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# TOWARD A MULTISTEP MECHANISM OF CYTOCHROME *c* REACTIVITY

## ANSWER TO A COMMENT *

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Koppenol's rejection (Biophys. Chem. 18 (1983) 203) of a model of polarity-dependent ferrocytochrome *c* oxidation (M. Fragata and F. Bellemare, Biophys. Chem. 15 (1982) 111) places emphasis on the role of the protein surface charges in reactivity but is at the same time too restrictive as it neglects largely the polarity (dielectric constant) of the aqueous and hydrophobic interfaces of the exposed heme edge and the inner cleft (heme crevice) of cytochrome *c* which appear to be the oxidation-reduction sites. It is suggested that a more general model should take into account (i) a recognition (or diffusion) step where the distance travelled by cytochrome *c* at the membrane surface and/or the Brownian displacements in the bulk solution are greatly influenced by ionic strength, and (ii) a redox step where low polarity effects prevail with concomitant weakening of ionic activity.

## 1. Introduction

Koppenol [23] argues that a model of polarity-dependent ferrocytochrome *c* oxidation which we developed recently [1] is incorrect because "It fails to show that the use of the 'overall dipole moment' is likely to be unreliable, and that the reactivity is best explained by a polarity effect on the dipole of the haem of cytochrome *c*". Hereinafter, we give first an overview answer to Koppenol's criticisms and show that his ionic strength model and our heme dipole hypothesis are but two aspects of a more general multistep mechanism of cytochrome *c* reactivity.

## 2. Koppenol's approximation and the heme dipole hypothesis

Kirkwood's theory [2] of solutions of molecules containing widely separated charges in a solvent of dielectric constant $\epsilon$, applies basically to solutions of low concentration of a spherical complex ion of radius $b$, having a dielectric constant $\epsilon_i$, within which are situated $M$ discrete charges $e_M$. Kirkwood's development accounts also for an electrolyte of ions of charge $Z_i e$, where $Z_i$ is the number of charges on the ion $i$ present in the solution at the molar concentration $C_i$, the charge distributions of which are spherically symmetrical. Moreover, the ions are assumed not to influence the dielectric constant $\epsilon$ of the solvent and their mean distance of closest approach to the complex ion is $a$.

The interaction of the complex ion with the electrolyte ions produces a mean space charge distribution in the solution outside the sphere $a$. Their mutual electrostatic energy ($\Delta G$) arises from the ionic space charge in the solution outside the sphere $a$, and from the surface charge of polarization on the sphere $b$ which is dependent upon the difference between $\epsilon_i$ and $\epsilon$. The result is

$$\Delta G = \Delta G_0 + \Delta G_\kappa, \qquad (1)$$

* Answer to the preceding paper by W.H. Koppenol [23].

where the free energy of charging the sphere of radius $b$ in the absence or at high dilution of the electrolyte is

$$\Delta G_0 = \frac{1}{2}\sum_{n=0}^{\infty} \frac{(n+1)Q_n(\epsilon_i - \epsilon)}{\epsilon_i b^{2n+1}[(n+1)\epsilon + n\epsilon_i]}. \quad (2)$$

and the free energy of charging in the presence of the electrolyte is

$$\Delta G_\kappa = -(Q_0/2\epsilon)\kappa/(1+\kappa a) - \frac{1}{2}\sum_{n=1}^{\infty} \frac{Q_n}{\epsilon a^{2n-1}} \frac{2n+1}{2n-1}\left[\frac{\epsilon}{(n+1)\epsilon + n\epsilon_i}\right]^2 \times [\kappa^2 K_{n-1}(\kappa a)]\left[K_{n+1}(\kappa a) + \frac{n(\epsilon - \epsilon_i)}{(n+1)\epsilon + n\epsilon_i} \times \left(\frac{b}{a}\right)^{2n+1} \frac{\kappa^2 a^2 K_{n-1}(\kappa a)}{(2n-1)(2n+1)}\right]^{-1}. \quad (3)$$

