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THE ELECTRIC POTENTIAL FIELD AROUND CYTOCHROME *c* AND THE EFFECT OF IONIC STRENGTH ON REACTION RATES OF HORSE CYTOCHROME *c*

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Summary

1. The electric potential fields around tuna ferri- and ferrocytochrome *c* were calculated assuming that (i) all of the lysines and arginines are protonated, (ii) all of the glutamic and aspartic acids and the terminal carboxylic acid are dissociated, and (iii) the haem has a net charge of +1*e* in the oxidized form.

2. Near the haem crevice high values for the potential ($>+2.5\ kT/e$) are found. Consequently, electron transfer via the haem edge is favored if the oxidant or reductant is negatively charged.

3. The inhomogeneous distribution of charges leads to a dipole moment of 244 and 238 debye for oxidized and reduced tuna cytochrome *c*, respectively. Horse cytochrome *c* has dipole moments of 303 (oxidized) and 286 (reduced) debye.

4. A line through the positive and negative charge centres, the dipole axis, crosses the tuna cytochrome *c* surface at Ala 83 (positive part) and Lys 99 (negative part). The direction of the dipole axis of horse cytochrome *c* is very similar. Since the centre of the domain on the cytochrome *c* surface, which is involved in the binding to cytochrome *c* oxidase, is found at the β-carbon of the Phe 82 in horse cytochrome *c* (Ferguson-Miller, S., Brautigan, D.L. and Margoliash, E. (1978) J. Biol. Chem. 253, 149—159) it is suggested that the direction of the dipole is of physiological importance.

5. The activity coefficients of horse ferri- and ferrocytochrome *c* were calculated as a function of ionic strength using a formula derived by Kirkwood (Kirkwood, J.G. (1934) J. Chem. Phys. 2, 351—361).

6. Due to the high net charge at pH 7.5 the influence of the dipole moments

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of horse ferri- and ferrocytochrome *c* on the respective activity coefficients can be neglected at $I \leqslant 50$ mM.

7. Using the Brønsted relation the effect of ionic strength on reaction rates of horse cytochrome *c* was calculated. Good agreement is found between theory and experimental results reported in the literature.

Introduction

Tuna cytochrome *c* is a relatively small protein which is composed of 103 amino acids linked together in a single polypeptide chain wrapped around a haem. The crystal structures of oxidized and reduced tuna cytochrome *c* have been resolved recently by X-ray crystallography [1,2]. It was concluded that these structures were identical within the limits of resolution [3], although there are 'chemical' differences between the ferri and ferro form [4].

Quite a few amino acid residues are ionized at pH 7.5. The resulting charges are not homogeneously distributed over the protein surface; in two recent review articles [5,6], the role of positive groups around the haem crevice and negative groups at the 'back' has been stressed with regard to the reaction of cytochrome *c* with its physiological redox partners. This particular charge distribution is also important for the reaction of small, charged molecules with cytochrome *c*.

We present here the results of calculations which quantitatively show that the charge distribution is asymmetric. From an electrostatic point of view one can consider cytochrome *c* to be a superposition of a monopole, a dipole, a quadrupole, etc. We calculated the dipole moment and neglected quadrupole and higher order moments. Then, using Kirkwood's theory [7], we computed the activity coefficients of cytochrome *c* as a function of ionic strength. According to this theory the activity coefficient is determined by the net charge, the dipole moment and higher order moments of the molecule. This calculation was made for horse cytochrome *c*, since this cytochrome is more often used in experiments.

Part of this work has been presented at the 174th National Meeting of the American Chemical Society [8].

