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DIELECTRIC CONSTANT DEPENDENCE OF BIOLOGICAL OXIDATION-REDUCTION

1. A MODEL OF POLARITY-DEPENDENT FERROCYTOCHROME *c* OXIDATION

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A theoretical model for the effect of the dielectric constant (ϵ) of the solvent medium on ferrocytochrome *c* oxidation by ferricyanide is developed to account for the observed variations of the rate constant (k) of reactions in aqueous binary mixtures with alcohols ($<5-10$ mol% ethanol and propranol). A correlation between k and ϵ is found if $\ln k$ is expressed as a function of the Kirkwood parameter $(\epsilon-1)/(2\epsilon+1)$. The results of calculations indicate that the use of the 'overall dipole moment' of cytochrome *c* in oxidoreduction studies is likely to be unreliable. Instead, the decrease in k in alcohol/water mixtures is best explained—in conformity with Onsager's theory of the reaction field—by a polarity effect on the dipole moment of the cytochrome *c* heme upon diffusion of the polar solvent molecules into the low dielectric constant heme crevice.

1. Introduction

The studies of Mayerle et al. [1] and Job and Bruice [2] concerning solvent effects on the redox properties of iron-sulfur proteins provide convincing evidence that the redox potentials of synthetic iron-sulfur cluster complexes in nonaqueous solutions are up to 0.85 V more negative than the potentials of analogous clusters and clusters in proteins present in aqueous solutions. A theoretical model developed by Kassner and Yang [3] to explain these effects revealed that the factors which may be relevant in describing the solvent-dependent variations of the redox potentials are the difference in the charging energies (see ref. 4) in media of different dielectric constants, the charge interactions which are dependent on the local dielectric constant and the dielectric constant of a shell shielding the cluster from the surrounding medium. The model predicts that the negatively charged cluster redox potentials are more negative in solvents of lower dielectric constant. These conclusions set forth clearly the importance of the polarity of the molecular environment of redox couples in biological oxidoreduction reactions. They are especially interesting inasmuch as most of such reactions take place at water/lipid and water/lipoprotein interfaces of the biomembrane, or at its inner interfaces like the hydrophobic core and the polar head/hydrocarbon chain interface.

An aspect of this question that we wish to analyze in the present paper is the effect of the dielectric constant of the aqueous solvent on the rate constants of ferrocytochrome *c* oxidation by ferricyanide. A polarity-dependent effect associated with this type of redox reaction is suggested by a number of experiments performed in water and in aqueous binary mixtures with alcohols (ethanol, propanol) [5–10]. The work of Lebon [9] and Ilan and Shafferman [10] is most significant for the theory developed here because the authors avoided alcohol/water mixtures exceeding 5–10 mol% alcohol. In brief, it is known that below such concentrations the alcohols bring about modifications of the cytochrome *c* structure which are

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rather slight and thus exclude the possibility of protein denaturation [5,7,11].

Ilan and Shafferman [10] observed that the fast and slow rate constants of cytochrome *c* activity decreased with increasing concentration of ethanol in the aqueous medium, and concluded that the observed variations of activity are the result of structural changes of water. This conclusion, however, does not preclude the polarity effect hypothesis; the present work shows that the rate constants (k) of ferrocytochrome *c* oxidation vary, at least partly, because of polarity variations caused by the decrease in the dielectric constant (ϵ) of the aqueous medium upon addition of ethanol. We found that a correlation between k and ϵ can be made if the natural logarithm of the rate constants is expressed as a function of $(\epsilon - 1)/(2\epsilon + 1)$, in conformity with Kirkwood's theory of solutions of molecules containing widely separated charges [12]. In conjunction with this, we recognized a low dielectric constant effect on the dipole moment of cytochrome *c* in accordance with Onsager's theory of the reaction field [13,14].

2. Theory

2.1. Dielectric constant dependence of a bimolecular reaction

It is remarked first that ionic strength effects [15] and polarity-induced shifts of the dissociation constant of the solute [16,17] are not considered in the approximation discussed hereinafter. This will be the object of a second paper in this series.

