Biophysical Chemistry 18 (1983) 203–205
Elsevier

BPC 00798

IS CYTOCHROME *c* REACTIVITY DETERMINED BY DIPOLE MOMENT OR BY LOCAL CHARGES?

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Received 10th October 1982
Accepted 16th May 1983

Key words: Cytochrome c, Dipole moment; Electron transfer; Active site charge; Electrostatics

A criticism of a recent paper by M. Fragata and F. Bellemare (Biophys. Chem. 15 (1982) 111) is presented. These authors developed a model of polarity-dependent ferrocytochrome *c* oxidation which is shown to be incorrect. It fails to show that the use of the 'overal dipole moment' is likely to be unreliable, and that reactivity is best explained by a polarity effect on the dipole of the haem of cytochrome *c*.

1. Introduction

Recently, Fragata and Bellemare [1] presented a model of polarity-dependent ferrocytochrome *c* oxidation in which the use of overall dipole moments of proteins in redox studies [2,3] was called 'an unreliable simplification'. This conclusion is based on the results of a calculation which shows that the dipole moment of cytochrome *c*, increased by the reaction field, should vary with changes in the dielectric constant of the solvent, while it was found to be constant. This analysis is based on experimental data obtained by others, namely, the oxidation of ferrocytochrome *c* by ferricyanide in the presence of various amounts of an alcohol [4,5]. With ethanol this reaction is biphasic at pH 8.5 [4], and the rate constants of both the fast and the slow step were both found to decrease by approx. 50% when the alcohol concentration was increased to 2.5 M.

As shown below, a basic assumption underlying this analysis is incorrect. Furthermore, in the calculations themselves various omissions and mistakes were found which invalidate the above-mentioned conclusion.

2. Criticisms

At an ionic strength of 0.1 M horse ferricytochrome *c* undergoes a conformational change at pH 9.2 (see ref. 6) which is shifted to lower pH by alcohols [4,7]. Horse ferrocytochrome *c* changes conformation above pH 12. When the alkaline form of ferricytochrome *c* is reduced by hydrated electrons 'unrelaxed' ferrocytochrome *c* is formed. Ferricyanide oxidizes unrelaxed ferrocytochrome *c* faster than native ferrocytochrome *c*, which causes biphasicity when both forms of cytochrome *c* are present [8]. Since a calculated dipole moment is dependent on the structure of the molecule, it cannot be used in calculations involving the alkaline conformation of cytochrome *c*. Thus, the fast reaction cannot be analyzed as proposed by Fragata and Bellemare [1]. Due to the design of the pulse radiolysis experiments [4] it is quite likely that both forms of cytochrome *c* have ferricyanide bound to them, as well as phosphate [9]. Binding of anions would change the dipole moment and prevent an explanation as attempted [1].

Eq. 1* of Fragata and Bellemare [1]

$$\Delta G = \frac{RT}{N} \ln \gamma = \frac{-\mu^2}{b^3}\left[\frac{\epsilon - 1}{2\epsilon + 1}\right] \qquad \text{(FB1)}$$

represents the difference in work of charging a dipolar molecule at low concentration in a medium with dielectric constant ϵ relative to a medium with $\epsilon = 1$ in the absence of electrolytes. This equation does not describe cytochrome *c* or ferricyanide, since it does not take into account net charges. Furthermore, it has a peculiar standard state as reference ($\epsilon = 1$) such that eq. FB1 cannot be applied to eq. FB4

$$k = k_0 \frac{\gamma_A \gamma_B}{\gamma_{AB}^{\ddagger}} \qquad \text{(FB4)}$$

where k_0 represents the rate constant in water, extrapolated to zero ionic strength [3]. Therefore, eq. FB3

$$\ln k = \ln k_0 - \frac{N}{RT}\left[\frac{\mu_A^2}{b_A^3} + \frac{\mu_B^2}{b_B^3} - \frac{\mu_{AB^{\ddagger}}^2}{b_{AB^{\ddagger}}^3}\right]\left[\frac{\epsilon - 1}{2\epsilon + 1}\right] \qquad \text{(FB3)}$$

is not expected to describe the reaction of ferrocytochrome *c* with ferricyanide at an ionic strength of 0.1 M [4] as a function of the dielectric constant of the medium. Since the centre of positive charge does not coincide with the centre of mass, the use of eq. FB5

$$\mu = Yre \qquad \text{(FB5)}$$

is not permitted. It should have been [3]:

$$\mu = p r_P e - n r_N e \qquad (1)$$

The dipole moment of the transition-state complex calculated by Fragata and Bellemare [1], approx. 930 debye, is also incorrect. Since ferricyanide reacts at the solvent-accessible haem edge [10], the transition complex would have a dipole moment much smaller [3] than that of horse ferrocytochrome *c*, 308 debye [11]: The dipole vector of the latter makes an angle of approx. 30° with the haem plane [11] which can be resolved in a component of 265 debye in the plane of the haem and a component of 155 debye perpendicular to it. In the transition complex the distance between the centre of ferricyanide and the centre of mass of cytochrome *c* is estimated to be 18 Å. A charge $-3e$ located at that distance in the plane of the haem represents a dipole of 260 debye with respect to the centre of mass of the transition-state complex, with a direction opposite to that of the haem component of the dipole moment of cytochrome *c*. They cancel and only the vector of 155 debye remains. Even if ferricyanide reacted at the back where the negative end of the dipole axis crosses the protein surface, the dipole moment of the transition complex could not be larger than 600 debye.