Methods and Results

We have assumed that tuna ferricytochrome *c* has 28 surface charges *e* at pH 7.5:

10 negative charges: Asp 2, 50, 62, 93; Glu 21, 44, 66, 69, 90; C-terminal end (Ser 103)
18 positive charges: Lys 5, 7, 8, 13, 25, 27, 39, 53, 55, 72, 73, 79, 86, 87, 88, 89; Arg 38, 91

The haem has a charge of only $+1e$ because the propionic side chains are involved in hydrogen bonding [2]. Thus ferri- and ferrocytochrome *c* have net charges of $+9e$ and $+8e$, respectively. Using atomic coordinates obtained from the Protein Data Bank, Brookhaven National Laboratories [3] we calculated

absolute values for the electric potential field outside the ferricytochrome *c* molecule according to Eqn. 1:

$$V(\vec{r}) = \frac{1}{4\pi\epsilon_0 D} \sum_{i=1}^{19} \frac{+e}{|\vec{r} - \vec{r}_i|} + \sum_{j=1}^{10} \frac{-e}{|\vec{r} - \vec{r}_j|} \quad \text{(S.I. units)} \tag{1}$$

in which $V(\vec{r})$ is the potential at $\vec{r}$; $|\vec{r} - \vec{r}_i|$ is the distance between $\vec{r}$ and $\vec{r}_i$; $\vec{r}_i$ is the location of charge i; ϵ_0 is the permittivity constant, $8.85 \cdot 10^{-12}$ F/m; e is the elementary charge, $1.60 \cdot 10^{-19}$ C, and D is the dielectric constant of water, 80.

To calculate the potential field around ferrocytochrome *c* (c^{2+}) we used the coordinates of the 28 charges on the surface of the ferricytochrome *c* (c^{3+}) molecule, but we left out the single positive charge at the haem.

The results of these calculations are expressed in units of kT/e in which k is Boltzmann's constant and T the temperature in K. Figs. 1 and 2 show equipotential lines in three parallel cross-sections perpendicular to the crystallographic Z axis.

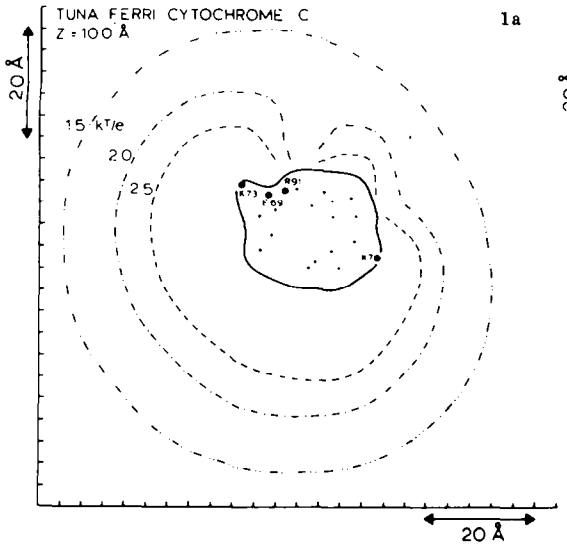
The 1 kT/e surface (not shown) is almost spherical and is found at a distance of approx. 50 Å from the protein surface of ferricytochrome *c*. The 1.5 kT/e surface is indented at one place, near serine 103. The 2 kT/e and the higher kT surfaces have irregular shapes at the 'back' of the molecule, where they cross the protein surface several times. At the front and at the methionine 80 side the 2.5 kT/e surface is 10—15 Å away from the outside of the protein, indicating that high values for the potential are reached near the protein surface. Ferrocytochrome *c* is surrounded by a similar field, but the kT/e lines are closer to the protein surface. These results are valid for the unrealistic case when the ionic strength is zero. A correction factor to include the effect of ionic strength is shown in Eqn. 2 [9].

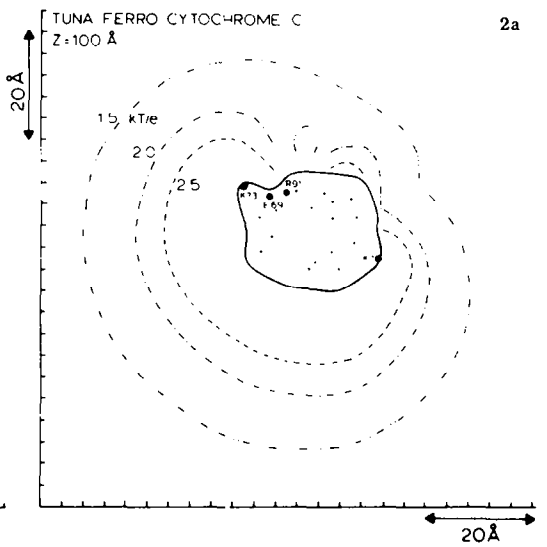
$$V(\vec{r})_I = V(\vec{r})_{I=0} \cdot \frac{e^{\kappa(a - |\vec{r} - \vec{r}_c|)}}{1 + \kappa a} \tag{2}$$