$\kappa^2 = (4\pi N e^2/1000\epsilon k_B T)\sum_i Z_i^2 C_i$ is the Debye-Hückel parameter, where $N$ is Avogradro's number, $e$ the electronic charge, $k_B$ Boltzmann's constant and $T$ the temperature (in K). The polynomials $K_n(x)$ of eq. 3 are given by the explicit expressions of eq. 22 of ref. 2. The relations between the charges and their positions on the sphere are defined by the $Q_n$ functions

$$Q_n = \sum_{k=1}^{n}\sum_{l=1}^{n} e_k e_l r_k^n r_l^n P_n(\cos\theta_{kl}).$$

where the $e$ terms are the charges on the sphere, the $r$ terms their distances from the center, and $P_n(\cos\theta_{kl})$ is the $n$th order Legendre polynomial. In particular, $P_0(x) = 1$ and $P_1(x) = x$.

Eq. 1 applies to reactions between molecular or ionic species in solution, say A (e.g., ferricyanide) and B (e.g., cytochrome $c$), since (i) the reaction rate constant ($k$) is related to that in the gas phase ($k_g$) or in very dilute solution ($k_0$) by the expressions $k = k_g(\alpha_A\alpha_B/\alpha_{AB^\ddagger})$ and $k = k_0(\gamma_A\gamma_B/\gamma_{AB^\ddagger})$ where $\alpha$ and $\gamma$ are activity coefficients (see discussions in refs. 3 and 4) and $AB^\ddagger$ is the activated complex resulting from the reaction between A and B, and (ii) $\Delta G$, $\Delta G_0$ and $\Delta G_\kappa$ (eq. 1) depend linearly on the natural logarithm of the activity coefficient multiplied by $k_B T$. Now, our polarity-dependent model of cytochrome $c$ reactivity [1] as well as Koppenol's model [5,6] are special cases of Kirkwood's theory [2]. The former is for $\Delta G_\kappa = 0$ and the latter for $\Delta G_0 = 0$, $\epsilon_i = 1$ and $\epsilon > 25$.

Whether these two sets of conditions are applicable is so far an unsettled matter. For example, with $\epsilon_i = 1$ and accepting only the first two terms of eq. 2 one has

$$\ln k = \ln k_0 - \frac{e^2}{k_B T}\left(\frac{\epsilon - 1}{\epsilon}\right) \times \left[\frac{z_A^2}{b_A} + \frac{z_B^2}{b_B} - \frac{(z_A + z_B)^2}{b_{AB^\ddagger}}\right] - \frac{1}{k_B T}\left(\frac{\epsilon - 1}{2\epsilon + 1}\right)\left[\frac{\mu_A^2}{b_A^3} + \frac{\mu_B^2}{b_B^3} - \frac{\mu_{AB^\ddagger}^2}{b_{AB^\ddagger}^3}\right]. \quad (4)$$

where $ze$ is the charge on the ion and $\mu$ the dipole moment. The second term of eq. 4 is neglected in a reaction between two dipoles having no net charge, in which case the last term represents the solvent effect. Both terms have to be included, however, in reactions between an ion and a dipole or between two dipoles with net charges, but in most cases the main effect of the dielectric is given entirely by the last term [4]. This may explain the linear relationships of fig. 1 (cf. also fig. 2 of ref. 1) which present the plots of $\ln k$ vs. $(\epsilon - 1)/(2\epsilon + 1)$, where $k$ is the rate constant of the slow and fast reactions of ferrocytochrome $c$ with ferricyanide (data from ref. 7). An interesting aspect of this question is that $\ln k$ is thereby related through the polarity parameter $(\epsilon - 1)/(2\epsilon + 1)$ to Onsager's reaction field theory of a dipole in a dielectric (see section 2.2 of ref. 1).

