Ionic-strength-dependence of the oxidation of native and pyridoxal 5'-phosphate-modified cytochromes *c* by cytochrome *c* oxidase

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The ionic-strength-dependences of the rate constants ($\log k$ plotted versus $\sqrt{I}$) for oxidation of native and pyridoxal 5'-phosphate-modified cytochromes *c* by three different preparations of cytochrome *c* oxidase have complex non-linear character, which may be explained on the basis of present knowledge of the structure of the oxidase and the monomer–dimer equilibrium of the enzyme. The wave-type curve (with a minimum and a maximum) for oxidation of native cytochrome *c* by purified cytochrome *c* oxidase depleted of phospholipids may reflect consecutively inhibition of oxidase monomers (initial descending part), competition between this inhibition and dimer formation, resulting in increased activity (second part with positive slope), and finally inhibition of oxidase dimers (last descending part of the curve). The dependence of oxidation of native cytochrome *c* by cytochrome *c* oxidase reconstituted into phospholipid vesicles is a curve with a maximum, without the initial descending part described above. This may reflect the lack of pure monomers in the vesicles, where equilibrium is shifted to dimers even at low ionic strength. Subunit-III-depleted cytochrome *c* oxidase does not exhibit the maximum seen with the other two enzyme preparations. This may mean that removal of subunit III hinders dimer formation. The charge interactions of each of the cytochromes *c* (native or modified) with the three cytochrome *c* oxidase preparations are similar, as judged by the similar slopes of the linear dependences at *I* values above the optimal one. This shows that subunit III and the phospholipid membrane do not seem to be involved in the specific charge interaction of cytochrome *c* oxidase with cytochrome *c*.

INTRODUCTION

Charge interactions of cytochrome *c* with cytochrome *c* oxidase (EC 1.9.3.1) have been shown to be important for proper orientation, binding and electron transfer between the two macromolecules [1–3]. On the basis of chemical modification studies, a ring of highly conserved positively charged lysine residues in cytochrome *c* [4–9] has been proposed to interact specifically with negatively charged carboxy groups on subunit II of cytochrome *c* oxidase [10,11]. Bound cytochrome *c* is proposed to be also in contact with subunit III of cytochrome *c* oxidase [12], the subunit that in addition to participation in proton translocation [13] was also proposed to play an important role in stabilizing the dimers of the enzyme [14].

Since charge interactions are affected by ionic strength, the influence of the latter on the pre-steady-state [15–17] and steady-state [18] reaction has now been investigated. Studies on the ionic-strength-dependence of the rate constants for oxidation of cytochrome *c* by cytochrome *c* oxidase at a fixed cytochrome *c* concentration have shown deviations from linearity [16,17,19,20]. Attention has been focused on the apparently linear parts of the curves at intermediate or higher ionic-strength range (0.08–0.22) to estimate the net charge of cytochrome *c* oxidase [17].

The present paper describes studies on the complex cytochrome *c* oxidase reaction predominantly at lower ionic strengths. Three different cytochrome *c* oxidase

preparations (purified phospholipid-depleted enzyme [21], enzyme reconstituted into phospholipid vesicles [22] and subunit-III-depleted enzyme [23]) were chosen to analyse the interaction with native and pyridoxal 5'-phosphate-modified cytochromes *c* [24], thus providing different model systems. The derivatives of cytochrome *c* modified by negatively charged pyridoxal 5'-phosphate, namely [P-Pxy-Lys-79]cytochrome *c*, [P-Pxy-Lys-86]cytochrome *c* and [P-Pxy-Lys-79,P-Pxy-Lys-86]cytochrome *c*, have the same overall conformation and redox potentials as native cytochrome *c*, but their abilities to restore respiration in cytochrome *c*-depleted mitochondria are different: native cytochrome *c* > [P-Pxy-79]cytochrome *c* > [P-Pxy-Lys-86]cytochrome *c* > [P-Pxy-Lys-79,P-Pxy-Lys-86]cytochrome *c* [24].