To calculate the free energy of transfer (ΔG) of a strong dipole from a medium of dielectric constant unity to one of dielectric constant ϵ, one may use Kirkwood's approximation [12] which neglects the short-range van der Waals' forces and attributes the deviation from ideal behavior solely to electrostatic forces. This quantity is related to the activity coefficient γ for which a medium of dielectric constant unity is the standard state.

For a single molecule with an asymmetric charge distribution required to generate a dipole moment, the result is

$$\Delta G = \frac{RT}{N} \ln \gamma = -\frac{\mu^2}{b^3}\left[\frac{(\epsilon-1)}{(2\epsilon+1)}\right] \tag{1}$$

where μ is the dipole moment, b the radius of the molecule, T the absolute temperature (K), R the gas constant (erg K^{-1} mol^{-1}) and N Avogadro's number.

For a bimolecular reaction of the type

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AB^{\ddagger} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{products}, \tag{2}$$

which represents reasonably well the reaction of ferrocytochrome *c* with ferricyanide [18], and where $AB^{\ddagger}$ is the activated complex, one obtains

$$\ln k = \ln k_0 - \frac{N}{RT}\left(\frac{\mu_A^2}{b_A^3} + \frac{\mu_B^2}{b_B^3} - \frac{\mu_{AB^{\ddagger}}^2}{b_{AB^{\ddagger}}^3}\right)\left(\frac{\epsilon-1}{2\epsilon+1}\right). \tag{3}$$

in which, according to Brønsted [19] and Bjerrum [20], the rate constant (k) of the reaction and the specific reaction rate (k_0) are related by the expression

$$k = k_0 \frac{\gamma_A \gamma_B}{\gamma_{AB^{\ddagger}}}. \tag{4}$$

The accuracy of eq. 3 has been confirmed with chemical reactions in solvents of high dielectric constant [21–23], whereas with reactions in low dielectric constant media it is observed that $\ln k$ versus $(\epsilon - 1)/(2\epsilon + 1)$ may deviate from linearity (e.g., see ref. 24). Although the reasons for this behavior are not well understood, we anticipate that this effect is probably the result of special solute-solute interactions in solvent media where forces of the London–van der Waals' type predominate. The analysis of this question is under study in our laboratory.

To apply eq. 3 to redox reactions between a protein having an asymmetric charge distribution and a small ion it is necessary to consider the coordinates of the center of positive charge (p), negative charge (n) and mass (m). The dipole moment of the protein is given by [25]

$$\mu = Yre, \tag{5}$$

in which r is the vector from n to p assuming that m coincides with p, Y the total number of dipoles and e the elementary charge in electrostatic units.

Hence, for a reaction between a small ion A (i.e., ferricyanide) where Y_A is taken as being equal to unity and a macromolecule B (i.e., cytochrome

c), eq. 3 is found to be

$$\ln k = \ln k_0 - \frac{Ne^2}{RT}\left[\frac{r_A^2}{b_A^3} + \frac{Y_B^2 r_B^2}{b_B^3} - \frac{Y_{AB^\ddagger}^2 r_{AB^\ddagger}^2}{b_{AB^\ddagger}^3}\right]\left[\frac{\epsilon - 1}{2\epsilon + 1}\right] \quad (6)$$

The term r_A^2/b_A^3 vanishes if one assumes that $r_A \leq 1$ Å (i.e., $\mu_A \leq 4.8$ debye *), $b_A = 4.19$ Å (obtained from crystallographic data [43]), $r_B = 4.59$ Å, $b_B = 18.5$ Å, $Y_B = 13$ and $Y_{AB^\ddagger} = 14$ (obtained from ref. 32), $r_{AB^\ddagger} = 13.75$ Å (see section 3.2 and table 1) and $b_{AB^\ddagger}$ is equal to b_B; thus, the ratio $(r_A^2/b_A^3):(Y_B^2 r_B^2/b_B^3):(Y_{AB^\ddagger}^2 r_{AB^\ddagger}^2/b_{AB^\ddagger}^3)$ is about 0.01:0.56:5.85. Under these conditions, for values of ϵ greater than approx. 20 (because of reasons discussed above) a plot of $\ln k$ against $(\epsilon - 1)/(2\epsilon + 1)$ is represented by a straight line with a slope given by

$$\frac{d \ln k}{d[(\epsilon - 1)/(2\epsilon + 1)]} = -\frac{Ne^2}{RT}\left[\frac{Y_B^2 r_B^2}{b_B^3} - \frac{Y_{AB^\ddagger}^2 r_{AB^\ddagger}^2}{b_{AB^\ddagger}^3}\right]. \quad (7)$$

