized groups of the heme–protein system cannot be approximated by a single charge. The interaction between the heme charges and the ionized groups of the protein $V_{QQ'}$ is evaluated by

$$V_{QQ'} = 332\sum_{ij} Q_i Q_j / r_{ij} \qquad (12)$$

Note that this is done with the vacuum dielectric constant ($\epsilon = 1$) since the actual dielectric effect is reproduced by changes in solvation energies.

The combination of all the contributions included in eq 5 provides the overall electrostatic energy of the system. This energy can be used as an approximation for the free energy of the system [see Discussion in Russell & Warshel (1985)]. We will describe below the use of the PDLD model for evaluation of the energetics of the oxidation of cytochrome *c*.

RESULTS

Solvation Energy Contributions to the Oxidation Potential of the Heme in Cytochrome c and in Octapeptide. To examine the role of the protein in determining the heme redox potential, we compare the calculated solvation energy of eq 5 for the heme complex in cytochrome *c* and heme in octapeptide. We define the "heme complex" to be the iron porphyrin of the heme plus histidine-18 and methionine-80, taking the atomic coordinates from the protein structure (Takano & Dickerson, 1981). In cytochrome *c*, the heme complex is surrounded by a polypeptide of 103 residues in which the porphyrin is linked to the protein by thioether bonds to cysteine-14 and cysteine-17 and by the axial bonds between the iron and histidine-18 and methionine-80. The porphyrin is located in a groove that keeps

Table I: Components of the Heme Solvation Energy (kcal/mol)[a]

	$\Delta G_{Q\mu}$	$\Delta G_{Q\alpha}$	ΔG_{QL}	ΔG_{bulk}	ΔG_{sol}
cytochrome *c*					
$\Delta G_{\text{sol}}^{\text{ox}}$	−51.18	−2238.2[b]	−19.25	−9.93	−2318.56
$\Delta G_{\text{ox}}^{\text{red}}$	−47.36	−2225.8	−19.36	0.0	−2292.52
$\Delta\Delta G_{\text{sol}}$	−3.82	−12.4	0.09	−9.93	−26.04
octapeptide					
$\Delta G_{\text{sol}}^{\text{ox}}$	−9.78	−153.4	−41.21	−9.93	−214.32
$\Delta G_{\text{sol}}^{\text{red}}$	−7.24	−154.3	−17.11	0.0	−178.65
$\Delta\Delta G_{\text{sol}}$	−2.54	0.9	−24.10	−9.93	−35.67

[a] The grid of the Langervin dipoles was centered on the heme iron and truncated at a radius of 15 Å. Accordingly, $\bar{b}$ in eq 11 is 16.5 Å, $\Delta G_{\text{sol}}^{\text{ox}}$ and $\Delta G_{\text{sol}}^{\text{red}}$ are the indicated contributions to the solvation energy of the oxidized and reduced systems, respectively. $\Delta\Delta G_{\text{sol}}$ is the difference between the corresponding contributions from the oxidized and reduced systems. [b] This very large number reflects the interaction between the induced dipoles and the permanent dipoles. However, the difference between the oxidized and reduced contributions is insensitive to the absolute value of $\Delta G_{Q\alpha}$ and to the effect of the partial atomic charges that do not change during the oxidation process.