The complex non-linear ionic-strength-dependence of the observed first-order rate constants for oxidation of native and pyridoxal 5'-phosphate-modified cytochromes *c* by the three different cytochrome *c* oxidase preparations is described in detail, and an attempt is made to explain it on the basis of the existing model for the structure of cytochrome *c* oxidase [12] and the functional model of homotropic negative co-operativity [25,26], taking into account the equilibrium between the oxidase monomers and dimers.

MATERIALS AND METHODS

Cytochrome *c* (type VI) and asolectin (L- α -phosphatidylcholine, type II-S) were obtained from

Abbreviation used: P-Pxy-, 5'-phosphopyridoxyl.

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Fig. 1. Ionic-strength-dependence of the apparent first-order rate constants for the oxidation of native and pyridoxal 5'-phosphate-modified ferrocycytochromes *c* by purified phospholipid-depleted cytochrome *c* oxidase

The concentration of the oxidase was 0.6 nM. The initial concentrations of native cytochrome *c* (●), [*P*-Pxy-Lys-79]cytochrome *c* (○), [*P*-Pxy-Lys-86]cytochrome *c* (▼) and [*P*-Pxy-Lys-79, *P*-Pxy-Lys-86]cytochrome *c* (△) were adjusted to 1 μM. The ionic strength varied from 0.01 to 0.21 (0–0.2 M-KCl) in a buffer containing 0.5 % Tween-80, 5 mM-Tris/HCl and 5 mM-Hepes with pH 6.4 (a), pH 7.4 (b) and pH 8.2 (c).

Fig. 2. Ionic-strength-dependence of the apparent first-order rate constants for the oxidation of native and pyridoxal 5'-phosphate-modified ferrocycytochromes *c* by cytochrome *c* oxidase reconstituted into phospholipid vesicles

The concentration of the enzyme was 0.85 nM. The vesicles were uncoupled with 0.25 μM-valinomycin and 2.5 μM-carbonyl cyanide *m*-chlorophenylhydrazine. The initial concentrations of native cytochrome *c* (●), [*P*-Pxy-Lys-79]cytochrome *c* (○), [*P*-Pxy-Lys-86]cytochrome *c* (▼) and [*P*-Pxy-Lys-79, *P*-Pxy-Lys-86]cytochrome *c* (△) were adjusted to 1 μM. The ionic strength varied from 0.01 to 0.11 (0–0.1 M-KCl) in a buffer containing 5 mM-Tris/HCl and 5 mM-Hepes with pH 6.4 (a), pH 7.4 (b) and pH 8.2 (c).

Sigma Chemical Co. Tris, Triton X-100 and pyridoxal 5'-phosphate were from Fluka, and Amberlite CG-50 was from BDH Chemicals. Sephadex G-75 and activated thiol-Sepharose 4B (Sepharose-glutathione-2-pyridyl disulphide) were from Pharmacia. Cholic acid (Merck) was purified by recrystallization from acetone by acidification with HCl. All other reagents were of the highest analytical grade commercially available.

Bovine heart mitochondria were prepared by the method of Smith [27] and were kept frozen. Purified cytochrome *c* oxidase was isolated from them by the method of Yu *et al.* [21] and stored highly concentrated in small portions in 50 mM-sodium phosphate buffer, pH 7.4, at -80°C . Cytochrome *c* oxidase reconstituted

into phospholipid vesicles was obtained by the method of cholate dialysis [22]. Subunit-III-depleted cytochrome *c* oxidase was prepared by affinity chromatography [23]. Total protein concentration was determined by the method of Wang & Smith [28].

Pyridoxal 5'-phosphate-modified derivatives of cytochrome *c* were prepared as described in ref. [24]. Native and modified cytochromes *c* were reduced with dithionite. The reducing agent and any polymeric material were removed on a Sephadex G-75 column, as described by Ferguson-Miller *et al.* [7].

