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THE REACTION OF CYTOCHROME aa_3 WITH (PORPHYRIN) CYTOCHROME *c* AS STUDIED BY PULSE RADIOLYSIS

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(1) Using the pulse-radiolysis and stopped-flow techniques, the reactions of iron-free (porphyrin) cytochrome *c* and native cytochrome *c* with cytochrome aa_3 were investigated. The porphyrin cytochrome *c* anion radical (generated by reduction of porphyrin cytochrome *c* by the hydrated electron) can transfer its electron to cytochrome aa_3. The bimolecular rate constant for this reaction is $2 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ (5 mM potassium phosphate, 0.5% Tween 20, pH 7.0, 20°C). (2) The ionic strength dependence of the cytochrome *c*-cytochrome aa_3 interaction was measured in the ionic strength range between 40 and 120 mM. At ionic strengths below 30 mM, a cytochrome *c*-cytochrome aa_3 complex is formed in which cytochrome *c* is no longer reducible by the hydrated electron. A method is described by which the contributions of electrostatic forces to the reaction rate can be determined. (3) Using the stopped-flow technique, the effect of the dielectric constant (ϵ) of the reaction medium on the reaction of cytochrome *c* with cytochrome aa_3 was investigated. With increasing ϵ the second-order rate constant decreased.

Introduction

Understanding the mechanism of the interaction between cytochrome *c* and cytochrome aa_3, the two terminal components of the mitochondrial respiratory chain, requires definition of the relationship between chemical structure and biological activity. One of the approaches which has been widely used to provide information on the role of different residues in the mechanism of the cytochrome *c* action is chemical modification of the protein moiety [1–5]. A result of these studies is that the areas on the cytochrome *c* surface that are involved in its interaction with mitochondrial cytochrome *c* oxidase [1–5] and reductase [6] contain a large section of the exposed heme edge. Hence, these studies provide circumstantial evidence that the pathway of electron transfer to and from the heme iron is via the exposed heme edge. Another approach, complementary to the studies mentioned above, is to investigate directly the role the heme iron plays by examining the reaction of a metal-substituted cytochrome *c* with cytochrome aa_3 [7]. However, substitution of the heme iron by cobalt affects the integrity of the protein structure [7]. As a more suitable method we have chosen to investigate the electron-transfer reaction of iron-free cytochrome *c* (which maintains the native structure [8–10]) with cytochrome aa_3. It has been demonstrated that iron-free (porphyrin-) cytochrome *c* can be reduced by the strongly reducing hydrated electron (e_{aq}^-), to yield the porphyrin-cytochrome *c* anion radical [11]. With the pulse-radiolysis technique it is possible to produce e_{aq}^- within a microsecond and to follow the subsequent absorbance changes spectrophotometrically [12]. We have used this technique to investigate the reaction of the porphyrin-cytochrome *c* anion radi-

cal with isolated cytochrome aa_3.

In a previous paper, the application of the pulse-radiolysis technique to the study of the pre-steady-state reaction of (native) cytochrome *c* with cytochrome aa_3 was described [12]. Using this technique, it is possible to study the pre-steady-state reaction of cytochrome *c* with cytochrome aa_3 at a 100-fold higher time resolution than with conventional fast-mixing techniques. With the pulse-radiolysis technique we have studied this reaction at ionic strengths between 60 and 100 mM. From the ionic strength dependence of the reaction rate constant, it is possible to determine the value of the bimolecular rate constant at $I = 0$ and at $I = \infty$; the difference between these two values yields information about the contribution made to the reaction by electrostatic interactions between the two proteins [13–15].

Materials and Methods

Cytochrome aa_3 was isolated from beef heart according to the methods of Fowler et al. [16] and MacLennan and Tzagoloff [17], as modified by Van Buuren [18]. Cytochrome *c* was prepared from horse heart as described by Margoliash and Walasek [19]. Reduced cytochrome *c* was obtained by gel filtration after incubation with ascorbate. Iron-free (porphyrin) cytochrome *c* was obtained as reported by De Kok et al. [11]. The preparation did not contain any ascorbate-reducible contaminations of native cytochrome *c*.

Absorbance coefficients used for cytochrome aa_3 (reduced minus oxidized) were 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [20], for cytochrome *c* (reduced minus oxidized) 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm [21], and for porphyrin-cytochrome *c* 13.0 $mM^{-1} \cdot cm^{-1}$ at 504 nm [8]. The experimental procedures for measuring the pre-steady-state reaction between cytochrome *c* and cytochrome aa_3 with the stopped-flow technique have been described before [22].