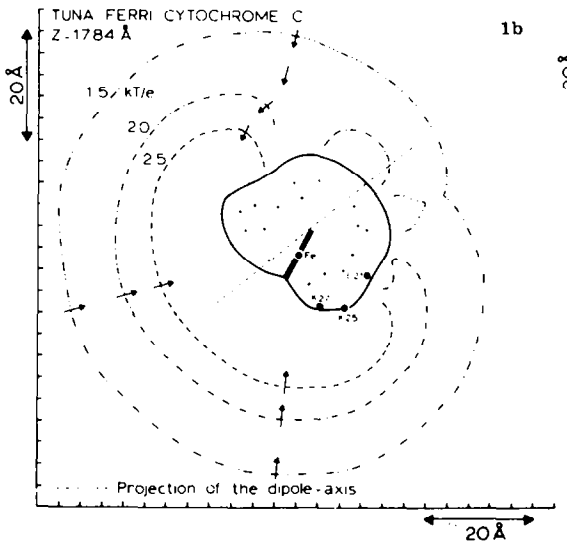
in which κ is proportional to the square root of ionic strength, a is the radius of cytochrome *c* plus the radius of a water molecule, 18.5 Å, and $\vec{r}_c$ is the centre of the molecule. This correction factor can be used only for spherically symmetrical kT/e surfaces, i.e. $kT/e < 1.5$. Some cross-sections show a nearly circular 1.5 kT/e line. We have applied Eqn. 2 to one of these lines and the numerical results are presented in Table I.

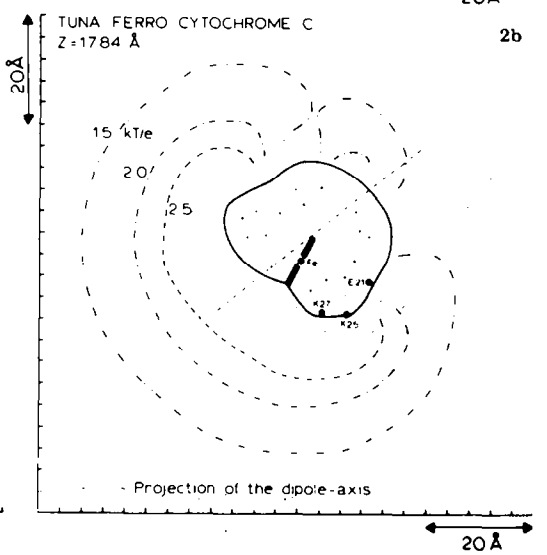
The 1.5 kT/e line is indented at the 'back' of the molecule (see for instance Fig. 1c) as a result of the inhomogeneous charge distribution on the surface of the protein. This distribution of charges leads to a dipole and higher order multipoles superimposed on a monopole.

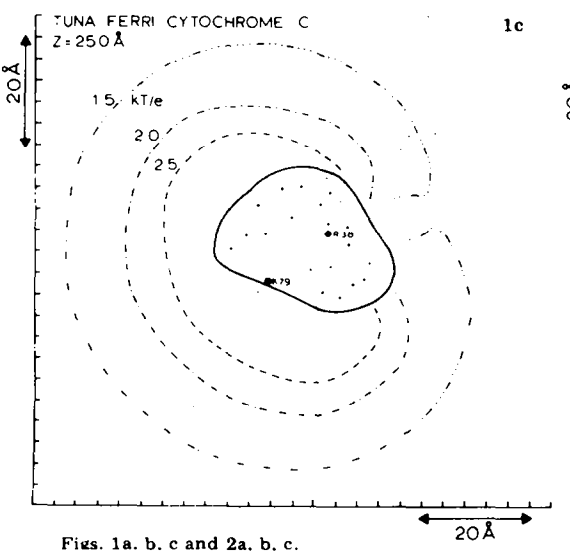
We calculated the coordinates of the charge centres of all positive and all negative charges. The distance between these two centres is 5.09 Å in c^{3+} and 4.97 Å in c^{2+}. There are 10 negative charges and hence the dipole moments are: $0.81 \cdot 10^{-27}$ C · m (244 debye) and $0.79 \cdot 10^{-27}$ C · m (238 debye), respectively. A line through the two centres in tuna c^{3+}, the dipole axis, crosses the protein surface at Ala 83 (positive part) and at Lys 99 (negative part).