Spectral analysis was performed with an Aminco DW-2a spectrophotometer. The absorption coefficients (reduced-minus-oxidized) used for the haem *a* content of cytochrome *c* oxidase and cytochrome *c* were $12\text{ mm}^{-1}\cdot\text{cm}^{-1}$ at 605 nm–630 nm [29] and $21.1\text{ mm}^{-1}\cdot\text{cm}^{-1}$ at 550 nm–540 nm [30] respectively. The activity of the different oxidase preparations was assayed spectrophotometrically in the same spectrophotometer at 550 nm–540 nm (double-wavelength mode) at 25°C [31]. The activities were expressed as observed first-order rate constants, k (s^{-1}) [32,33], determined from semi-logarithmic plots of the entire time courses.

The ionic strength was adjusted by the addition of appropriate amounts of KCl and precisely checked by conductimetric measurements. The statistical processing of the results was carried out by linear-regression analysis.

RESULTS

A typical effect of the ionic strength on the oxidation of native and pyridoxal 5'-phosphate-modified cytochromes *c* by purified cytochrome *c* oxidase is shown in Fig. 1 at three different pH values: (a) pH 6.4, (b) pH 7.4 and (c) pH 8.2. Though there are some quantitative differences (increase of pH leads to a slight decrease in activity), the curves for each of the cytochromes have the same course at the three pH values. The dependences of $\log k$ on $\sqrt{I}$ are not linear even within a relatively narrow range of ionic strengths. At low ionic strength the activity for native cytochrome *c* at first goes down. Further increase of ionic strength leads to a peak activity at I 0.06. For [P-Pxy-Lys-79]cytochrome *c* and [P-Pxy-Lys-86]cytochrome *c* an initial descending part is not seen. The maxima of the curves are shifted towards lower ionic strengths. In the case of [P-Pxy-Lys-79, P-Pxy-Lys-86]cytochrome *c* at pH 6.4 and 7.4 only the linear parts of the dependences, without

Fig. 3. Ionic-strength-dependence of the apparent first-order rate constants for the oxidation of native and pyridoxal 5'-phosphate-modified ferrocytochromes *c* by subunit-III-depleted cytochrome *c* oxidase

The concentration of the enzyme was 1.14 nM. The initial concentrations of native cytochrome *c* (●) and [P-Pxy-Lys-79]cytochrome *c* (○) were adjusted to 1 μM . The ionic strength varied from 0.01 to 0.21 (0–0.2 M-KCl) in a buffer containing 0.5% Tween-80 and 10 mM-Tris/HCl with pH 7.4.

Table 1. Comparison of preparations of purified cytochrome *c* oxidase, cytochrome *c* oxidase reconstituted into phospholipid vesicles and subunit-III-depleted cytochrome *c* oxidase

The values of the rate constants at zero ionic strength (k_0) and the slopes are determined from the linear parts of the curves in Figs. 1–3 at I values higher than at the apparent maxima, at pH 7.4. Abbreviation: N.D., not determined.

Cytochrome <i>c</i>	Purified cytochrome <i>c</i> oxidase		Reconstituted cytochrome <i>c</i> oxidase		Subunit-III-depleted cytochrome <i>c</i> oxidase	
	k_0 (s^{-1})	Slope	k_0 (s^{-1})	Slope	k_0 (s^{-1})	Slope
Native cytochrome <i>c</i>	0.285	–4.3	1.106	–5.1	0.229	–4.6
[P-Pxy-Lys-79]cytochrome <i>c</i>	0.035	–5.2	0.070	–5.2	0.025	–4.6
[P-Pxy-Lys-86]cytochrome <i>c</i>	0.022	–7.5	0.054	–8.5	N.D.	N.D.
[P-Pxy-Lys-79, P-Pxy-Lys-86]cytochrome <i>c</i>	0.004	N.D.	0.033	–9.1	N.D.	N.D.

maxima, can be observed. At pH 8.2 the enzyme activity for this derivative is considerably lowered. It may be proposed that the curves for [P-Pxy-Lys-79, P-Pxy-Lys-86]-cytochrome *c* also have maxima but that the shifts towards lower ionic strength do not allow them to be registered.