Hydrated electrons were generated by delivering a 0.5 μs pulse of 2-MeV electrons from a Van de Graaff accelerator to a matrix solution. This solution consisted of anaerobic, argon-saturated phosphate buffer (pH 7.0) in triple-distilled water, to which 0.5% Tween 20 was added to solubilize cytochrome aa_3 and to scavenge the $OH^{\cdot}$ radicals formed [23]. The reactions were followed by fast spectrometry essentially as described previously [24]. The concentration of e_{aq}^- was determined by using an extinction coefficient of 14.1 $mM^{-1} \cdot cm^{-1}$ at 650 nm, or by dosimetry with a 10 mM KCNS solution (oxygen-saturated) using $G\epsilon = 2.1 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 480 nm.

Results

Fig. 1 shows the absorbance changes at 444 and 550 nm after the generation of e_{aq}^- in a solution containing the cytochrome *c*-cytochrome aa_3 complex at low ionic strength (traces A and B) and in the presence of excess ferricytochrome *c* (traces C

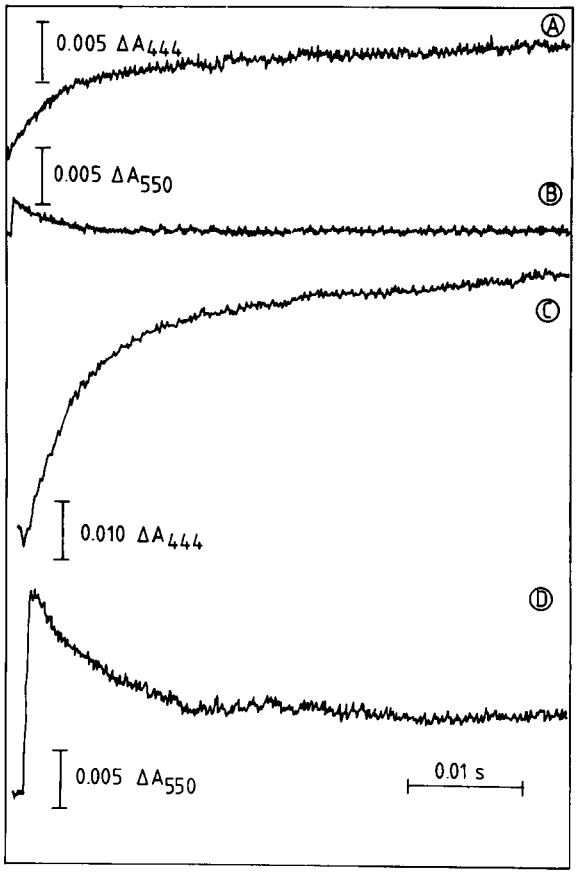

Fig. 1. Redox changes of cytochrome aa_3 at 444 nm and of cytochrome *c* at 550 nm after generation of e_{aq}^-. Reaction conditions: 5 mM potassium phosphate, 0.5% Tween 20, 20°C. (A, B) 5.7 μM cytochrome *c*-cytochrome aa_3 complex, 5.8 μM e_{aq}^-. (C, D) 5.4 μM cytochrome *c*-cytochrome aa_3 complex, 4.3 μM cytochrome *c*, 3.8 μM e_{aq}^-.

and D). Under conditions when all cytochrome *c* is bound to cytochrome aa_3 (i.e., at low ionic strength and [cytochrome aa_3] = [cytochrome *c*]), small absorbance changes were observed at both wavelengths (Fig. 1A and B) after the generation of e_{aq}^- in the solution. From these absorbance changes it can be calculated that less than 2% of the e_{aq}^- directly reduces cytochrome *c*, which subsequently reduces cytochrome aa_3. The fast ($t_{1/2} < 10$ μs) absorbance change at 550 nm is attributed to the reduction of free cytochrome *c* by e_{aq}^-; this reaction occurs rapidly ($k_1 \approx 10^{10}$ $M^{-1} \cdot s^{-1}$) and with a high yield (80–100%) [25]. It is therefore concluded that in the cytochrome *c*-cytochrome aa_3 complex, neither heme *c* nor heme *a* is easily accessible to e_{aq}^-.