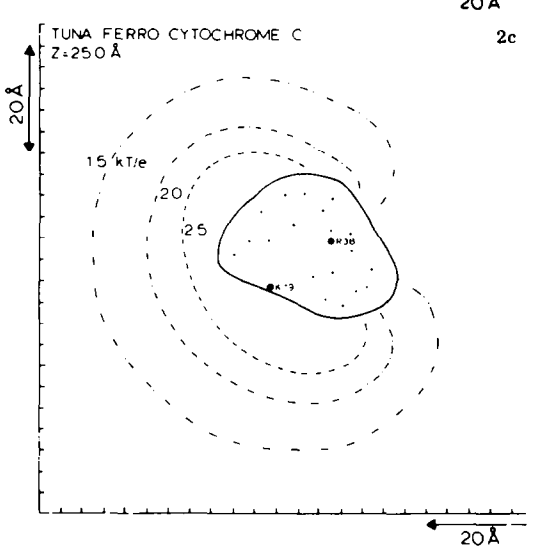












Figs. 1a. b. c and 2a. b. c.

TABLE I

EFFECT OF THE IONIC STRENGTH ON THE 1.5 kT/e LINE AROUND FERRICYTOCHROME c AT Z = 17.84 Å. a = 18.5 Å and $a - |r - r_c|) = -27.5$ Å.

I (M)	$\frac{e^{\kappa(a - \lvert r - r_c \rvert)}}{1 + \kappa a}$	1.5 kT/e becomes
0.001	0.630	0.95 kT/e *
0.002	0.525	0.79 kT/e *
0.005	0.369	0.55 kT/e *
0.010	0.252	0.38 kT/e *
0.020	0.150	0.23 kT/e *
0.050	0.056	0.08 kT/e *
0.100	0.020	0.03 kT/e *

* The correction factor was obtained on the assumption that $eV(k)_I << kT$. Therefore the results marked with an asterix are likely to be unreliable.

The direction of the dipole axis through c^{2+} is very similar. We have not yet calculated higher order multipole moments.

For horse cytochrome c we made the same calculation. Assuming that at pH 7.5 all lysines and all arginines are protonated, and that all carboxyl chains are dissociated except the propionic acid side chains of the haem [2], horse c^{2+} and c^{3+} have the same net charges as tuna c^{2+} and c^{3+}, $+8e$ and $+9e$. We calculated the charge centres of all positive and all negative charges using the atomic coordinates of horse c^{3+} obtained from an X-ray diffraction study at 2.8 Å resolution [10]. The interpretation of these crystallographic data is now thought to be erroneous as far as the location of Phe 82 is concerned [2], but fortunately this is not a charged residue. We found a distance of 4.87 Å between the two centres in c^{3+} and 4.59 Å in c^{2+}. There are 13 negatively charged groups, so the dipole moments are $1.01 \cdot 10^{-27}$ C · m (303 debye) and $0.95 \cdot 10^{-27}$ C · m (286 debye), respectively.

