A Theoretical Model for the Effects of Local Nonpolar Heme Environments on the Redox Potentials in Cytochromes

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Abstract: A theoretical model for the effects of local heme environments of a nonpolar nature on the redox potentials of cytochromes is formulated to account for observed potentials of *c*-type cytochromes relative to the potentials of model heme–ligand complexes. The results of calculations based on the difference in charging energy associated with the difference between the interaction of the ferric heme–ligand complex with polar and nonpolar molecules are presented. The results indicate that the high redox potentials of *c*-type cytochromes relative to model heme complexes and the difference between the redox potentials of these cytochromes may be accounted for by the nonpolar nature of the heme environment.

The oxidation–reduction potential, E_0' (pH 7.0), of cytochrome *c* has been reported to be 0.250 V.[1] Potentials of 0.365,[2] 0.360,[3] and 0.381 V[4] have been reported for other *c*-type cytochromes from plants, algae, and bacteria. By contrast, redox potentials of −0.05[5] and −0.11 V[6] have been reported for the model complex between methionine and the hemeoctapetide from cytochrome *c* and the histidine-methionine derivative of mesoheme, respectively. Values of 0.300–0.431 V characterize the difference between the redox potentials of these cytochromes and the corresponding model heme complexes. Thus, a fundamental question has remained as to the physical basis for the high oxidation–reduction potentials of cytochrome *c* and other *c*-type cytochromes based on a comparison with the potentials of iron porphyrins in the presence of ligands thought to typify groups found in these proteins. A large positive potential difference was recently observed[7] for a heme complex in a nonpolar solvent by comparison with reported values for the corresponding heme complex in aqueous solution. It was therefore suggested that the redox potentials of many high-potential cytochromes may be accounted for by a local heme environment of a nonpolar nature, and that such an environment may play a dominant role in determining the redox potentials of these cytochromes. In this report a theoretical model is advanced which provides a basis for understanding the effects of nonpolar environments on the redox potentials of the heme–ligand complexes in cytochromes.

Formulation of Model and Computational Results

The redox potentials of *c*-type cytochromes, like all oxidation–reduction couples, are dependent on the difference between the free energies of the oxidized and reduced states (eq 1), since $\Delta G^\circ_{\text{hydrogen}}$ is zero by definition. The difference between the redox potentials of *c*-type cytochromes and the redox potential of the hemepeptide plus methionine or the histidine-methionine heme derivative may be related to the difference between the microenvironment of the heme–ligand complex in the protein as compared with water. The local heme environment in ferricytochrome *c* consists of a layer of nonpolar amino acid side chains,[8] while that of the model heme complexes a polar aqueous environment as schematically represented in Figure 1a and b, respectively. Consider the free energy changes associated with the reduction of a heme–ligand complex (Fe–P) in polar (P) and nonpolar (N) environments (eq 2 and 3). The difference between the redox potentials of the two systems is then given by eq 4 and 5.

$$-nFE_0 = \Delta G^\circ_{\text{Fe-Cyt}} - \Delta G^\circ_{\text{hydrogen}} = G^\circ_{\text{Fe(II)-Cyt}} - G^\circ_{\text{Fe(III)-Cyt}} \quad (1)$$

$$\Delta G^\circ_{\text{Fe-P}_P} = G^\circ_{\text{Fe(II)-P}_P} - G^\circ_{\text{Fe(III)-P}_P} \quad (2)$$

$$\Delta G^\circ_{\text{Fe-P}_N} = G^\circ_{\text{Fe(II)-P}_N} - G^\circ_{\text{Fe(III)-P}_N} \quad (3)$$

$$-nFE_{0_P} - (-nFE_{0_N}) = nF(E_{0_N} - E_{0_P}) = \Delta G^\circ_{\text{Fe-P}_P} - \Delta G^\circ_{\text{Fe-P}_N} \quad (4)$$

$$nF\Delta E_{0_{(N-P)}} = (G^\circ_{\text{Fe(II)-P}_P} - G^\circ_{\text{Fe(III)-P}_P}) - (G^\circ_{\text{Fe(II)-P}_N} - G^\circ_{\text{Fe(III)-P}_N}) \quad (5)$$