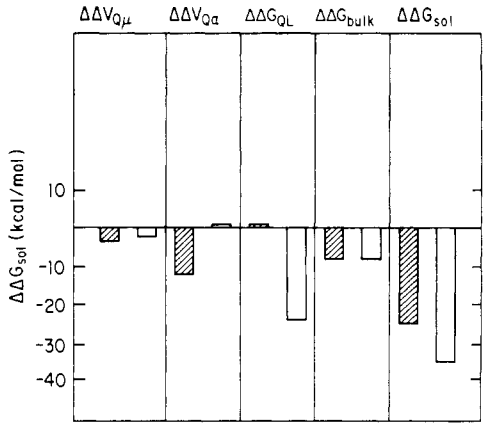

FIGURE 4: Comparison of the solvation energy contributions for oxidation of cytochrome *c* (■) and octapeptide (□) system. $\Delta\Delta G_{\text{sol}}$ is the difference between the ΔG_{sol} of the corresponding oxidized and reduced system [$\Delta\Delta G_{\text{sol}} = \Delta G_{\text{sol}}(\text{ox}) - \Delta G_{\text{sol}}(\text{red})$]. The individual components (see eq 5) are the changes in the contributions from the protein permanent dipoles ($\Delta\Delta V_{Q\mu}$), protein-induced dipoles ($\Delta\Delta V_{Q\alpha}$), Langevin dipoles ($\Delta\Delta G_{QL}$), and the solvation by the bulk water ($\Delta\Delta G_{\text{bulk}}$).

water molecules at a distance larger than 9 Å (see below). On the other hand, in the octapeptide system (consisting of the heme, residues 14–21, and *N*-acetyl-DL-methionine replacing methionine-80), water can approach heme atoms at van der Waals or H-bonding distances and stabilize the oxidized heme (Figure 3). Since residues 14 and 17 are covalently bonded to the heme and histidine-18 is liganded to the iron, we use the corrdinates of residues 14–21 of cytochrome *c* in modeling the octapeptide structure. This procedure is clearly an approximation since the heme–octapeptide system is likely to relax to a geometry that is somewhat different from the geometry of the corresponding residue in the completely folded protein. However, as much as the stabilization of the oxidized heme is concerned, the calculations using the unrelaxed octapeptide provide an *upper limit* for the stabilization by the relaxed octapeptide!

Figure 4 and Table I summarize the calculations of the change in the solvation energy upon oxidizing the neutral heme complex in the cytochrome and in the octapeptide environments. The calculation gives the quantity $\Delta\Delta G_{\text{sol}} = \Delta G_{\text{sol}}(\text{heme}^{\text{ox}}) - \Delta G_{\text{sol}}(\text{heme}^{\text{red}})$, where the reduced heme is a neutral species (the effect of ionizing the propionates and other groups will be considered in the next section), and the oxidized heme has charge of 1+. The largest contributions to the heme solvation energy in the cytochrome are from the protein-in-

duced dipoles and the bulk water, while for the octapeptide the Langevin and bulk water contributions are the largest. The total $\Delta\Delta G_{sol}$ for the cytochrome is −26 kcal/mol, whereas for the octapeptide it is −35 kcal/mol. This is in agreement with the experimental finding (Figure 1) that oxidization of the heme in cytochrome *c* is ~7 kcal/mol less favorable than that in the octapeptide system.

The dominant contribution of protein induced dipoles to $\Delta\Delta G_{sol}$ in cytochrome *c* indicates that in this case the protein microenvironment destabilizes the heme charges (relative to water) and can be considered as an effective low dielectric environment. In general, proteins cannot keep a nonpolar environment near ionized groups of a small radius (Warshel & Russell, 1984). However, when the radius of the charged group is sufficiently large, the protein-folding forces can keep the charge in a low dielectric region. The effective radius of the solvated heme and the surrounding nonpolar protein can be gauged from the following considerations: The Born solvation energy of a charge q of radius $\bar{a}$ centered in an oil drop of radius $\bar{b}$ and a dielectric $\epsilon_1 \sim 2$ embedded in a high dielectric medium $\epsilon_2 \gg \epsilon_1$ is (in kcal/mol)

$$\Delta\Delta G_{sol} \simeq -166\frac{q^2}{\bar{a}}\left(1-\frac{1}{\epsilon_1}\right) - 166\frac{q^2}{\bar{b}}\left(\frac{1}{\epsilon_1}-\frac{1}{\epsilon_2}\right) \quad (13)$$

The effective radiuis $\bar{a}$ can be evaluated by considering the solvation energy of the heme–octapeptide system ($\Delta\Delta G_{sol} \simeq$ −35 kcal/mol) as an approximation for the heme in water where $\bar{a} \simeq \bar{b}$. This gives $-166q^2/\bar{a} = -35$, which is satisfied by $\bar{a} \simeq 4.7$ Å. Using this estimate of $\bar{a}$ and the value of $\Delta\Delta G_{sol}$ $\simeq$ −26 kcal/mol (as found for the cytochrome system), one obtains $\bar{b} \simeq 9$ Å. Qualitatively, the difference in heme redox potential between the cytochrome *c* and aqueous systems is accounted for by regarding the protein as an oil shell of 11-Å radius surrounded by water. This low dielectric environment destabilizes the heme relative to water. While the above values for $\bar{a}$ and $\bar{b}$ should not be taken literally, it is interesting to note that 5 and 10.5 Å are the distances of closest approach to the iron by the water molecules (closer Langevin grid points are in van der Waals contact with the protein) in the octapeptide and cytochrome, respectively.