When e_{aq}^- was prepared in a solution that contained the cytochrome *c*-cytochrome aa_3 complex in the presence of excess cytochrome *c* (Fig. 1C and D), larger absorbance changes occurred at 550 and 444 nm, which can be ascribed to reduction of heme *c* and heme *a*, respectively. The initial fast increase in absorbance at 550 nm had a half-life characteristic of the reduction of ferricytochrome *c* by e_{aq}^-. The absorbance at 550 nm decayed within 1 s to its equilibrium value, indicating a reoxidation of the initially formed ferrocytochrome *c*. The decrease in absorbance at 550 nm was concomitant with the increase in absorbance at 444 nm, at which wavelength predominantly the reduction of heme *a* was observed. These experiments indicate that electron transfer takes place from reduced cytochrome *c* to the cytochrome *c*-cytochrome aa_3 complex. This finding confirms results of previous studies on the reaction of cytochrome *c* with cytochrome aa_3, indicating the existence of two catalytically active sites for cytochrome *c* on cytochrome aa_3 [22,26–28].

Fig. 2 shows the absorbance changes at 444 nm when e_{aq}^- was generated in a solution containing equimolar amounts of cytochrome *c* and cytochrome aa_3 at $I = 100$ mM. It has been shown that at this ionic strength the cytochrome *c*-cytochrome aa_3 complex dissociates readily [15]. From the observed absorbance changes at 416 nm (not shown) it could be calculated that under these conditions the yield of the reaction of e_{aq}^- with cytochrome *c* is 10%. This relatively low yield is ascribed to competitive reactions of e_{aq}^- with the

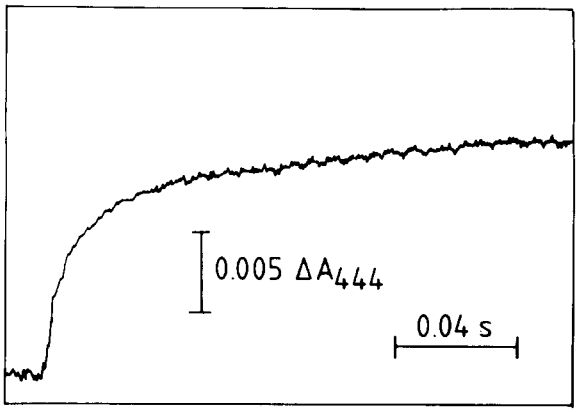

Fig. 2. Reduction of cytochrome aa_3 in a solution containing equimolar amounts of cytochrome *c* and cytochrome aa_3 in high ionic strength, after generation of e_{aq}^-. Reaction conditions as in Fig. 1, 1000 mM $NaClO_4$ was added. 1.9 μM e_{aq}^-, 2 μM cytochrome aa_3 and 2 μM cytochrome *c*.

matrix solution and with the protein moiety of cytochrome aa_3. Although e_{aq}^- cannot reduce heme *a* in cytochrome aa_3 directly, it has been shown that it reacts with the protein moiety of the molecule with a rate constant of 10^{10}–10^{11} $M^{-1} \cdot s^{-1}$ [12].

Fig. 3A shows the absorbance changes at 650 nm after 2.3 μM e_{aq}^- was generated in a solution containing 10 μM porphyrin-cytochrome *c*. These absorbance changes can be ascribed to the formation of the porphyrin-cytochrome *c* anion radical, which is formed after reduction of porphyrin-cytochrome *c* by e_{aq}^- [11].

When e_{aq}^- was generated in a mixture of cytochrome aa_3 (3.4 μM) and porphyrin-cytochrome *c* (7.5 μM) at low ionic strength, the absorbance changes at 650 nm observed instantaneously after the pulse returned with in 0.1 s to the original level (Fig. 3B); this indicates that the porphyrin-cytochrome *c* anion radical is oxidized. Fig. 3C shows the absorbance changes at 444 nm under the conditions of the experiments shown in trace B. The initial rapid change in absorbance shown in Fig. 3C is ascribed to the formation of the porphyrin-cytochrome *c* anion radical. The slower secondary absorbance change occurred at the same rate as the slower absorbance change at 650 nm and is thus attributed to the reduction of heme *a* by the porphyrin-cytochrome *c* anion radical. The