Rearranging terms gives

$$nF\Delta E_{0_{(N-P)}} = (G^\circ_{\text{Fe(III)-P}_N} - G^\circ_{\text{Fe(III)-P}_P}) - (G^\circ_{\text{Fe(II)-P}_N} - G^\circ_{\text{Fe(II)-P}_P}) \quad (6)$$

Each parenthetical term in eq 6 corresponds to the free energy change associated with the transfer of the complex from one environment to another. The difference between the energies of interaction of the oxidized and reduced complexes with polar and nonpolar environments may be divided into electrostatic (el) and nonelectrostatic (non-el) terms. Thus, for the oxidized complex

$$(G^\circ_{\text{Fe(III)-P}_N} - G^\circ_{\text{Fe(III)-P}_P}) = \Delta G^\circ_{\text{el}} + \Delta G^\circ_{\text{non-el}} \quad (7)$$

$$= (G^\circ_{\text{Fe(III)-P}_N} - G^\circ_{\text{Fe(III)-P}_P})_{\text{el}} + (G^\circ_{\text{Fe(III)-P}_N} - G^\circ_{\text{Fe(III)-P}_P})_{\text{non-el}} \quad (8)$$

and for the reduced complex

$$(G^\circ_{\text{Fe(II)-P}_N} - G^\circ_{\text{Fe(II)-P}_P}) = (G^\circ_{\text{Fe(II)-P}_N} - G^\circ_{\text{Fe(II)-P}_P})_{\text{el}} + (G^\circ_{\text{Fe(II)-P}_N} - G^\circ_{\text{Fe(II)-P}_P})_{\text{non-el}} \quad (9)$$

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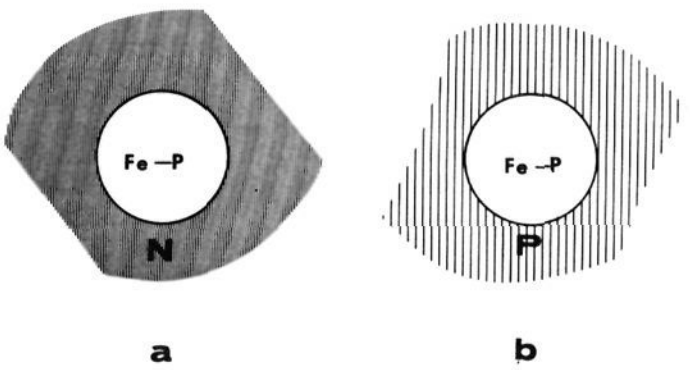

Figure 1. Schematic representation of the heme–ligand complex in nonpolar (a) and polar environments (b).

Then combining eq 6, 8, and 9 gives eq 10.

$$nF\Delta E_{0(\text{N-P})} = (G^\circ_{\text{Fe(III)-P}_\text{N}} - G^\circ_{\text{Fe(III)-P}_\text{P}})_{\text{el}} + (G^\circ_{\text{Fe(III)-P}_\text{N}} - G^\circ_{\text{Fe(III)-P}_\text{P}})_{\text{non-el}} - (G^\circ_{\text{Fe(II)-P}_\text{N}} - G^\circ_{\text{Fe(II)-P}_\text{P}})_{\text{el}} - (G^\circ_{\text{Fe(II)-P}_\text{N}} - G^\circ_{\text{Fe(II)-P}_\text{P}})_{\text{non-el}} \quad (10)$$

The Fe(III)–P complex has a formal charge of +1 relative to the Fe(II)–P complex which is considered electrically neutral due to the anionic state of two of the pyrrole nitrogen atoms. The electrostatic free energy change associated with the transfer of the neutral Fe(II)–P complex from a polar to a nonpolar environment should approach zero. Thus

$$nF\Delta E_{0(\text{N-P})} \approx (G^\circ_{\text{Fe(III)-P}_\text{N}} - G^\circ_{\text{Fe(III)-P}_\text{P}})_{\text{el}} + (G^\circ_{\text{Fe(III)-P}_\text{N}} - G^\circ_{\text{Fe(III)-P}_\text{P}})_{\text{non-el}} - (G^\circ_{\text{Fe(II)-P}_\text{N}} - G^\circ_{\text{Fe(II)-P}_\text{P}})_{\text{non-el}} \quad (11)$$