2.2. Onsager's model of a dipole in a dielectric

To assess the effect of the polarity of the solvent medium on a molecule having an asymmetric charge distribution, we used Onsager's theory of the reaction field applied to liquid media [13,14]. This theory predicts that the field of an ideal point electric dipole of moment μ at the center of a spherical cavity of radius a, immersed in a continuous medium of dielectric constant ϵ, polarizes its molecular environment. This inhomogeneous polarization of the surrounding matter gives rise to an electric field at the dipole, i.e., Onsager's reaction field (R_f), having the same direction as the dipole vector μ and given by the expression:

$$R_f = f\mu. \quad (8)$$

in which f, the factor of the reaction field, is

$$f = \frac{2}{a^3}\left[\frac{(\epsilon - 1)}{(2\epsilon + 1)}\right]. \quad (9)$$

* To the best of our knowledge there are no available data in the literature concerning the dipole moment of ferricyanide. Therefore, the μ_A value was estimated by comparison with μ data for similar molecules. We remark that it may be possible that $\mu_{\text{ferricyanide}} = 0$ (in which case $r_A = 0$ in eq. 6) because ferricyanide is a symmetrical molecule with the six cyanide groups arranged octahedrally about the iron atom (cf. p. 1030 of ref. 42).

For a polarizable point dipole the reaction field R_f induces a dipole αR_f and is given by the expression:

$$R_f = f(\mu + \alpha R_f). \quad (10)$$

where α is the average polarizibility of the permanent dipole. The moment of the dipole under the influence of the reaction field (μ^*) increases to a value

$$\mu^* = \mu + \alpha R_f. \quad (11)$$

An approximate calculation of this increase in the dipole moment is obtained from the expression [14]:

$$\frac{\mu^*}{\mu} = \left[\frac{(2\epsilon + 1)}{(2\epsilon + n_D^2)}\right]\left[\frac{(n_D^2 + 2)}{3}\right], \quad (12)$$

where n_D is the refractive index for the Na_D line. When the dipole is not surrounded by molecules of the same kind, n_D refers to the pure dipole compound, whereas ϵ is the dielectric constant of the binary mixture of solute and solvent.

Eq. 12 is useful to evaluate the dipole moment of a spherical molecule in a medium of known dielectric constant. However, it is emphasized that an approximation based on a spheroidal model instead of a sphere is generally more realistic, since the shape of almost all molecules can be best represented by an ellipsoid. The following expression was derived for ellipsoid particles [14,26]:

$$\frac{\mu^*}{\mu} = \frac{[\epsilon + (1 - \epsilon)A_\beta][1 + (\epsilon_i - 1)A_\beta]}{[\epsilon + (\epsilon_i - \epsilon)A_\beta]}. \quad (13)$$

where ϵ_i is the dielectric constant of a homogeneous ellipsoid, and A_β is a factor given by

$$A_\beta = \frac{abc}{2}\int_0^\infty \frac{ds}{(s + \beta^2)R}, \quad (14)$$

with $\beta = a,b,c$, and $R^2 = (s + a^2)(s + b^2)(s + c^2)$, where a, b and c are the ellipsoid axes. Extensive tabulations of A_β as a function of a, b and c are available in the literature [14,27,28], thus avoiding difficulties inherent to the evaluation of the integral of eq. 14. It is remarked that eq. 13 can be applied in particular to oblate and prolate particles if $a/b = a/c$ and μ lies along the a-axis of the ellipsoid [14].