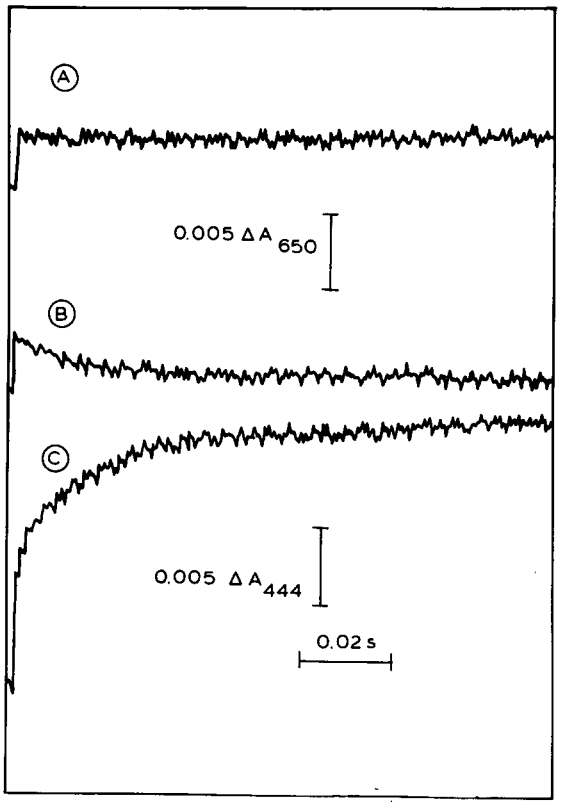

Fig. 3. Redox changes of porphyrin-cytochrome *c* at 650 nm and cytochrome aa_3 at 444 nm after generation of e_{aq}^-. Conditions as described in Fig. 1. (A) 10 μM porphyrin-cytochrome *c*, 2.3 μM e_{aq}^-, (B) 7.5 μM porphyrin-cytochrome *c*, 3.4 μM cytochrome aa_3, 5.3 μM e_{aq}^-. (C) as B, 2.5 μM e_{aq}^-.

low ionic strength promotes the formation of the porphyrin-cytochrome *c*-cytochrome aa_3 complex, in which heme *a* or porphyrin-cytochrome *c* is not directly reducible by e_{aq}^- (not shown). The experiments shown in Fig. 3 indicate that electron transfer between the porphyrin-cytochrome *c* anion radical and the porphyrin-cytochrome *c*-cytochrome aa_3 complex occurs at a rate that is comparable with that of the reaction of the native protein ($k = 2 \cdot 10^7\ M^{-1} \cdot s^{-1}$).

The pulse-radiolysis technique can be used to study the electron transfer from cytochrome *c* to cytochrome aa_3 at a 100-fold higher time resolution than with the conventional flow techniques, which are limited by the time needed for mixing the reactions (1–2 ms). Using the pulse-radiolysis technique, we have studied the reaction of cytochrome *c* with cytochrome aa_3 at ionic strengths below 120 mM. Fig. 4 shows the second-order rate constant of the reaction of cytochrome *c* with cytochrome aa_3 as a function of the ionic strength (I) in terms of Eqn. 1.

$$\log k_I = \log k_{I=0} + CI^{1/2} \quad (1)$$

where k_I is the second-order rate constant at ionic strength I, $k_{I=0}$ the second-order rate constant at $I = 0$, and C a constant that is a function of the effective charge of the molecules, the dielectric and Boltzmann constants, the radii of both cytochromes and of the encounter complex, and the temperature. Although this equation is not applicable to a reaction between two large proteins [26], a linear relationship was obtained in the ionic strength range between 60 and 120 mM; by linear extrapolation to $I = 0$, a value of $k_{I=0}$ of 10^{11} $M^{-1} \cdot s^{-1}$ was estimated. At ionic strengths below