Effect of Ionized Groups on Cytochrome c Redox Potential. The ionized groups of cytochrome *c* and other proteins have been the subject of considerable attention [for example, see Margalit & Schejter (1973), Koppenol et al. (1978), Koppenol & Margoliash (1982), Schejter & Eaton (1984), Moore (1983), and Moore et al. (1980)]. In particular, the propionic acid groups of heme *c* are thought to be important in controlling the redox energetics because they are inside the heme cleft. Unfortunately, the pK_a's of the propionic acids in cytochrome *c* have not been assigned, since no change in ionization state of the proprionates has been observed over the pH range of 4.5–9.0 (Ångström et al., 1982). This has been taken to indicate that the propionic acids are in hydrophobic sites with pK_a > 9 (Koppenol & Margoliash, 1982) although recent work (Moore, 1983) suggested that the pK_a of propionate-7 is smaller than 4.5. The present study (see below) indicates that both propionates are in hydrophylic rather than hydrophobic sites (see below). However, the aim of this work is not a study of the pK_a's of the propionates [we feel that such a study should be made first in cases such as cytochrome *c*-551 where the pK_a is known (Moore (1983)] but a demonstration of the energy balance associated with the interaction between a surface charge and the ionized heme.

Figure 5 summarizes our microscopic calculations for the effect of charging the propionic groups on the oxidation potential of the heme. As seen from the figure, the very large interaction between the oxidized heme and propionate charges is largely compensated by the change in solvation energy upon reduction. The largest compensation effect is due to the polarization of the water molecules around the protein. As mentioned repeatedly (Warshel, 1978; Warshel & Russell, 1984; Warshel et al., 1984), a large compensation of charge–charge interactions by changes in solvation energy is equivalent to a large dielectric constant. The distance between the propionate O^- and the charge center of the oxidized heme is about 8 Å, while the effective radius of the protein extends ~11 Å from the iron. Thus, the surrounding water molecules are quite effective in leading to high dielectric function for charge–charge interaction.

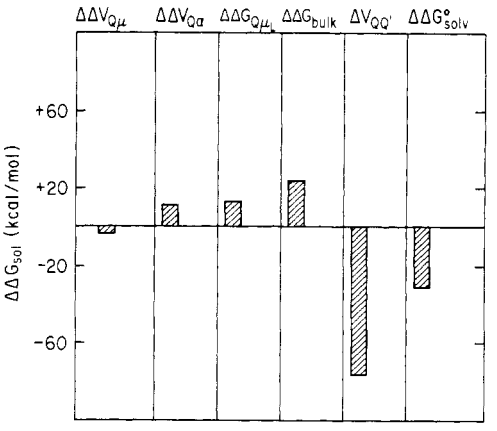

FIGURE 5: Energy balance associated with the interaction between the charges of the propionates and the heme. Notation is as in Figure 4, where $\Delta V_{QQ'}$ is the vacuum interaction between the propionates and the heme (eq 12).

The nature of the local environment of the heme propionates was explored here only in a preliminary way, since the corresponding pK_a's are not yet assigned experimentally. The present study gave a solvation energy [$\Delta\Delta G_{sol}$(AH→A$^-$)] of −70 ± 5 to both groups, indicating [see Russell & Warshel (1985)] that the propionates are stabilized by the protein–water environment as much as they would be stabilized in bulk water. This finding, which clearly requires further studies, indicates that the environment of the ionized propionates is polar rather than hydrophobic.