3. Data analyses and interpretations

3.1. The Kirkwood model

Kinetic data obtained by Lebon [9] for the oxidation of ferrocytochrome *c* by ferricyanide in aqueous binary mixtures with propanol (≤ 10 mol%) are illustrated in fig. 1. It is seen that a plot of the natural logarithm of the pseudo-first-order reaction rate constant (k') as a function of $(\epsilon - 1)/(2\epsilon + 1)$ obeys a linear relation (regression correlation coefficient = 0.97), in agreement with Kirkwood's theory (see section 2). A similar plot concerning the results of Ilan and Shafferman [10] on the fast and slow reactions of ferrocytochrome *c* oxidation (see also ref. 29) is shown in fig. 2. $\ln k$ versus $(\epsilon - 1)/(2\epsilon + 1)$, where k is the reaction rate constant for the oxidation of cytochrome *c* by $Fe(CN)_6^{3-}$, yields two straight lines within reasonable limits of error. The lines were obtained by a linear least-squares curve-fitting program, and are represented by the equations

$$\ln k_{fast} = -1339.04 + 2770.14(\epsilon - 1)/(2\epsilon + 1), \quad (15)$$

and

$$\ln k_{slow} = -1375.48 + 2836.99(\epsilon - 1)/(2\epsilon + 1). \quad (16)$$

The linear regression correlation coefficients were calculated to be 0.98 for the two equations.

Note that the reaction rate constants k_{fast} and k_{slow} (cf. fig. 2, and Table II of ref. 10) vary from 2.9×10^8 to 1.3×10^8 M^{-1} s^{-1} and from 7.8×10^6 to 3.2×10^6 M^{-1} s^{-1}, respectively—an approx. 2-fold change—while the dielectric constant decreases from 77.9 (0.18 mol% ethanol) to 75.4 (5.0 mol% ethanol). Excluding any denaturation of the cytochrome *c* molecule at ethanol concentrations up to 5 mol% (see section 1), the reason for the decrease in k with decreasing ϵ should be sought in microenvironmental effects represented in eqs. 15 and 16 by the Kirkwood parameter $(\epsilon - 1)/(2\epsilon + 1)$ and the dipolar terms of their respective angular coefficients (cf. eq. 3). In other words, one has to look for variations of the dipole moment of cytochrome *c* with changes in polarity of the solvent medium as predicted by eq. 11.

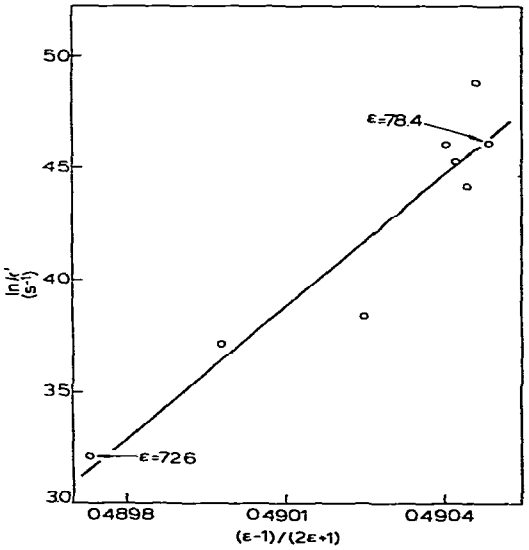

Fig. 1. Natural logarithm of pseudo-first-order rate constant k' versus $(\epsilon - 1)/(2\epsilon + 1)$ for reactions of ferrocytochrome *c* with ferricyanide in binary mixtures of propanol and H_2O at 25°C (data from ref. 9).

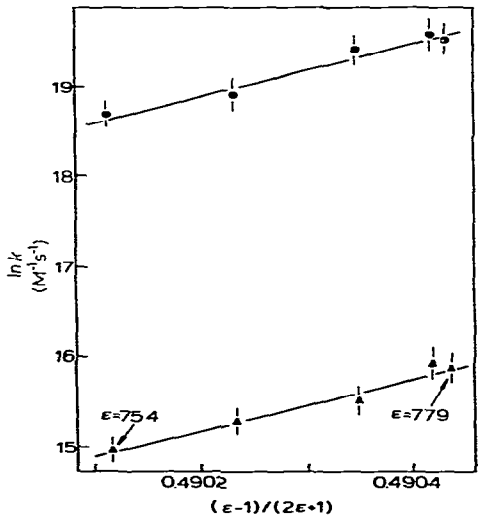

Fig. 2. Natural logarithm of the rate constants k_{fast} (●) and k_{slow} (▲) as a function of $(\epsilon - 1)/(2\epsilon + 1)$ for reactions of ferrocytochrome *c* with ferricyanide in binary mixtures of ethanol and H_2O at 22°C (data from ref. 10).