Let us recall at this point that the oxidation of ferrocytochrome $c$ by ferricyanide is a two-fold mechanism involving an increase in the electrostatic charge of the buried heme iron from +2 to +3 and electron exchanges with ferricyanide at the exposed here edge (see discussion in ref. 1). In this connection, Koppenol's refutation that the cytochrome $c$ heme does not have a permanent dipole of any significance could be unjustified. As remarked previously (cf. section 3.3 and fig. 4 of ref. 1), a polarity effect over the heme ring may influence its dipole moment as a result of fluctuations of the charge distribution of the conjugated $\pi$-orbital system. In a close related system, the chlorophyll $a$ molecule, the tetrapyrrole dipole is about 5 debye (A.F. Antippa, J.-P. Dodelet, M. Fragata, R.M. Leblanc, L. Pazdernik and P.

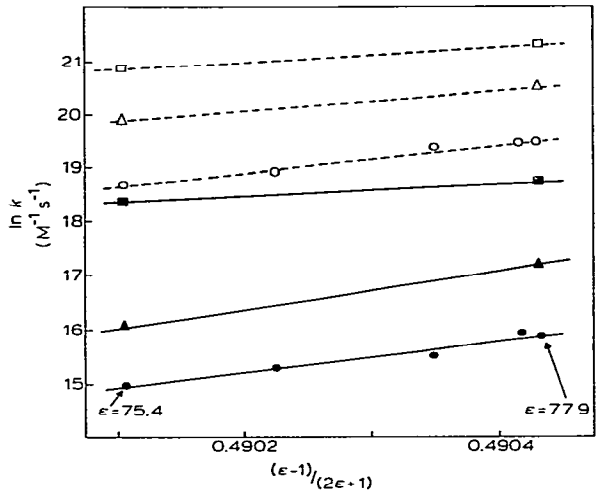


Fig. 1. Natural logarithm of the rate constants $k_{fast}$ (○, △, □) and $k_{slow}$ (●, ▲, ■) as a function of $(\epsilon - 1)/(2\epsilon + 1)$ and ionic strength ($I$) for reactions of ferrocytochrome $c$ with ferricyanide in binary mixtures of ethanol and $H_2O$ at 295 K (data from ref. 7) $I = 0.1$ (○, ●), 0.02 (△, ▲), 0.005 (□, ■). $k_{fast}$ and $k_{slow}$ are accurate to within ±10%.

Tancrède, unpublished results) and nonetheless fairly important to the pigment function. This also argues in favor of an oblate model of the heme crevice to explain cytochrome $c$ reactivity instead of the spherical symmetry model suggested by Koppenol in the preceding paper.

Note further that the condition $\epsilon > 25$ (see above), put forward by Kirkwood (cf. p. 356 and eq. 21 of ref. 2) and tacitly adopted by Koppenol (compare, e.g., eq. 9 of ref. 6 with eq. 21 of ref 2), is strictly applicable in such cases where $\epsilon_i/\epsilon$ is small in comparison with unity which is possible if $\epsilon_i = 1$ (cf. ref. 2) and $\epsilon \approx 78$ (dielectric constant of water at 298 K). But this accounts insufficiently for local polarities at the active sites of the protein and the small ion (or molecule). An alternative view consists of taking $\epsilon$ as the dielectric constant of the outer surfaces that intervene in the oxidation-reduction processes (see discussion in section 4 of ref. 1 and references therein), and $\epsilon_i$ as the polarity of the inner cleft where the tetrapyrrole macrocycle is located (heme crevice). If so, $\epsilon$ is smaller than 78 because of dielectric saturation effects [8,9] at short distances from the active surfaces of cytochrome $c$ and ferricyanide. A typical polarity value for the surface of contact or active site of the protein (most probably the heme edge) seems to be $15 \pm 5$ (ref. 10, and J.A. Watkins and M. Cusanovitch, personal communication), whereas at the ferricyanide surface $\epsilon$ might be as low as 5 up to distances of 2–3 Å [4,8]. On the other hand, the most probable value of $\epsilon_i$ cannot be easily predicted. It is acceptable to speculate a low value, but nonetheless higher than 2 on account of the finding of Takano and Dickerson [11,12] that the heme crevice may contain about three $H_2O$ molecules *. Of special interest in this respect is the suggestion of Watkins and Cusanovitch (personal communication) that the average interior dielectric constant of cytochrome $c$ could be 7.

