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The effects of pH and ionic strength on cytochrome *c* oxidase steady-state kinetics reveal a catalytic and a non-catalytic interaction domain for cytochrome *c* *

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The influence of pH and ionic strength on the steady-state kinetics of purified bovine cytochrome *c* oxidase was studied by spectrophotometry. At low ionic strength, increasing the pH in the range between 5.4 and 8.6 resulted in a slight decrease in maximal turnover numbers of the high-affinity and the low-affinity reactions. The high-affinity K_m was also found to decrease with increasing pH. The ionic-strength dependence of the steady-state kinetics of positively charged cytochrome *c* oxidase at pH 6.2 and that of negatively charged cytochrome *c* oxidase at pH 7.8 were similar; in both cases, high-affinity K_m values and high-affinity and low-affinity TN_{max} values increased with ionic strength. The low-affinity K_m was independent of both pH and ionic strength. Above $I = 100$ mM, no low-affinity reaction could be observed. A description of the electrostatic interactions between cytochrome *c* and cytochrome *c* oxidase, based on the overall monopoles and overall dipoles of the two proteins, could not explain our data. We propose that at $I \geqslant 25$ mM such an approximation cannot be used for electrostatic interactions between large proteins, since the assumption that all charges on the surfaces of the reacting proteins would contribute equally to the electrostatic interaction is not valid. A qualitative description of electrostatic interactions between the two cytochromes based on limited electrostatic interaction domains on the cytochrome *c* oxidase surface was found to be in good agreement with all our data and supports the model of Speck et al. (Speck, S.H., Dye, D. and Margoliash, E. (1984) Proc. Natl. Acad. Sci. USA 81, 347–351), who proposed one catalytic and one non-catalytic cytochrome *c* binding site. It is proposed that the allosteric effect of the cytochrome *c* at the non-catalytic site is of an electrostatic nature. At high ionic strength (occurring in vivo), this cytochrome *c* molecule would then no longer affect the catalytic site, resulting in the absence of the low-affinity reaction.

Introduction

The kinetics of cytochrome *c* oxidase (EC 1.9.3.1) have been investigated extensively. In studies using an extended range of cytochrome *c* concentrations, the steady-state reaction rate was shown not to be first-degree in substrate concentration [1–4]. At low ionic strength, the observed concave Eadie-Hofstee plots could be described by two reactions between cytochrome *c* and cytochrome *c* oxidase: a high-affinity reaction with an apparent K_m of 10^{-8} M and a low-affinity reaction of $K_m = 2 \cdot 10^{-5}$ M [5].

* This paper is dedicated to the memory of Gerrit J.A. Schilder, gratefully recalling his many inspiring contributions to our discussions on the mechanism of cytochrome *c* oxidase.

Abbreviation: Mops, 4-morpholinepropanesulphonic acid.

An important item in cytochrome *c* oxidase studies has been the effect of pH and ionic strength on its kinetics. The results of these studies have been rather contradictory. Many authors studied the ionic-strength effects at one cytochrome *c* concentration only [6–10] and observed an optimum in enzyme activity. Studies at a series of high cytochrome *c* concentrations revealed a maximal turnover number independent of ionic strength, while the K_m increased when the ionic strength was raised [11–13]. Furthermore, Brooks et al. [14] reported an increase of high-affinity maximal turnover numbers with increasing ionic strength.

In studies of pH effects on cytochrome *c* oxidase kinetics where only one substrate concentration was used, an optimum was observed [7–9], whereas in studies with a series of high cytochrome *c* concentrations the observed K_m and TN_{max} were found to decrease when the pH was raised [11,13].

To explain these incoherent results we studied the dependence of cytochrome *c* oxidase steady-state kinetics on pH and ionic strength, using a broad range of cytochrome *c* concentrations. We analysed the effects on high-affinity and low-affinity K_m and TN_{max} values separately.

Since ionic strength affects the cytochrome *c* oxidase reaction rate, it is generally accepted that electrostatic interactions play an important role in the reaction between cytochrome *c* and the oxidase. Different types of electrostatic interaction have been considered. The monopole–monopole interaction describes the electrostatic forces between the two proteins resulting from their total electrical charges. As was argued by Koppenol and Margoliash [15], also the distribution of charge over the protein surfaces must be considered. They proposed that electrostatic forces resulting from the asymmetric distribution of charges help the molecules to attain the proper orientation for electron transfer. This was also suggested in studies of Matthew et al. [16]. Dipole moments, describing the charge distributions, have been calculated for a number of proteins, including tuna fish cytochrome *c* [17] and horse cytochrome *c* [15].