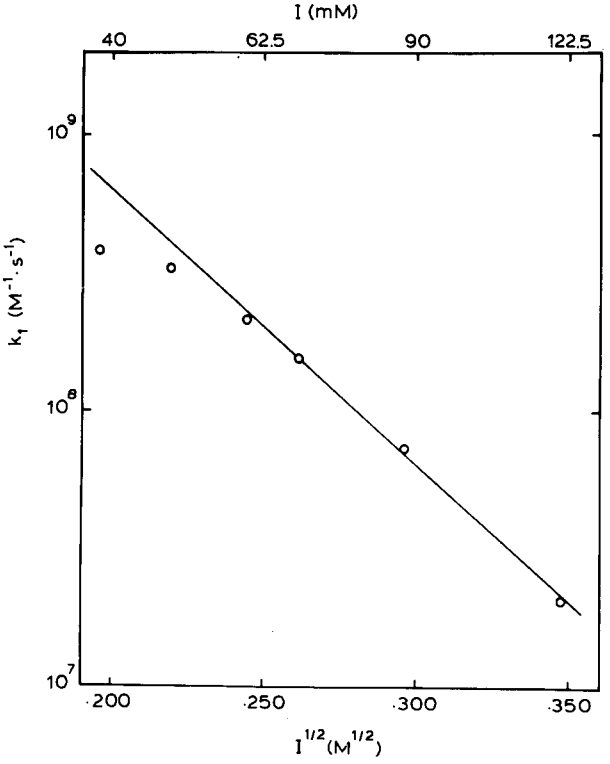

Fig. 4. Effect of ionic strength on the rate constant of the reaction of cytochrome *c* with cytochrome aa_3. Conditions as described in Fig. 1. 6.9 μM cytochrome aa_3, 3.2 μM cytochrome *c*. Ionic strength was provided by $NaClO_4$.

30 mM the reaction could not be measured; this is caused by the fact at these low ionic strengths under these conditions when [cytochrome aa_3] > [cytochrome c], all cytochrome c is bound to cytochrome aa_3. The same can be concluded from Fig. 5, which shows the ratio $\Delta A_{444}/[e_{aq}^-]$ as a function of I. This ratio remained constant above $I = 70$ mM, whereas it decreased below this value, indicating the formation of the cytochrome c-cytochrome aa_3 complex. From Fig. 1A and B it can be inferred that in the complex, neither heme c nor heme a is easily accessible to e_{aq}^-; hence the decrease in the ratio $\Delta A_{444}/[e_{aq}^-]$ upon diminishing the ionic strength is attributed to a shift of cytochrome c from the freely dissolved state to the cytochrome aa_3 bound form. The deviation of the rate constant from the linear relationship in the low ionic strength region of Fig. 4 is similarly ascribed to a part transformation of cytochrome aa_3 into the less active cytochrome c-cytochrome aa_3 complex.

Fig. 6 shows the ionic strength dependence of k_1 of the reaction of cytochrome c with the cytochrome c-cytochrome aa_3 complex at ionic strengths between 4 and 30 mM. The rate of this reaction increased with higher ionic strengths. This indicates the formation of the more active cytochrome aa_3 upon increasing the ionic strength.

The dependence of the reaction rate on the dielectric constant was determined by studying the reaction of cytochrome c with cytochrome aa_3 in

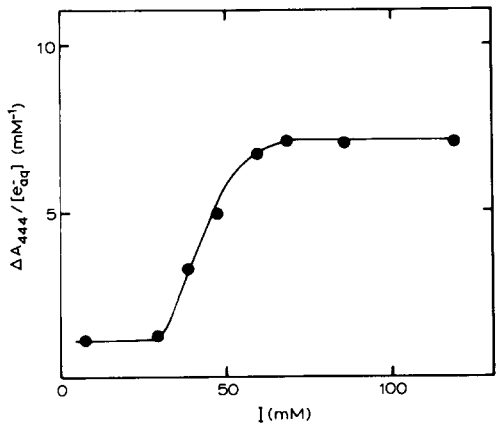

Fig. 5. Ratio of $\Delta A_{444\ nm}$ and e_{aq}^- as a function of ionic strength. The absorbance change at $\lambda = 444$ nm was determined 2 s after the pulse was given. Other conditions as described in Fig. 4.

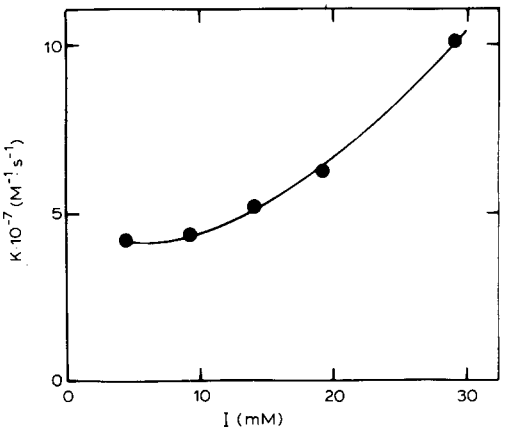

Fig. 6. Effect of ionic strength on the rate constant of the reaction of cytochrome c with the cytochrome c-cytochrome aa_3 complex. 3.1 μM cytochrome c, 6.9 μM cytochrome c-cytochrome aa_3 complex. Reaction conditions: 2 mM potassium phosphate, 0.5% Tween 20, pH 7.0, 20°C. Ionic strength was provided by $NaClO_4$.

media containing various concentrations of glycine in order to provide the desired dielectric constant [29]. These experiments were performed using the stopped-flow technique, since the pulse-radiolysis technique is less suitable for these experiments due to side-reactions of e_{aq}^- with glycine.