Table 1

Dipole moments[a] of ferrocytochrome c (B) and transition state complexes ($AB^{\ddagger}$) of its reaction with ferricyanide (A) in binary mixtures of ethanol and water (data from ref. 10) of ϵ between 75 and 78 (average $\mu^*/\mu = 1.27$)

		γ[b]	r[c]	r^*	μ	μ^*	μ^*_{max}
B[d]	fast and slow reactions	13	4.59	5.83	286	363	365
$AB^{\ddagger}$	fast reaction	14		13.75		924	925
	slow reaction	14		13.89		933	934

[a] Dipole moments (μ, μ^*, μ^*_{max}) are expressed in debye (1 debye $= 1.10^{-18}$ esu cm); μ^*_B was calculated according to eq. 13 with $a/b = a/c = 1.25$ and $\epsilon_i = 2$; $\mu^*_{AB^{\ddagger}}$ values were calculated from eq. 7 using the angular coefficients given by eqs. 15 and 16 and the calculated μ^* value of B.

[b] Number of dipoles.

[c] All distances (r, r^*) are given in Å.

[d] In this approximation r and r^* for the fast and slow reactions are assumed to be identical.

3.2. Extent of the polarity effect on the overall dipole moment

To this end, we will describe the results of some calculations done according to eqs. 7 and 13. Cytochrome *c* is essentially a globular protein [30] which could be represented crudely as a prolate ellipsoid of protein enclosing the tetrapyrrole macrocycle. In the calculation of μ^*_B (dipole moment of cytochrome *c*) and $\mu^*_{AB^{\ddagger}}$ (dipole moment of the activated complex), we used, as a first approximation, a spheroidal model with an axial ratio $a/b = a/c = 1.25$. The dielectric constant ϵ_i of the inner part of the molecule was chosen to be 2 (see ref. 31). Horse cytochrome *c* has a maximum number of 13 negatively charged groups, and thereby 13 dipoles [32]. According to Koppenol et al. [32], the distance between the centers of negative and positive charges is 4.59 Å in ferrocytochrome *c*. The dipole moments were calculated as follows: μ^*_B was obtained from eq. 13 and the data given above; $\mu^*_{AB^{\ddagger}}$ was obtained from eq. 7 using the angular coefficients given by eqs. 15 and 16 and the calculated μ^*_B values. The results are presented in table 1. It is seen that for ϵ between 75 and 78 (cf. fig. 2), μ^*_B has an almost constant value (≈ 363 debye) which is 1.27-times the value of the dipole moment of reduced cytochrome *c* ($\mu_B = 286$ debye) obtained by Koppenol et al. [32] using the atomic coordinates of the protein. Under these conditions, $\mu^*_{AB^{\ddagger}}$ is equal to 924 debye for the fast reaction of cytochrome *c* oxidation and 933 debye for the slow reaction *. These values are practically identical to their respective maxima (cf. fig. 3) which are obtained at very large values of the dielectric constant, i.e., $\mu^*_B = 365$ debye and $\mu^*_{AB^{\ddagger}} = 925$ debye (fast reaction), and $\mu^*_{AB^{\ddagger}} = 934$ debye (slow reaction).

It is obvious from these calculations that the rather marked invariance of the global dipole moment of ferrocytochrome *c* in water/ethanol mixtures does not explain the linear plots of $\ln k$ versus $(\epsilon - 1)/(2\epsilon + 1)$ represented in fig. 2. This suggests that the use of the overall dipole moment of redox polypeptides in biological oxidoreduction studies (as an example, cf. recent works by Koppenol et al. [32] and Koppenol [25]) is an unreliable simplification. Further evidence to reinforce this assumption is the finding that the apparent minimum charge at the site of electron transfer in cytochrome *c* is +1.3 [41], in contrast with the average net protein charge of +8 to +13 [32]. This discrepancy was noted by Miller and Cusanovich [41]. Cusanovich [18] remarked correctly that it might indicate that the site of electron transfer does not involve the net protein charge but that a specific site of electron transfer should be involved.

* The reason for this increase in μ^* of about 3-fold seems to be a displacement of charges upon formation of the transition state complex (cf. table 1).