Fig. 2 shows the ionic strength dependence of the oxidation of native and pyridoxal 5'-phosphate modified cytochromes *c* at (a) pH 6.4, (b) pH 7.4 and (c) pH 8.2 by cytochrome *c* oxidase reconstituted into phospholipid vesicles. In contrast with the purified oxidase, no initial descending part is seen for native cytochrome *c* at any of the pH values. Its peak activity is at the same ionic strength (I 0.06) as for the purified enzyme. The curve for [P-Pxy-Lys-79]cytochrome *c* has a maximum, shifted to lower ionic strengths. For [P-Pxy-Lys-86]cytochrome *c* and [P-Pxy-Lys-79, P-Pxy-Lys-86]cytochrome *c* only linear dependence is registered.

The ionic-strength-dependence of the oxidation of native cytochrome *c* by subunit-III-depleted cytochrome *c* oxidase (Fig. 3) differs from that for purified enzyme (Fig. 1) or reconstituted enzyme (Fig. 2). Only remnants of the peak activity are observed. The last linear descending part of the curve is clearly expressed at higher ionic strength. The curve for [P-Pxy-Lys-79]cytochrome *c* is without a maximum. Measurements with [P-Pxy-Lys-86]cytochrome *c* and [P-Pxy-Lys-79, P-Pxy-Lys-86]cytochrome *c* were not carried out because of the considerably lowered activity.

The apparently linear ionic-strength-dependences at I values higher than the optimum (Figs. 1–3) were analysed by using eqn. (1) [34]:

$$\log k = \log k_0 + 2AZ_0Z_c\sqrt{I} \quad (1)$$

where $A = 0.509$ at 25 °C and Z_0 and Z_c are the net charge numbers of the oxidase and cytochrome *c* respectively.

As Veerman *et al.* [17] have shown, although eqn. (1) [34] is known to be applicable to reactions between small ions at low ionic strength [35], a useful comparison of the charge interactions of cytochrome *c* oxidase with different cytochrome *c* derivatives can be made by evaluating the rate constants at zero ionic strength and the effective charges of the reacting proteins from the apparently linear ionic-strength-dependences of the rate constants [17].

Taking into account the critical remarks made on the application of eqn. (1) [34] to protein–protein interactions in refs [17], [36] and [37], we consider that the apparently linear ionic-strength-dependences in Figs. 1–3 may be useful only in a comparative qualitative evaluation of charge interactions between cytochrome *c* and different cytochrome *c* oxidase preparations. The slopes of these linear dependences at I values higher than the optimum, which reflect the number of the charges involved, are presented in Table 1. The slopes for the three cytochrome *c* oxidase preparations and a given form of cytochrome *c* are similar. This could mean that the charge interactions of cytochrome *c* with the different oxidase preparations are similar. The modification by pyridoxal 5'-phosphate, depending on the position of the substituted lysine residue, alters the slopes not more than 2-fold. Table 1 also contains the values of k_0 , which were estimated by extrapolation to zero ionic strength. The values of k_0 for the cytochrome *c* derivatives are considerably lower than for native cytochrome *c*.

Scheme 1. Equilibria for enzyme–substrate binding and monomer–dimer transition in the interaction of cytochrome *c* and cytochrome *c* oxidase

The **bold** arrows show the direction of the equilibrium shifts with increase of ionic strength. Abbreviation: Cyt. *c*, cytochrome *c*.

DISCUSSION

From studies on the ionic-strength-dependence of the rate constants for the cytochrome *c* oxidase reaction it has long been known that this dependence has a complex character [16,17,19,20]. The wave-type curve for native cytochrome *c* and purified cytochrome *c* oxidase (Fig. 1), with its minimum and maximum within the pH range 6.4–8.2, cannot be explained solely by salt shielding and charge repulsion between substrate molecules. The molecular form of the enzyme (monomeric or dimeric) should also be taken into consideration, as well as the fact that the monomer–dimer equilibrium is salt-dependent, too. Superposition of both equilibria (enzyme–substrate binding and monomer–dimer transition) is presented in Scheme 1.