The nonelectrostatic free energy terms correspond to the difference between the chemical interactions of the heme–ligand complexes with the molecules of the two different environments. Thus, it should be apparent that to a first approximation the non-electrostatic free energy change for the transfer of the Fe(III)–P complex from a polar to a nonpolar environment should be equivalent to that for the corresponding transfer of the Fe(II)–P complex (eq 12).

$$(G^\circ_{\text{Fe(III)-P}_\text{N}} - G^\circ_{\text{Fe(III)-P}_\text{P}})_{\text{non-el}} \approx (G^\circ_{\text{Fe(II)-P}_\text{N}} - G^\circ_{\text{Fe(II)-P}_\text{P}})_{\text{non-el}} \quad (12)$$

Then

$$nF\Delta E_{0(\text{N-P})} \approx (G^\circ_{\text{Fe(III)-P}_\text{N}} - G^\circ_{\text{Fe(III)-P}_\text{P}})_{\text{el}} \quad (13)$$

Thus the difference between the redox potentials of the heme–ligand complex in the two different environments should be primarily dependent on the electrostatic free energy change, $\Delta G^\circ_{\text{el}}$, associated with the transfer of the Fe(III)–P complex from a polar to a nonpolar environment. This change reflects the difference between the electrostatic interactions of the complex with the molecules of the two different environments and should thus be equivalent to the difference between the energies required to charge an ion in two different dielectrics. The free energy change may be estimated by the Born equation[9] for a spherical ion of radius r immersed in a continuum of uniform dielectric as given by the following equation

$$\Delta G^\circ_{\text{el}} = \frac{Nz^2e^2}{2r}\left(\frac{1}{D_\text{N}} - \frac{1}{D_\text{P}}\right) \quad (14)$$

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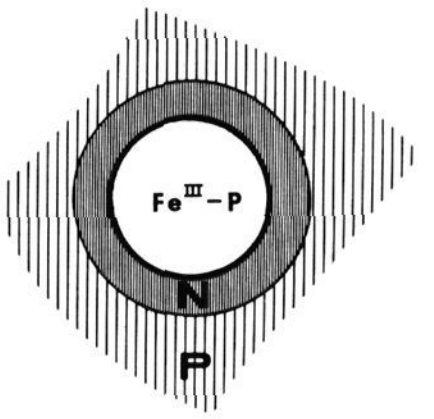

Figure 2. Schematic representation of the environment of the heme–ligand complex in cytochrome *c*.