We wish to emphasize that a polarity effect on ionic dissociation ($K$) has long been the object of a number of studies (see, e.g., refs. 14 and 15). It is found in general that $K$ decreases with decreasing polarity. Therefore, it is not unreasonable to assume, at variance with Koppenol, that a linear relationship between $\ln k$ and $(\epsilon - 1)/(2\epsilon + 1)$ at constant ionic strength of the bulk solution (see fig. 1) reflects the preponderance of polarity over ionic strength effects at the reaction site. It is clear, however, that the $\epsilon$ values used to draw fig. 1 (as well as figs. 1 and 2 of ref. 1) are the dielectric constant values of the bulk solutions. It is not easy to give a straightforward explanation of the results (cf. section 3 of ref. 1). Nevertheless, we hypothesize tentatively that the movement of mixed solvent ($H_2O$/ethanol or $H_2O$/propanol) molecules through the active interfaces leads to polarities lower than those obtained in the presence of pure aqueous solvent. An interesting point that comes out is that a calculation of the tunneling distance ($d$) according to ref. 16 (refuted by Koppenol but accepted by others [17–19]) shows that, e.g., a slight variation of $d$ from 24.67 and 24.77 Å corresponds to an increase of ethanol in the bulk

* The possibility is not excluded, however, that this figure may be higher (see ref. 13 for a discussion on buried $H_2O$ molecules).

solution from 0.18 to 1.5 mol% (cf. table 2 of ref. 1).

## 3. The multistep model

Koppenol schematized in a suggestive model (cf. fig. 5 of ref. 5) the reaction of cytochrome *c* with cytochrome *c* oxidase. In brief, cytochrome *c* is first attracted to the cytochrome *c* oxidase molecule and then aligns its dipole along a preferential direction in the latter protein. This hypothesis is supported by the finding [29] that electrostatic interactions are necessary to orient flavodoxin with respect to the cytochrome *c* molecule during electron transfer. Another example of this type of mechanism is provided by plastocyanin, a surface protein which is involved in photosynthetic oxidation-reduction. It is known that the monomeric protein from poplar has a single type-1 Cu atom buried in a pocket surrounded by 6–7 hydrophobic residues and a negatively charged patch that spreads from residues 42–44 to residues 59–61 [21]. Farver et al. [22] suggested that the anionic hydrophilic patch and the hydrophobic region are instrumental in the recognition of the plastocyanin-binding sites on P-700 and cytochrome *f*, respectively. Most interesting, since it is directly related to ref. 1, is the conclusion [20] that in cytochrome *c* tightly bound water and counterions are excluded from the site of intermolecular contact. From this and the discussions of section 2 we suggest that any model mechanism of cytochrome *c* reactivity, and probably of other redox proteins, has to account for (i) a recognition (or diffusion) step where the distance travelled by the protein at the membrane surface and/or the Brownian displacements in the bulk solution are greatly influenced by ionic strength, and (ii) a redox step where low polarity effects predominate with concomitant weakening of ionic activity.

Therefore, the oxidation-reduction of cytochrome *c* in solution is dependent on a set of conditions that is expressed at least by $\Delta G_0$ (eq. 2) and $\Delta G_\kappa$ (eq. 3). At the membrane surface, however, one may eventually have to consider parameters that cannot be included in Kirkwood's development [2]. These are, e.g., protein diffusion and viscous drag as a result of constraints dictated by the membrane exoskeletal framework. The analysis of these questions will be the object of a forthcoming paper.

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