These values enable us to make detailed calculations of the activity coefficient γ using Eqn. 1 [7]:

$$\ln \gamma = \frac{-Q_0}{2DkT} \cdot \frac{\kappa}{1 + \kappa a} - \frac{\kappa^2}{2DkT} \left\{ \frac{3Q_1}{4a\left(1 + \kappa a + \frac{\kappa^2 a^2}{3} + \frac{b^3 \kappa^2}{6a}\right)} \right\} \quad \text{(c.g.s. units)} \qquad (3)$$

in which D is the dielectric constant of water, 80; k is Boltzmann's constant; $Q_0 = Z^2 q^2$ is the square of the net charge; Q_1 is the square of the dipole moment; b is the radius of c^{3+}, $17.0 \cdot 10^{-8}$ cm; a is the radius of c^{3+} plus

Fig. 1. (a, b and c) Equipotential lines around ferricytochrome c in three parallel cross-sections perpendicular to the crystallographic Z-axis as indicated. Only positive kT/e lines are shown. Arrows show the direction of the force acting on negatively charged molecules. The location of positive and negative charges and α-carbon atoms lying between the planes Z +2.5 Å and Z −2.5 Å are shown as ● and •, respectively. E = Glu; K = Lys and R = Arg. Methionine 80 is to the left of the haem, histidine 18 to the right.

Fig. 2. (a, b and c) Equipotential lines around ferrocytochrome c. See legend of Fig. 1.

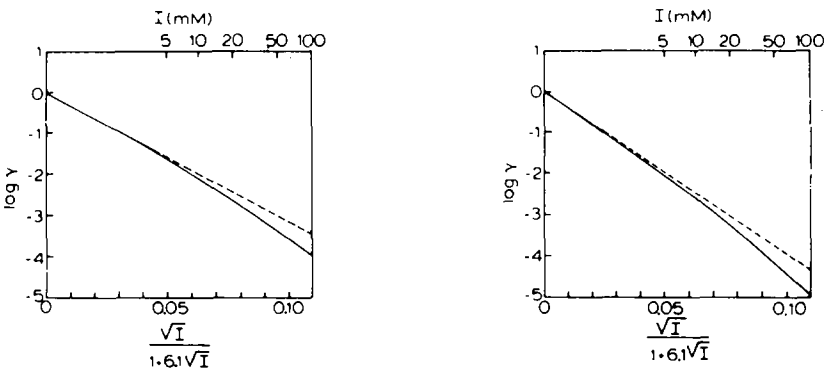


Fig. 3. Activity coefficient of horse ferrocytochrome *c* as a function of ionic strength, *I*. $6.1\sqrt{I} = \kappa a$, Eqn. 1. The dashed line was obtained by omitting the effect of the dipole on the activity coefficient.

Fig. 4. Activity coefficient of horse ferricytochrome *c*. See legend of Fig. 3.

radius of water molecule, $18.5 \cdot 10^{-8}$ cm; T is the temperature; $\kappa^2 = 8\pi N q^2 I / 1000\, DkT$; N is Avogadro's number; $q = 4.80 \cdot 10^{-10}$ e.s.u., the elementary charge; $I = \frac{1}{2}\Sigma_i Z_i^2 m_i$, the ionic strength; m_i is the molality of ion i and Z_i is the number of elementary charges on ion i. The first term of the formula describes the effect of the monopole on the activity coefficient, the second the effect of the dipole. The effect of quadrupoles and higer order poles has been neglected.

The results for c^{2+} and c^{3+} are shown in Figs. 3 and 4. It can be seen that below 50 mM ionic strength the dipole does not have much effect on the activity coefficient.

Discussion

The electric potential field around tuna cytochrome c

The results of the electric potential field calculations shown in Figs. 1 and 2 are important for the reaction of cytochrome *c* with small charged molecules. If the latter are negatively charged they are attracted and during drifting towards cytochrome *c* they will tend to follow the electric field lines, which are orthogonal to the equipotential lines. In so doing they have a higher probability of finding the haem edge than in the case of an uncharged surface. We have found a field of similar shape around horse cytochrome *c* (Koppenol, W.H. and Vroonland, C.A.J., unpublished). This electric field configuration would explain the high yield found for (i) the reaction of the radicals e^-_{aq}, CO_2^- and O_2^- [11—13] with ferricytochrome *c*, (ii) the reaction of e^-_{aq} with ferrocytochrome *c* [14], and (iii) the reaction of e^-_{aq} with porphyrin cytochrome *c* [15]. The presence of an inert electrolyte obscures the electric field: the equipotential lines draw closer round the molecule, as follows from Table I.