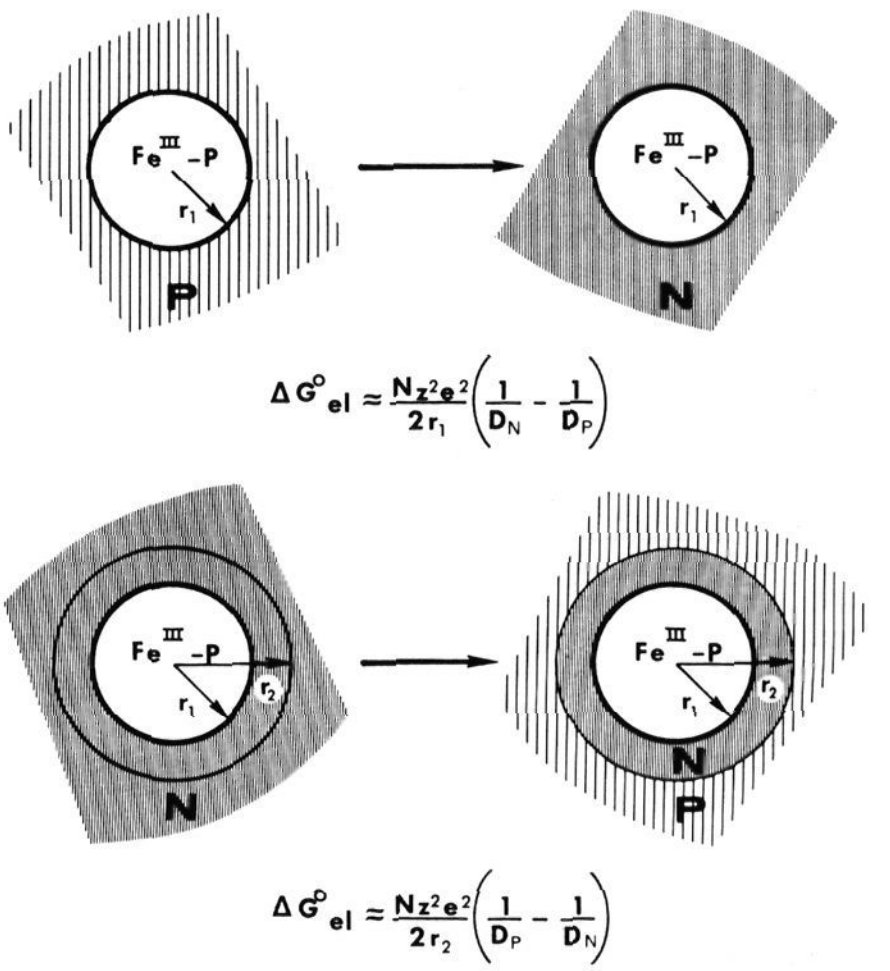

Figure 3. Schematic representation of the hypothetical, stepwise transfer reactions associated with the difference in free energy between the heme–ligand complex in the polar aqueous environment and the environment of the complex in the protein.

where N is Avogadro's number, z the charge number, e the electronic charge, and D_N and D_P the dielectric constants of the nonpolar and polar environments.

The total environment of the heme–ligand complex in cytochrome *c* can be approximated by an inner region of nonpolar amino acid side chains and an outer region corresponding to the aqueous environment of the protein (Figure 2). The inner region may be represented by a solid spherical volume element concentric to the positively charged complex and characterized by a low dielectric constant while the outer region is characterized by the dielectric constant of water. The difference between the electrostatic free energy of the Fe(II)–P complex associated with the protein in aqueous solution and the electrostatic free energy of the Fe(III)–P model complexes in aqueous solution may be equated to the sum of two free energy terms corresponding to the stepwise transfer reactions represented in Figure 3. The first term is associated with the transfer of the Fe(III)–P complex from the polar to the nonpolar en-

vironment as given by

$$\frac{Nz^2e^2}{2r_1}\left(\frac{1}{D_N}-\frac{1}{D_P}\right)$$

where r_1 is the radius of the Fe(III)–P complex. The second term is the free energy change associated with the transfer of the Fe(III)–P complex together with the nonpolar concentric volume element from the nonpolar to the polar aqueous environment as given by

$$\frac{Nz^2e^2}{2r_2}\left(\frac{1}{D_N}-\frac{1}{D_P}\right)$$

where r_2 is the radius of a sphere enclosing the nonpolar region of the protein. Thus

$$\Delta G^\circ_{el} \approx \frac{Nz^2e^2}{2r_1}\left(\frac{1}{D_N}-\frac{1}{D_P}\right)+\frac{Nz^2e^2}{2r_2}\left(\frac{1}{D_P}-\frac{1}{D_N}\right) \quad (15)$$

Rearranging terms gives eq 16 and 17.

$$\Delta G^\circ_{el} \approx \frac{Nz^2e^2}{2D_N}\left(\frac{1}{r_1}-\frac{1}{r_2}\right)-\frac{Nz^2e^2}{2D_P}\left(\frac{1}{r_1}-\frac{1}{r_2}\right) \quad (16)$$

$$\Delta G^\circ_{el} \approx \frac{Nz^2e^2}{2}\left(\frac{1}{r_1}-\frac{1}{r_2}\right)\left(\frac{1}{D_N}-\frac{1}{D_P}\right) \quad (17)$$

Thus it is seen that the electrostatic free energy change corresponds to the difference between the interaction of the Fe(III)–P complex with the concentric volume element of water and the interaction of the Fe(III)–P complex with the nonpolar volume element of the protein. Finally, the difference between the redox potentials of the model heme–ligand complexes and the *c*-type cytochromes is given by the following equation

$$\Delta E_0 \approx \frac{\Delta G^\circ_{el}}{nF} \approx \frac{Nz^2e^2}{2nF}\left(\frac{1}{r_1}-\frac{1}{r_2}\right)\left(\frac{1}{D_N}-\frac{1}{D_P}\right) \quad (18)$$