While the issue of the interaction between the propionic acids and the heme requires more experimental and theoretical studies, those interactions that were assigned experimentally (Bosshard & Zurrer, 1980; Smith et al., 1977) seem to be well described by a macroscopic Coulomb's law with a large dielectric constant (Warshel et al., 1984). In this way, one should evaluate (as was done here) the solvation energy (self-energy) of the heme in the natural (uncharged) protein using the microscopic PDLD model and only then estimate the charge–charge interactions using the macroscopic dielectric constant [see Discussion in Warshel & Russel (1984)].

Discussion

This work made an attempt to evaluate the microscopic effect of the protein environment on the redox potential of cytochrome *c*. The main conclusions drawn from our studies are summarized in Figure 6. The figure indicates that a major source of the difference between the redox potential of cytochrome *c* and the octapeptide system is due to the *destabilization* of the oxidized heme by its local microenvironment, which can be viewed as a low dielectric medium (see discussion under Results). It should be noted, however, that the protein can retain a low dielectric environment around the heme be-

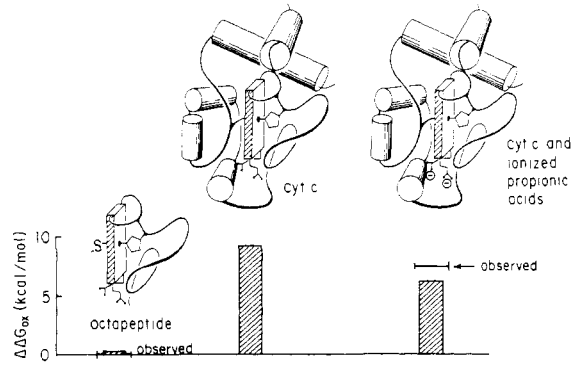

FIGURE 6: Summary of calculations. The figure presents the calculated energy for oxidizing the heme in octapeptide, in neutral cytochrome *c*, and in cytochrome *c* with ionized propionates. The horizontal lines represent the observed energetics.

cause the heme charges are delocalized over a large region (thus applying relatively small forces on the protein permanent dipoles and surrounding water). In cases of localized charges (such as ionized acidic and basic groups) at equilibrium, the electrostatic forces constrain the protein permanent dipoles and bound water to stabilize the charged groups as much as water does.

The cytochrome *c* case provides an instructive demonstration of the risks of considering the protein as a uniform dielectric medium with a single dielectric constant for all properties. That is, as was argued before (Warshel & Russell, 1984; Warshel et al., 1984), one can use the concept of an effective dielectric constant in many cases, but a different constant might be needed for different properties. For example, while the self-energy of the heme can be modeled by Born's formula (eq 11) with a low dielectric constant, the effect of surface charges can be modeled by using Coulomb's law with a large dielectric constant. It is also instructive to note that the free energy of bringing the positively charged heme and a given surface charge from water (at infinite separation) to their actual site in the protein gives a *negative* effective dielectric constant. That is, as seen from Figure 28 of Warshel and Russell (1984), the two charges at infinite separation are more stable than at their shorter distance in the protein.

This work has concentrated on evaluating the "self-energy" of the heme in its actual microenvironment. The effect of charge–charge interactions between the heme and the surface charges was considered only for the propionate groups. Although we did not calculate the effects of other surface groups, it appears from the available experimental information that these effects can be approximated by using Coulomb's law with a large dielectric constant (Warshel et al., 1984). The effect of changing ligands is not the subject of this paper. The evaluation of this "chemical" effect does not require calculations since it can be determined experimentally by using model compounds in solutions. Note, however, that the calculations of the environmental effects should take the relevant model compound in solution as a reference (as was done in this work with the octapeptide system).

The possible role of steric effects was not considered in this paper. These effects can be divided into two classes: (a) The first class is the possible effect of stretching bonds (Moore & Williams, 1977) and deforming bond angles. This effect is expected to be very small since the corresponding small geometrical changes can be relaxed by the motion of several protein atoms that costs very little energy [see, for example, examination of steric effects on much larger geometry changes by Warshel & Weiss (1981)]. (b) The second class is the effect of holding the axial ligands in different chirality (Senn et al., 1984). This type of effect might contribute to the control of the redox potential and should be explored theoretically, calculating the energy required to deform the protein to accommodate the motion between different ligand configurations (this will provide an upper limit for the change in redox potential).