There is evidence that electrostatic interactions are involved in the binding of cytochrome *c* to cytochrome *c* oxidase [4—6]. If this is the case, the electrostatic interaction energy should be at least of the order of 1 kT at a distance of about 100 Å. Assuming a net charge of $-20e$ on cytochrome *c* oxidase and an ionic strength of zero, we calculated an electrostatic interaction of approx.

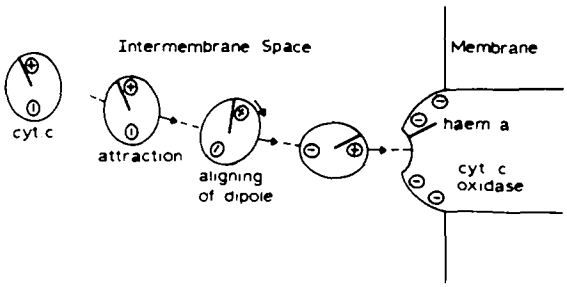


Fig. 5. Schematic representation of the reaction of cytochrome *c* with cytochrome *c* oxidase (not drawn to scale). Cytochrome *c* is first attracted and then forced to align its dipole to the direction of the electric field vector which is assumed to point into the cytochrome *c* oxidase molecule. See text for discussion.

10 kT at a distance of 80 Å between cytochrome *c* and cytochrome *c* oxidase. Because of these assumptions this value is an upper limit. Recently the domain on the surface of horse cytochrome *c* involved in the binding to the high-affinity site [16] of cytochrome *c* oxidase has been determined by chemical modification of lysine residues [17,18]. This domain has been shown to be the top left front of the molecule [19] (normal orientation of cytochrome *c*, looking into the haem crevice, methionine 80 left, histidine 18 right). The centre of this binding domain is the β-carbon of phenylalanine 82, which is very near alanine 83 where the positive part of the dipole axis emerges from the protein. It seems likely therefore that the dipole is of physiological importance: it is suggested that it helps to align cytochrome *c* during its approach as shown in Fig. 5. According to the proposed mechanism the cytochrome *c* molecule can still rotate unimpeded around the dipole axis during drifting towards cytochrome *c* oxidase. We therefore follow the suggestion of Dickerson and Timkovich [5] that the negative charges at the top of the haem crevice, Glu 90 and Asp 93, serve to attain a final orientation prior to binding. Whether cytochrome *c* moves freely in the inner membrane space as suggested by Fig. 5 depends on the distribution of charges on the membranes on both sides of the intermembrane space. Diffusion can occur when the charges opposite each other are the same in sign and number. Then the electric field is zero everywhere except very close to the membrane surfaces. If this condition is not fulfilled, cytochrome *c* will tend to an orientation which is determined by the net electric field, making two-dimensional diffusion possible on the surface of a membrane [20,21].

The effect of ionic strength on reaction rates of horse cytochrome c

Brønsted [22] has proposed the following relation for the rate of a reaction between two charged species X and Y

$$k_I = k_{I=0} \frac{\gamma_X \gamma_Y}{\gamma_{XY}^{\neq}} \tag{4}$$

which is equivalent to

$$\log k_I = \log k_{I=0} + \log \gamma_X + \log \gamma_Y - \log \gamma_{XY}^{\neq} \tag{5}$$

in which k_I is the second-order rate constant at ionic strength I and $\gamma_{XY}^{\neq}$

is the activity coefficient of the transition complex. If we consider for instance the reaction of a small radical having a single negative charge, O_2^- for instance, with ferricytochrome *c* we can use for the calculation of the various activity coefficients the Debye-Hückel expression:

$$\log \gamma_i = -\frac{Z_i^2 A\sqrt{I}}{1 + a_i B\sqrt{I}} \tag{6}$$

in which $A = 0.434 \frac{q^2\kappa}{2D\mathbf{k}T}$ and $B\sqrt{I} = \kappa$.