Taking into account the existing structural model for cytochrome *c* oxidase [12] and the functional model for homotropic negative co-operativity [25,26], the following explanation is proposed for the complex ionic-strength-dependence of the rate constants.

At very low ionic strengths the cytochrome *c* oxidase molecules are monomeric owing to charge repulsion in absence of salts, as experimentally proven [26]. The negative slope of the first part of the curve for native cytochrome *c* (Fig. 1) might represent the simple shielding effect of ionic strength on the rate constants for the monomeric enzyme according to Brönsted–Debye–Hückel theory [34,35]. Further increase of ionic strength allows two different processes to occur: (1) inhibition of monomer activity as a result of diminished cytochrome *c* binding; (2) rise of formation of dimers. The occupation by one molecule of cytochrome *c* of one binding site in the oxidase dimer hinders occupation of the second site by the next molecule. Thus negative co-operativity creates conditions for increasing the 'off' rate constant of cytochrome *c* with consequent increase in $V_{\max}$. This increase of the velocity is bigger than the simple inhibitory effect of ionic strength.

At higher ionic strengths, where the dimers are already formed [26], further increase of the ionic strength decreases their activity (see the last descending part of the curves with maxima in Fig. 1).

From this point of view, the lack of an initial descending part in the dependence for cytochrome *c* oxidase reconstituted into vesicles (Fig. 2) is not surprising. In the phospholipid vesicles there are no pure monomers because the enzyme molecules are fixed on the membrane and association is preferred, as proven experimentally [26]. Thus the enzyme inserted into

phospholipid vesicles reacts predominantly as a dimer even at low ionic strength. Further increase of ionic strength accelerates the dimerization process. Thus the activity of the enzyme, passing through a maximum, is then inhibited.

Our proposal, that the non-linear ionic-strength-dependence of the cytochrome *c* oxidase reaction is strongly dependent on the monomer–dimer equilibrium, is further proven by the loss of maximum in the case of subunit-III-depleted enzyme (Fig. 3). This preparation of the enzyme has been shown to be monomeric and to demonstrate monophasic kinetics in Eadie–Hofstee plots at I 0.06 [14]. Our results provide evidence that the removal of subunit-III hinders dimer formation.

As mentioned above, the ionic-strength-dependences of the rate constants for the cytochrome *c* oxidase reaction have been used to evaluate the effective charges of the reacting proteins and the rate constants at zero ionic strength [17]. The analysis has been done with the linear descending parts of the curves over an intermediate range of I (0.08–0.22) by using the Brönsted [34] equation [17]. The values of the parameters estimated by these plots depend on the experimental conditions, and, as discussed in refs. [17], [36] and [37], these estimates may be grossly inaccurate.

We consider that these plots give, though not full and exact, nevertheless useful qualitative information about the charge environment in the different cytochrome *c* oxidase preparations. The apparent slopes of the linear parts of the curves in Figs. 1–3 at I values higher than the optimum (see Table 1) are in the range 4.3–9.1. This may mean that only two or three pairs of complementary charges might be crucial for the formation of a complex between any of the cytochrome *c* oxidase preparations and any of the forms of cytochrome *c*. This is in line with Sinjorgo *et al.* [18], who have postulated that the electrostatic interactions between cytochrome *c* and the oxidase can (at least above I 0.025) be approximated by the electrostatic interactions between the interaction domains on the proteins.

The almost equal values of the slopes show that the charge interactions of any of the forms of cytochrome *c* with the three oxidase preparations are similar. This could mean that the removal of subunit-III or the presence of phospholipids in the vesicles does not alter the charge interactions.