The findings of this work are consistent with the fact that the energy of unfolding of oxidized cytochrome *c* is smaller than that of the reduced cytochrome. That is, the folding energy can be expressed as

$$\Delta G_{\text{fold}} = \Delta\Delta G_{\text{pp}}^{\text{fold}} + \Delta\Delta G_{\text{sol}}^{\text{fold}} \qquad (14)$$

where $\Delta\Delta G_{\text{pp}}^{\text{fold}}$ is the change in the protein–protein interaction upon folding (this is approximately the folding energy of the protein without the heme) and $\Delta\Delta G_{\text{sol}}^{\text{fold}}$ is the change in the protein–heme interaction upon folding. $\Delta\Delta G_{\text{sol}}^{\text{fold}}$ is approximately zero for the neutral (reduced) heme, while for the oxidized heme this energy is about 7 kcal/mol (since the heme is destabilized by about 7 kcal/mol upon removal from water to its nonpolar site in the folded protein). Thus, the difference between $\Delta\Delta G_{\text{sol}}^{\text{fold}}$ of the reduced and oxidized cytochrome is directly related to the destabilization of the oxidized heme in the folded cytochrome. It is important to note in this respect that $\Delta\Delta G_{\text{sol}}^{\text{fold}}$ can manifest itself by a partial unfolding of the protein and penetration of water molecules to the heme site (these water molecules will stabilize the oxidized heme). Such an effect is expected to be of major importance in determining the difference in the rate of hydrogen exchange in reduced and oxidized cytochromes (Wand & Englander, 1985).

This work demonstrated that the key contribution to the control of the electrostatic energy of the heme in cytochrome *c* is not charge–charge interactions but the self-energy of the charged heme in its given environment. This crucial electrostatic effect cannot be evaluated by macroscopic models [e.g., Matthew et al. (1979)] that restrict themselves to evaluation of charge–charge interactions by placing the relevant charges on the surface of the protein [see Discussion in Warshel et al. (1984)]. In fact, even the much more physical semimacroscopic approach of Warwicker and Watson (1982) [which was used recently by Rogers et al. (1985) in a study of electrostatic energies in cytochrome *c*-551] might not give quantitative results for the self-energy of the heme. This approach evaluates the electrostatic potential by using a finite difference procedure to numerically integrate the Poisson's equation. Unfortunately, this equation involves the use of a macroscopic dielectric constant (a high dielectric constant is assigned to volume elements that can be occupied by water molecules, and a low dielectric constant is assumed elsewhere). Such a dielectric constant cannot be related uniquely to the microscopic structure of protein. This procedure correctly reproduces a high effective dielectric constant for the interactions between the heme and the surface charges. This result is expected in the case of cytochrome *c* from models that correctly represent the effect of the bulk water (in the cytochrome case most of the effective dielectric constant for charge–charge interactions is due to the water molecules around the protein). Difficulties are expected, however, in obtaining quantitative estimates of the self-energies of the heme in cytochrome *c* and in the octapeptide system. These absolute energies depend strongly on the distance between the charge centers and the closest water molecules. It is hard to simulate this effect with a continuum representation of the water molecules. In fact, the finite difference method is equivalent in many respects to a version of our Langevin dipoles method that uses a very small grid size (and correspondingly small dipoles). This fine-grid approach [see Warshel & Russell

(1984)] has encountered major problems in reproducing intrinsic pK_a's in proteins. The main problems appeared in cases where only one or two water molecules could approach the relevant group. It is quite likely that the finite difference method, as well as the fine-grid Langevin dipoles method, can be improved and parameterized to give reasonable results. However, our purpose in this discussion is not to explore possible alternatives to the PDLD model. We would simply like to point out that models which correctly obtain a high effective dielectric constant for charge–charge interactions are not automatically valid for studying key problems about electrostatic energies in proteins. Apparently, as we emphasized repeatedly and demonstrated in this work, electrostatic energies in proteins are not just charge–charge interactions.

Registry No. Cytochrome *c*, 9007-43-6; heme, 14875-96-8.

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