3.3. The heme crevice as a possible site of the polarity effect

We will show next that the polarity effect on ferrocytochrome *c* oxidation by ferricyanide is better explained while confining the redox center to the tetrapyrrole macrocycle, and thereby limiting the shell shielding the cluster to the low dielectric constant heme pocket. This question was approached previously by Kassner [31], who aimed at developing a theoretical model to explain the effects of the nonpolar heme environment on the redox potentials in cytochromes. Kassner's approximation, however, is a special treatment of the heme-ligand interactions based on considerations raised by Born's equation (cf. ref. 4, and also the discussion in ref. 33) which neglects the heme dipole moment, and will be therefore disregarded here.

Evidence for the participation of the cytochrome heme in redox processes comes from the recognition that this protein possesses the structural features necessary to undergo oxidoreduction by an outer-sphere reaction mechanism. First, there is considerable electron spin-density delocalization over the whole tetrapyrrole macrocycle [34]. Second, the heme group is embedded in a hydrophobic pocket with only one edge exposed and a ring of lysines around the edge [35]. Third, a body of physical and chemical data suggests [36] that the reactions of cytochrome *c* with small molecules take place near the exposed edge of the heme. Moreover, it is well established that ferricyanide forms a strongly bound complex with cytochrome *c*, presumably in the vicinity of the lysine cluster near the exposed edge. The presence of this edge at the surface of the protein could potentially allow π-π interactions, due to orbital overlap between the electron acceptor, the conjugated π-orbital system of the porphyrin ring and the aromatic residues on the cytochrome.

A polarity effect over the heme ring influence efficiently its dipole moment as a result of fluctuations of the charge distribution of the conjugated π-orbital system. To illustrate this effect, a plot of μ^*/μ versus ϵ according to eq. 13 for several model ellipsoids is presented in fig. 3. First, the results indicate that for $\epsilon > 20$—incidentally the

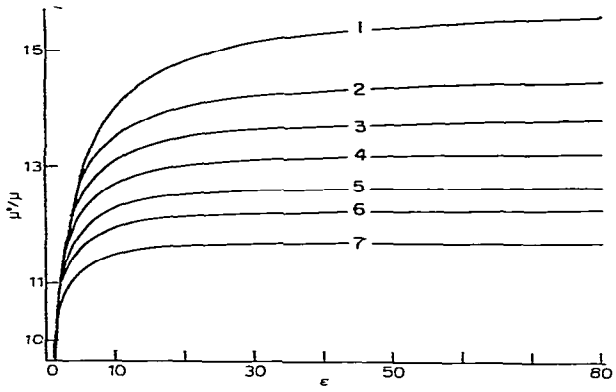

Fig. 3. Plot of dipolar ratios μ^*/μ vs. ϵ for spherical (curve 4), oblate (curves 1–3) and prolate particles (curves 5–7) with axial ratios $a/b = a/c$. The μ^*/μ values were obtained from eq. 13 with $\epsilon_i = 2$ and $A_\beta = 0.583$ (1), 0.464 (2), 0.394 (3), 0.333 (4), 0.276 (5), 0.233 (6), 0.174 (7). Axial ratios ($a/b = a/c$): curve 1, 0.40; curve 2, 0.60; curve 3, 0.80; curve 4, 1.00; curve 5, 1.25; curve 6, 1.50; curve 7, 2.00.

polarities which prevail at the polar head interfaces of lipid bilayers [23,37] and in aqueous media—the polarity of the dipole environment does not seem to influence significantly μ^*/μ. Instead, it is the shape of the particle that affects chiefly the magnitude of μ^*. Second, we observe that the major variations of μ^*/μ with ϵ, $d(\mu^*/\mu)/d\epsilon$, take place in low dielectric constant media ($\epsilon < 20$). It is seen, in addition, that oblate particles with axial ratios $a/b = a/c < 0.6$ are particularly sensitive to polarity variations. For instance, assuming that the tetrapyrrole macrocycle of cytochrome *c* can be modelled roughly as an oblate ellipsoid with an axial ratio $a/b = a/c \approx 0.4$, we conclude from fig. 3 (cf. curve 1) that slight polarity variations of the low dielectric constant porphyrin environment affect significantly the heme μ^*/μ ratio. This is best seen in fig. 4, which represents the rate of variation of μ^*/μ with ϵ for the axial ratio 0.4 given by the expression:

$$\frac{d(\mu^*/\mu)}{d\epsilon} = \frac{2.21}{(\epsilon+2.80)^2}. \qquad (17)$$

The figure indicates that the major variations of μ^*/μ occur at ϵ values smaller than about 20.