The expression for log γ_i is equal to the first term of Eqn. 3. Using Eqn. 6 to determine the activity coefficient of O_2^-, we neglect the second term in the denominator because the radius of O_2^- is small. This results in

$$\log \gamma(O_2^-) = -A\sqrt{I} \tag{7}$$

Use of Eqn. 6 for the calculation of the activity coefficients of ferricytochrome *c* and the transition complex implies the following: (i) the concentration of cytochrome *c* should be such that it does not contribute significantly to the ionic strength, and (ii) the effect of the dipoles of cytochrome *c* and the transition complex on the activity coefficients are neglected. This is perhaps not very serious because these effects are not large and they cancel each other partly (see Eqn. 5). We further assume that the radius of the transition complex is equal to that of ferricytochrome *c*. Substitutions of the three activity coefficients in Eqn. 5 leads to:

$$\log k_I = \log k_{I=0} - \frac{(a_{c^{3+}} B\sqrt{I} + 2Z_{c^{3+}})A\sqrt{I}}{1 + a_{c^{3+}} B\sqrt{I}} \tag{8}$$

We believe this equation to hold up to $I = 100$ mM. If we had used the equation found in most textbooks on physical chemistry:

$$\log k_I = \log k_{I=0} + \frac{2Z_X Z_Y A\sqrt{I}}{1 + aB\sqrt{I}} \tag{9}$$

we would have found:

$$\log k_I = \log k_{I=0} - \frac{2Z_{c^{3+}} A\sqrt{I}}{1 + a_{c^{3+}} B\sqrt{I}} \tag{10}$$

Eqn. 9 has been criticised by Perlmutter-Hayman [23] because the reacting species and the transition complex are assumed to have the same radii. It must be mentioned however that Eqns. 8 and 10 are not very different as long as $a_{c^{3+}} B\sqrt{I}$ is small compared to $2Z_{c^{3+}}$, i.e. at $I \leqslant 50$ mM. Therefore it is not surprising that we [13] found a net charge of +6.4*e* on ferricytochrome *c* at pH 7.1 by plotting log k_I (O_2^- + ferricytochrome *c*) vs. $\sqrt{I}/(1 + a_{c^{3+}} B\sqrt{I})$ and determining the slope of the line. This net charge is not unreasonable since probably two formate ions were bound to ferricytochrome *c*. Pecht and Faraggi [24] found a net charge of +7*e* on ferricytochrome *c* at pH 7.0 by studying the effect of the ionic strength on the reaction of ferricytochrome *c* with the hydrated electron. In a similar way Hoffmann and Hayon [25] determined a net charge of +6.4*e* on lysozyme.

There are several authors [26—30] who derived a charge from a plot of log k_I vs. $\sqrt{I}$. They always find a charge which is a fraction of the net charge. This result is clearly an artifact, since one cannot neglect the second term of the denominator in Eqn. 6 if the molecule has a large radius. This has been admitted by Cummins and Gray [31] in a recent paper. If one re-plots the data of Seki et al. [28], who studied the reaction of c^{3+} with O_2^- and CO_2^-, one finds a net charge of approx. $+9e$ on c^{3+} at pH 6.6. However, this value may not be reliable since these authors used sodium sulphate as an inert electrolyte. This salt is not fully dissociated over the concentration range used, as follows from the dissociation constant [32]:

$$K_D = \frac{[Na^+][SO_4^{2-}]}{[NaSO_4^-]} = 10^{-0.72} \tag{11}$$

The above-mentioned authors [27—30] concluded that the charge they found is the net charge at the site where electron transfer takes place. This conclusion is fundamentally incorrect: if a small ion with a single negative charge were to come so near to the cytochrome *c* surface that it would 'see' local charges, then the electrostatic interaction energy would be more than 1 kT. The Debye-Hückel expression for the activity coefficient however only holds for interaction energies less than 1 kT. The above criticism also applies to the formula used by Gray and co-workers [31,33] to calculate the electrostatic interaction energy of the reactants in the activated complex: the formula was derived using the Debye-Hückel theory, but the energies calculated through it are of the order of 1 kT at 0.1 M ionic strength [33].

Therefore, in order to explain the effects of ionic strength on reaction rates, we prefer the Brønsted equation, using suitable expressions for the activity coefficients.

Acknowledgements

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