The reaction field is indeed expected to increase the dipole moment of cytochrome *c*. Whether eq. FB13, which applies to an ellipsoidal cavity, is appropriate to calculate this effect is uncertain, because the direction of the dipole moment lies not along one of the unspecified axes ***a***, ***b*** or ***c***, as required, nor is it known whether the ***a***-axis is the principal axis of the polarizability tensor [12]. It would have been better to assume spherical symmetry and to multiply the dipole moment with the term $3\epsilon_w/(2\epsilon_w + \epsilon_i)$ [12], which is still primitive, but simpler. Such a correction for the reaction field would increase the dipole moment by 50%. However, in a model where the charges were situated in a polar environment [13] the total dipole moment was found to be reduced [14]. In the absence of a rigorous electrostatic description of macromolecules in solution, the use of an ellipsoidal model is unwarranted.

3. Discussion

The shortcomings in the approach by Fragata and Bellemare [1] undermine seriously their conclusion that the use of overall dipole moments is an unreliable simplification. As support they cite a local charge of $+1.3e$ near the site of electron transfer [1]. As discussed previously [2,3], such

* Symbols used: M, centre of mass; *N*, Avogadro's number; N, centre of negative charge; P, centre of positive charge; *R*, gas constant; *T*, temperature; *Y*, the smaller of the two parameters *n* and *p*; *Z*, net charge; *b*, radius of protein or ion in centimetres; *e*, elementary charge in electrostatic units; *n*, number of negative charges; *p*, number of positive charges, *r*, vector from N to P; r_P and r_N, radius vectors from M to P and M to N, respectively; γ, activity coefficient; ϵ_w, dielectric constant of water; μ, dipole moment in esu cm.

local charges are the result of the misuse of the simplified Brønsted-Debye-Hückel equation in ionic strength studies of small ions with proteins. Indeed, it would appear that it has been forgotten that the calculation of the charge at the active site 'involves the assumption that there is no effect on the rate constants due to the net charge of the protein molecule' [15]. If such results of ionic strength studies are properly treated, they yield to a first approximation the net charge of the protein, and not the charge at the site of electron transfer [2,3]. Changes in overall dipole moments of modified cytochromes *c* were recently found to correlate with enzymic activity, except when the charge modification was within the enzymic interaction domain. In those cases short-range effects, such as steric hindrance and changes in quadrupole and higher-order moments, become important and the activity predicted by the change in dipole moment only is too high [11,16], as one would expect.

Fragata and Bellemare [1] ascribe the reactivity of ferrocytochrome *c* in the ferricyanide-alcohol system to a polarity effect on the dipole moment of the haem of cytochrome *c*, in spite of the fact that the haem, due to its symmetrical structure, does not have a permanent dipole moment of any significance. No value for its magnitude is given [1]. The change in polarity is thought to be brought about by water molecules pervading the haem crevice. Although three water molecules have been found in native ferric and ferrous tuna cytochrome *c*, their distances to the iron are 6 Å or more and they do not displace the iron from its equilibrium position [17], as purported [1].

The fact that ethanol has an identical effect on both the rapid and the slow phase of the reaction argues against the involvement of dipole moments, which, as stated above, must be different for the native and alkaline conformation of the protein. Methanol and 1-propanol have been shown to associate with alkaline ferricytochrome *c* [18]. Binding of ethanol to both forms of cytochrome *c* could interfere with the oxidation by ferricyanide.

Finally, to calculate the distance between the redox centres, Fragata and Bellemare [1] use an equation which describes tunnelling of an electron in a well through a barrier to a continuum of electronic states. As shown by Grigorov and Chernavskii [19] and, independently, Hopfield [20], inclusion of vibronic coupling leads to smaller tunnelling distances and conservation of energy. The distances calculated by Fragata and Bellemare are consequently too large [21] for a ferricyanide molecule reacting at the exposed haem edge of ferrocytochrome *c* [10]. The use of the old theory of tunnelling has been severely criticized by Hopfield [22].

It is concluded that the equations employed by Fragata and Bellemare to explain the reactivity of cytochrome *c* are not applicable.

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