Variations of polarity of the hydrophobic

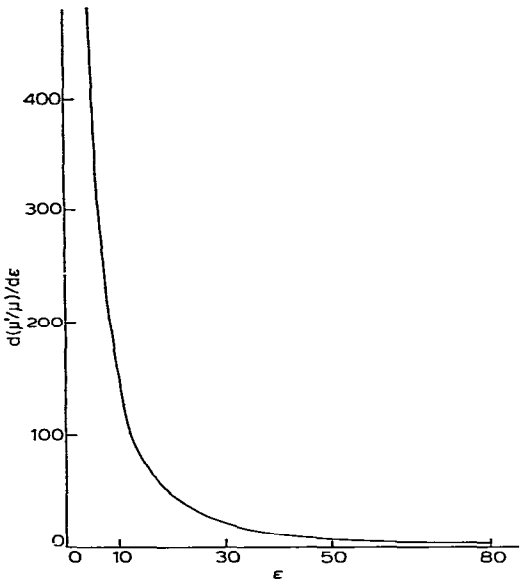

Fig. 4. Rate of variation of the dipolar ratios μ^*/μ with ϵ calculated according to eq. 17 for an oblate particle with an axial ratio $a/b=a/c=0.4$.

porphyrin environment can be conceived with reasonable certainty, since it is known that cytochrome *c* is susceptible to a number of discrete conformation changes [38,39] which may give rise to a sufficiently large opening of the heme crevice to allow the passage of permeants. This argument is supported by the suggestion of Cooper [40] that H_2O may pervade through the heme pocket. This is interesting because H_2O molecules may give rise to displacements of the iron atom within the heme framework, and thus be at the origin of changes in the distance separating the redox centers in the ferricyanide-ferrocytochrome complex. This situation is not unusual. For example, in related systems—hydrated chlorophyll aggregates—it is well established that water constrains the magnesium atom in a small region approx. 0.4 Å out of the plane of the tetrapyrrole macrocycle [44], and this in turn affects the function of the pigments in the photosynthetic membrane [45].

Accordingly, a simple mechanism which is consistent with discrete water-induced fluctuations of the Fe(II) atom about its average position concomitant with variations of electron-transfer activity is tunneling of electrons through a potential barrier [46–48]. This is mainly due to the high sensitivity of the tunneling time to slight variations of the barrier width (tunneling distance). Evidence in favor of the application of the tunnel hypothesis to the present studies comes from Ilan and Shafferman's work itself (see p. 131 of ref. 10), for these authors found that the kinetics of ferrocytochrome *c* oxidation by ferricyanide are not affected by temperature between 5 and 35°C. Now, temperature independence has been often taken as a reliable test for the function of tunneling [46,48].

To calculate the width (d) of the barrier through which tunneling occurs we use the following equation applied to a rectangular barrier [48]:

$$d=\left\{\hbar/\left[8m(V-E)\right]^{1/2}\right\}\ln\left[8E^2(V-E)/k_d\pi\hbar V^2\right]. \quad (18)$$

where $\hbar$ is Planck's constant divided by 2π, m the mass of the electron, V the potential energy of the electron inside the barrier with potential energy outside equal to zero, E the kinetic energy of the electron outside the barrier, $(V-E)$ the barrier height and k_d the probability of barrier penetration by tunneling, i.e., the tunneling rate constant. To a first approximation, let us put $E=1$ eV and $V-E=1$ eV (cf. refs. 47 and 48). Eq. 18 then simplifies to

$$d=33.47-0.97\ln k_d, \quad (19)$$

where d is in Å and k_d in s^{-1}. The results are summarized in table 2.