Fig. 7 shows the results of these experiments in terms of the equation:

$$\ln k = \ln k_i - \frac{z_A z_B e^2}{\epsilon k T r_{AB}} \qquad (2)$$

in which where k_i is the rate constant when no electrostatic interactions occur (at infinite ϵ or at infinite ionic strength), ϵ the dielectric constant, k Boltzmann's constant, T the temperature (K), r_{AB} the radius of the encounter complex ($r_{AB} = r_A + r_B$), and e the charge of the electron.

From the slope of the line in Fig. 7 a value of $z_A z_B$ of -15 can be determined. It is conceivable that under the conditions of the experiments, the effective net charges of both proteins will be diminished because of the screening by counterions. Hence, the real product $z_A z_B$ will be more negative than -15. By extrapolation to $1/\epsilon = 0$ a value of $2 \cdot 10^6\ M^{-1} \cdot s^{-1}$ for $k_{\epsilon=\infty}$ is obtained. However, when the reaction was measured at $I = 0.9$ M with the stopped-flow technique, a rate constant of

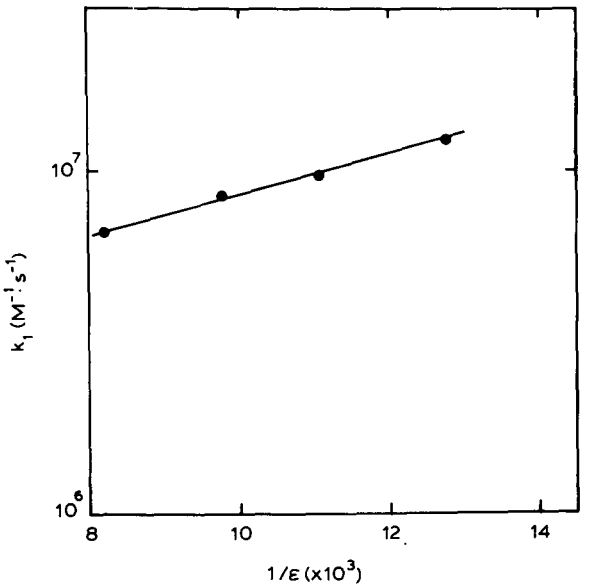

Fig. 7. Effect of the dielectric constant on the rate constant of the reaction of cytochrome *c* with cytochrome aa_3. The dielectric constant was varied by adding glycine (0–2 M) to the reaction medium. 5 μM cytochrome aa_3, 0.8 μM ferrocytochrome *c*. Other conditions: 100 mM potassium phosphate (pH 6.1), 0.5% Tween 20, 10°C.

$6 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ was found (not shown). It is reasonable to assume that $k_{1/\epsilon=0}$ and $k_{I=\infty}$ must be of the same order of magnitude, since in both cases the reactants behave as if they are uncharged. This discrepancy will be treated in more detail in the Discussion.

Discussion

The comparatively high stability of the porphyrin-cytochrome *c* anion radical has permitted the measurement of the electron-transfer reaction to ferricytochrome *c* [11]. The present study shows that the radical can also transfer its electron to the porphyrin-cytochrome *c*-cytochrome aa_3 complex. It is unlikely that in this electron-transfer reaction the electron leaves the porphyrin ring of porphyrin-cytochrome *c* via the residues that function in native cytochrome *c* as axial ligands. This suggests, then, that in the analogous electron-transfer reaction of native cytochrome *c* with cytochrome aa_3 the electrons can be transferred via the exposed part of the heme, which is in accordance with earlier proposals [28,30].