The equation may be evaluated for values of r_1, r_2, and D_N which are within limits consistent with crystal structure data for ferricytochrome *c*[10] and heme–ligand complexes.[11] The value of r_1 may be equated to the radius of a sphere of volume equivalent to that occupied by the heme–ligand complex. In this way, r_1 is estimated to be >4 and <5.5 Å. The average thickness of the spherical volume element, $r_2 - r_1$, is estimated to be >4 and <9 Å from dimensions of the 1-methylpropyl side chain of isoleucine and the benzyl group of phenylalanine. The nonpolar region consists of both aromatic and aliphatic hydrocarbon side chains and thus we estimate the lower limit of D_N to be between 2.28 and 1.89 corresponding to the dielectric constants of benzene[12] and hexane,[12] respectively. Values of ΔE_0 calculated within the above limits are recorded below in Table I.

Table I. Calculated ΔE_0 Values in Millivolts[a]

r_1, Å	$r_2 - r_1$, Å: 4.0	5.0	6.0	7.0	8.0	9.0
4.00	469	510	564	596	625	650
	384	417	461	489	512	532
4.25	428	477	516	549	576	626
	351	391	424	450	472	514
4.50	393	438	476	508	534	555
	322	358	390	415	437	455
4.75	361	405	440	470	495	517
	296	332	361	385	406	424
5.00	333	377	410	437	461	482
	273	307	336	358	378	395
5.25	309	349	381	408	431	451
	253	286	312	334	354	370
5.50	287	324	356	382	404	423
	235	266	292	313	331	347

[a] The first of the two numbers, corresponding to each value of r_1 and $r_2 - r_1$, is the ΔE_0 calculated for $D_N = 1.86$ and the second number the ΔE_0 calculated for $D_N = 2.27$.

Discussion

The calculated values for ΔE_0 in Table I indicate that the local nonpolar environment of the heme–ligand complex in cytochrome *c* could account for its observed redox potential relative to those for the model heme–ligand complexes. Thus it is observed for $D_N = 1.86$ that ΔE_0 is greater than 300 mV for values of $r_1 \leq 5.25$ Å and all values of $r_2 - r_1$, and for $D_N = 2.27$ that ΔE_0 is equivalent to 300 mV for values of r_1 less than about 4.75 Å or $r_2 - r_1$ greater than *ca.* 6 Å.

The redox potentials of *c*-type cytochromes which are much more positive than cytochrome *c* may also be accounted for within the present theoretical framework. It may be observed from Table I that for a given value of D_N the ΔE_0 increases as the value of $r_2 - r_1$ increases. In addition it may be noted that ΔE_0 also increases as the value of D_N decreases. Thus the model suggests that a local heme environment which is more extended than that in cytochrome *c* and/or is less polar than that of cytochrome *c* will lead to potentials which are more positive than that of cytochrome *c*. A more extended nonpolar environment could be achieved through multiple layers of nonpolar amino acid side chains resulting from a further wrapping of the polypeptide chain about the heme–ligand complex. A more extended nonpolar environment could also be accomplished through a less compact winding of the polypeptide chain about the heme–ligand complex such as to increase the effective value of $r_2 - r_1$. A third alternative would be the substitution of larger nonpolar amino acids for smaller nonpolar amino acids such as phenylalanine for valine, leucine, and isoleucine. A heme environment which is less polar than that in cytochrome *c* could be achieved through a protein structure which more completely surrounds the heme–ligand complex with nonpolar amino acid side chains.