First, note that the calculated potential barrier widths of about 25 Å do not differ significantly (except for Hopfield's results of 8–10 Å [49]) from data available in the literature; namely, 20 Å for electron transfer, from excited states of metalloporphyrins to acceptors in solution [50], 25 and 33 Å for cytochrome *c* photooxidation in *Rhodopseudomonas palustris* [47], and 30 Å for the light-induced EPR signal and optical changes in reaction centers of *Rhodopseudomonas sphaeroides* [48].

In addition, the table shows clearly that a slight change in d from 24.67 Å (cytochrome *c* reactions in 0.18 mol% ethanol [10], or in its absence [51]) to 25.45 Å (reactions in 5.0 mol% ethanol [10]) corresponds to a large variation of k_{fast} from 2.9×10^8

Table 2

Tunneling distances [a] (d) for the reactions of ferrocytochrome c with ferricyanide (fast phase of reaction rate constant k_{fast}) in binary mixtures of ethanol and water (data from ref. 10)

Ethanol concentration (mol%)	k_{fast} ($M^{-1}\,s^{-1}$) ($\times 10^{-8}$)	k_d^b (s^{-1}) ($\times 10^{-3}$)	d (Å)
0.18	2.9	8.7	24.67
1.5	2.6	7.8	24.77
3.2	1.6	4.8	25.25
5.0	1.3	3.9	25.45

[a] Calculated from eq. 19.

[b] The tunneling rate constants (k_d) were estimated by multiplying k_{fast} by an average 3×10^{-5} M concentration of ferricyanide (cf. Table II of ref. 10).

to 1.3×10^{8} $M^{-1}\,s^{-1}$, respectively. These data support earlier beliefs that cytochrome c oxidation-reduction is not accompanied by large conformational changes of the protein [38,39]. An explanation compatible with the results of table 2 is a mechanism involving essentially submolecular rearrangements at the inner portion and the hydrophilic edge of the heme macrocycle.

4. Conclusion

In a general way, we note that the dielectric constant variations of the redox center environment of cytochrome c are at the origin of free energy changes in the molecule manifested as changes in $\ln k$ versus $(\epsilon - 1)/(2\epsilon + 1)$ in conformity with Kirkwood's theory, and give rise therefore to perturbations of the oxidation levels of the protein. These variations seems to be the result of diffusion of solvent water into the hydrophobic heme crevice. In this respect, it is worthy of mention that Salemme [38] postulated previously that the complex formation between cytochrome b_5 and cytochrome c causes the bulk solvent water to be excluded from the intermolecular interface with a reduction in the dielectric constant along the path of interheme communication and an increase in the rate constant of electron transfer (cf. pp. 323–324, and fig. 13 of ref. 38). This is in agreement with the point made by Libby [52] that water probably has to be excluded from the vicinity of the donor (or receptor) iron-containing heme group of globular proteins with internal electron donor-receptor systems because of constraints imposed by the electronic Franck-Condon principle (see discussions in refs. 46 and 53). We conclude that transient inward-outward movements of solvent molecules alter to some extent the steric distribution of hydrophobic and hydrophilic groups at the heme crevice and thereby affect the rate constants of ferrocytochrome c oxidation.

In addition to the aforementioned bulk solvent-protein interactions, it is tempting to consider the curves of fig. 2 as the representation of an overall effect resulting from a dualism peculiar to cytochrome c function. That is, during oxidation of this protein by ferricyanide, the chemical changes are the increase in the electrostatic charge of the buried heme iron from $+2$ to $+3$ and the electron exchanges with ferricyanide at the exposed heme edge. Therefore, the actual situation may be represented by an electron-exchange reaction involving a hydrophilic pair of reactants and another exchange reaction involving a hydrophobic pair of reactants. The reaction rate constant would thus be under the control of a hydrophilic-hydrophobic interaction mechanism. This conclusion is in close agreement with that of Adman [35] on the structure-function relationships of the redox centers of electron-transfer proteins: i.e., a modulation of the energy levels is provided by placing the protein redox center in a hydrophobic environment with the possibility of formation of occasional hydrogen bonds.

The discussion above of the polarity properties of cytochrome c displays the complexity of interfacial phenomena occurring at the heme pocket and the heme hydrophilic edge. Changes in local surface charge distribution due to screening (or shielding) by ions present in the surrounding medium complicate further these phenomena. The present studies are now carried on with emphasis on the combined effects of solvent polarity and ionic strength.

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