In the cytochrome *c*-cytochrome aa_3 complex the heme group of cytochrome *c* is not accessible to e_{aq}^-. It is suggested that cytochrome *c* is reduced by e_{aq}^- via the solvent-exposed heme edge [31]. Hence, the inaccessibility for e_{aq}^- of the complex-bound cytochrome *c* indicates that the heme group is no longer exposed to the solvent, but is masked by the cytochrome *c*-binding site of cytochrome aa_3. This result is in accordance with the results of studies on chemically modified cytochrome *c* derivatives, which indicate that the areas on the cytochrome *c* surface that are involved in its interaction with cytochrome aa_3 contain a large section of the heme edge [1–5]. The ionic strength dependence of the reaction of cytochrome *c* with cytochrome aa_3 confirms earlier studies that indicated the involvement of electrostatic forces in the cytochrome *c*-cytochrome aa_3 interaction [13–15].

The reaction of cytochrome *c* with the cytochrome *c*-cytochrome aa_3 complex, however, is much less sensitive to the ionic strength than the reaction of cytochrome *c* with cytochrome aa_3. There are several explanations for this behavior. Due to the presence of the bound cytochrome *c* in the complex, the negative charge of cytochrome aa_3 will be partly neutralized [32]. This will decrease the effect of ionic strength on the reaction rate. Furthermore, in the high ionic strength region (above 30 mM) part of the complex will be dissociated, rendering the more active free cytochrome aa_3. In a similar way, in the low ionic strength region, part of the cytochrome *c*-cytochrome aa_3 complex might have bound a second cytochrome *c* molecule. It must be noted that in these studies ferricytochrome *c* was present in excess over cytochrome aa_3. For the binding of cytochrome *c* to the low-affinity site of the cytochrome *c*-cytochrome aa_3 complex, a dissociation constant of 0.1 μM has been reported by Ferguson-Miller et al. [26]. However, considerably higher values have also been reported by other authors [27,33].

It is clear that at low ionic strength the reaction between cytochrome *c* and cytochrome aa_3 occurs with a rate constant of at least 10^8 $M^{-1} \cdot s^{-1}$. This high value is due to the involvement of electrostatic forces in the reaction. Using different theoretical approaches, several investigators have related the ionic strength dependence of reaction

rate constants to the net charge of the reactants (cf. Ref. 34). However, only in limited cases can a quantitative relationship be derived, e.g., for reactions between small ions or between a charged protein and a small ion. In order to calculate the effective charges on the proteins under investigation, cytochrome *c* and cytochrome aa_3, we have used Eqn. 2. From the linear part of Fig. 4 the value of k can be extrapolated fairly accurately. The value of k_i is theoretically given by the value of the rate constant at infinite dielectric constant. However, Eqn. 2, which is used to determine the rate constant at infinite ϵ (cf. Fig. 7), is only applicable when the reaction occurs at zero ionic strength. Ions present in solution will screen the reacting proteins, and this screening will be more effective at low than at high ϵ. Neglect of this will lead to a serious overestimation of the value of $k_{1/\epsilon=0}$. Furthermore, in the determination of $k_{1/\epsilon=0}$ it was assumed that the value of the dielectric constant in the bulk of the solution is identical to that in the vicinity of the proteins. It is likely, however, that the effective dielectric constant in the vicinity of the protein surface is decreased due to the orientation of the water dipoles in the neighborhood of ionized groups of, e.g., cytochrome *c* [35].

In view of these considerations, we believe that the value of k obtained at 0.9 M will be a better approximation of the reaction rate when no electrostatic interactions are present between cytochrome *c* and cytochrome aa_3 than that obtained by extrapolation of the rate constant to $1/\epsilon = 0$. For the value of r_{AB} we take the sum of the radii of both cytochromes. The isolated cytochrome aa_3 can be approximated by a sphere with a radius of 40 Å [36]; the radius of the rather spherical cytochrome *c* molecule is 18 Å [28]; we therefore obtain a transition state radius r_{AB} of 58 Å. For the value of ϵ in the vicinity of the proteins we use 50 (cf. Ref. 35). Substitution of these values in Eqn. 2 yields -80 as a value for $z_A z_B$. If we introduce a value of $+6.5$ for the net charge of ferrocytochrome *c* at pH 7.0 [37], we obtain a value of -12 for the effective net charge of cytochrome aa_3.

The right-hand expression in Eqn. 2 represents the potential energy of cytochrome *c* in the electric field of cytochrome aa_3 when both proteins are in contact. It must be noted that the possible contributions that are made by ion-dipole and dipole-dipole interactions have not been taken into account. Since the exact charge distribution of cytochrome aa_3 is not yet known, it is difficult to predict to what extent corrections for these interactions will affect the results of this method.

Acknowledgements

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