Among the possible structural parameters which may affect redox potentials more positive than cytochrome *c*, that which appears most consistent with available information is a local heme environment which is less polar than that in cytochrome *c*. Thus it would appear that the formation of multiple layers of nonpolar amino acid side chains could only be accomplished in proteins of significantly higher molecular weight, which would have a much longer polypeptide chain. Yet the molecular weight of the *c*-type cytochrome having highest reported E_0' [4] is somewhat lower than that of cytochrome *c*.[13] Secondly, a less compact winding of the polypeptide chain about the heme–ligand complex would also appear to require a longer polypeptide chain

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in order to surround the complex to the same extent with nonpolar amino acid side chains. The alternative substitution of larger amino acids for smaller ones would require a large fraction of aromatic amino acids relative to aliphatic amino acids which is not evident from the reported amino acid analyses of the *c*-type cytochrome from bacteria.[13] With regard to a heme environment which is less polar than that in cytochrome *c*, it has been observed that the heme–ligand complex in cytochrome *c* sits in a crevice, the walls of which are composed of nonpolar amino acid side chains.[10] Yet there appears to be channels from the complex to the surface of the protein which are available to the solvent. It has been suggested on the basis of nmr spectra and sequence homologies between cytochrome *c* and bacterial cytochromes c_2 that additional sequences of amino acids in the bacterial cytochromes might fill one of the channels to the heme–ligand complex.[14] Such a structure would have the effect surrounding the complex to a greater extent with nonpolar groups, which would contribute to a heme environment which is less polar than that in cytochrome *c*. Consistent with this analysis is the observation that these cytochromes exhibit redox potentials which are significantly more positive than cytochrome *c*.

The influence of structural parameters other than the local nonpolar environment on the redox potential of cytochromes is not without notice. Significant free-energy changes may be associated with conformational changes attendant to oxidation and reduction. Such changes may include the transfer of apolar groups from an aqueous to a nonaqueous environment[8] or the transfer of charged groups from a polar to a nonpolar environment. The relationship between changes in conformation and the energetics of the redox process have been emphasized by Takano, *et al.*[15] Charged groups on the surface of the protein may also influence the redox potential, yet the high redox potentials of *c*-type cytochromes are not restricted to basic proteins but have also been observed for an acidic protein.[16] The ability of structural forces in the protein to affect the extent of coordination of the methionine sulfur to the heme iron in the oxidized state has also been discussed.[6,7] Yet the present theoretical treatment suggests that the high redox potentials of many *c*-type cytochromes may be accounted for by a heme environment of a nonpolar nature without structural changes attendant to the redox process, and that such an environment may have a profound effect on the redox potentials of all heme proteins.

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Reaction of Diethyl Pyrocarbonate with Nucleic Acid Components. Bases and Nucleosides Derived from Guanine, Cytosine, and Uracil

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Abstract: Diethyl pyrocarbonate, an enzyme inhibitor and bactericidal agent, reacts in aqueous solution with purine ribonucleosides to form products in which the imidazole ring has been opened, *e.g.*, compound **2** from adenosine, as previously reported, and compound **4**, 2-amino-5-carbethoxyamino-4-hydroxy-6-*N*-ribofuranosylaminopyrimidine, from guanosine. Unlike adenine, which does react, the free base guanine was not modified under the conditions employed. Cytosine and the "minor base" 5-hydroxymethylcytosine react with diethyl pyrocarbonate in aqueous solution to give respectively 1-carbethoxycytosine (**9**) and an unstable compound which appears to be the 1-carbethoxylated derivative **11**. The ribonucleoside cytidine reacted under the same conditions to give N^4-carbethoxycytidine (**13**). Uracil and thymine, in slightly basic aqueous media, give closely related ring-substituted products 1-carbethoxyuracil (**15**) and 1-carbethoxythymine (**17**). The ribonucleosides uridine and ribothymidine, by contrast, react in slightly basic solution to form unstable products which spectral data suggest to be 3-carbethoxyuridine (**19**) and 3-carbethoxyribothymidine (**21**). Pseudouridine C, a modified nucleoside found in transfer RNA, is converted by diethyl pyrocarbonate to an unstable compound which data suggest to be the 1-acylated **23**. Biological consequences of the use of diethyl pyrocarbonate as a nuclease inhibitor are considered, in view of the observed reactions of the pyrocarbonate with the individual nucleic acid components.

Diethyl pyrocarbonate (ethoxyformic anhydride or diethyl dicarbonate) has been used as a preservative in wines and other beverages.[1] The reagent is used to deactivate enzymes, presumably by blocking exposed nucleophilic functions,[2–7] thereby changing the chemical and structural integrity. Recent reports indicate,

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