Data for k_0 in Table 1 are in line with our previous results for restoration of respiration in cytochrome *c*-depleted mitochondria by native and pyridoxal 5'-phosphate-modified cytochromes *c* [24]. The activity of any of the three cytochrome *c* oxidase preparations towards the different forms of cytochrome *c* decreases in the order native cytochrome *c* > [*P*-Pxy-Lys-79]-cytochrome *c* > [*P*-Pxy-Lys-86]-cytochrome *c* > [*P*-Pxy-Lys-79,*P*-Pxy-Lys-86]-cytochrome *c* (Figs. 1–3 and Table 1), though the derivatives have the same redox potential and overall conformation as native cytochrome *c* [24]. The higher affinity of the oxidase for [*P*-Pxy-Lys-79]-cytochrome *c* than for [*P*-Pxy-Lys-86]-cytochrome *c* (Figs 1 and 2) can be explained by the different positions of Lys-79 and Lys-86 relative to the dipole axis of the charged multipole of cytochrome *c* [3].

The results in Figs. 1 and 2 do not show a strong pH-dependence of the rate constants. From this it may be assumed that no histidine residue takes part in the ionic cluster that is important for binding to the oxidase.

Numerous studies on the oxidation of cytochrome *c* by cytochrome *c* oxidase have given rise to structural [12] and functional models [25,26,38–40] of the enzyme. The biphasic steady-state kinetics of electron transfer from ferrocytochrome *c* to O₂ were interpreted as two active sites per monomer of oxidase [38]. The electrostatic influence of a single bound molecule of cytochrome *c* on the binding of others to non-catalytic sites on cytochrome *c* oxidase was subsequently discussed [39]. The single-catalytic-site model was proposed by Speck *et al.* [40] to account for the multiphasic kinetics of oxidation of ferrocytochrome *c* by cytochrome *c* oxidase. This model involves non-productive binding of substrate to sites near the catalytic site on cytochrome *c* oxidase for cytochrome *c*, decreasing the binding constant for cytochrome *c* at the catalytic site. The resulting increase in the first-order rate constant for the dissociation of the ferricytochrome *c*–cytochrome *c* oxidase complex gave the observed multiple kinetic phases [40]. The model of homotropic negative co-operativity [25,26] extends the single-catalytic-site model [40] and takes into account the structure [12] and the molecular form of the enzyme (monomeric or dimeric). It was proposed that dimers of cytochrome *c* oxidase contain two equivalent binding sites (one per monomer), located in the cleft between monomers [12,25,26]. Binding of one molecule of cytochrome *c* to any of those two sites makes the other one inequivalent, with a lowered affinity for cytochrome *c* due to steric and energetic hindrances.

The data presented in this paper are in line with present structural [12] and functional [25,26,40] models of cytochrome *c* oxidase. They support the idea that the effective enzyme activity depends not only on substrate binding but also on monomer–dimer equilibrium of the oxidase. Increase of ionic strength has opposing effects on these protein–protein interactions: the dimerization of the enzyme is increased, while the enzyme–substrate binding is decreased. By these effects, the complex ionic-strength-dependence of the rate constants for the cytochrome *c* oxidase reaction is explained above. The removal of subunit III does not seem to change the nature of the electrostatic interaction of the oxidase with cytochrome *c* but seems to hinder dimer formation. In the binding domains between cytochrome *c* and cytochrome *c* oxidase a histidine residue is not expected, as judged by the lack of strong pH-dependence of the rate constants in the pH range 6.4–8.2. Data from comparison between [*P*-Pxy-Lys-79]-cytochrome *c* and [*P*-Pxy-Lys-86]-cytochrome *c* are in line with the idea that the local interactions in the binding domains are mainly responsible for the specific interaction of cytochrome *c* oxidase with cytochrome *c*.

The financial support of the Schweizerische Nationalfonds (no. 3.739–0.80), a grant from the World Health Organisation for G. K. and partial support from the Bulgarian Science Council (no. 549/87) are gratefully acknowledged.

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Received 28 December 1988/28 March 1989; accepted 17 April 1989