On the basis of chemical modification studies [18–23], a number of highly conserved lysine residues surrounding the cytochrome *c* haem crevice has been proposed to interact specifically with carboxyl groups on subunit II of cytochrome *c* oxidase [24]. Several authors ascribed the observed ionic-strength dependence of cytochrome *c* oxidase kinetics to a number of the complementary-charge interactions [12,25].

The results of our pH and ion-strength dependence studies could explain the differences in published data of other authors and are discussed in the light of the role of the electrostatic interactions mentioned.

Materials and Methods

Bovine heart cytochrome *c* oxidase was purified according to the method of Fowler et al. [26], as modified in our laboratory [27]. Cytochrome *c* was prepared from horse heart as described by Margoliash and Walasek [28]. Ferrocytochrome *c* was obtained by incubating cytochrome *c* with ascorbate, followed by gel filtration on Sephadex G-50 superfine (Pharmacia) in Tris-Mops/1 mM EDTA. The pH and ionic strength of this buffer were always equal to those of the medium in which the steady-state kinetic experiments were performed.

Tris-Mops buffers of varyous pH values and ionic strength were prepared by mixing the two components up to the required pH. Ionic strengths were calculated from the two volumes used and, if necessary, adjusted by adding solid KCl.

The absorbance coefficients (reduced-minus-oxidised) used for cytochrome aa_3 and cytochrome *c* were 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm and 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm [29,30], respectively.

The steady-state activity of cytochrome *c* oxidase was determined spectrophotometrically at 25°C in Tris-Mops buffers of various pH and ionic strength, containing 1 mM EDTA and 0.05% laurylmaltoside. Reduced cytochrome *c* was added to a final concentration of 0.05–100 μM. For the higher cytochrome *c* concentrations reaction rates were determined at 520 nm (35–100 μM) or 550 nm (1–35 μM), using a Cary-14 spectrophotometer modified in our laboratory. Apparent first-order rate constants are determined from semi-logarithmic plots of the entire time-courses and displayed directly on this apparatus. Reactions at cytochrome *c* concentrations of 0.05–10 μM (usually showing very short half-times) were

followed at 416 nm by using a Durrum stopped-flow apparatus. In this case the apparent first-order rate constants, mean values of 5–10 traces, were determined using a Hewlett-Packard 2100A computer, as described before [31].

The final concentration of cytochrome *c* oxidase was 1 nM. In order to equilibrate the cytochrome *c* oxidase with laurylmaltoside, 16 h prior to each experiment the enzyme was transferred to a medium consisting of 100 mM Tris-Mops/1 mM EDTA/0.25 M sucrose (pH 7.8, 0°C) containing 0.5% of the detergent. This resulted in high and stable cytochrome *c* oxidase activities.

K_m and TN_{max} values of high-affinity and low-affinity reactions were determined by computer analysis of the steady-state kinetic data, based on independent catalytic sites [5]. Subsequently, we used $TN_{max\,1} + TN_{max\,2}$ for low-affinity TN_{max} values, in accordance with the cooperative-site model of Speck et al. [32].

Results

Under all conditions of pH and ionic strength we found that the oxidation of cytochrome *c* catalysed by cytochrome *c* oxidase showed strictly first-order kinetics [33,34] in contrast to the report of Yonetani and Ray [35]. Fig. 1 shows the steady-state reaction of cytochrome *c* oxidase, determined spectrophotometrically over a broad range of cytochrome *c* concentrations at $I = 25$ mM, at different pH values (5.4–8.6). In order to determine the effect of pH on the high-affinity and low-affinity components of the reaction, the curves were resolved into the two separate reactions, as described before [5]. TN_{max} and K_m values are given in Table I. Maximal turnover numbers of both the high-affinity and the low-affinity reactions, and the apparent high-affinity K_m were found to decrease when the pH was raised. Between pH 6.2 and 8.6, the low-affinity K_m showed almost no pH dependence. At pH 5.4, only a small part of the low-affinity reaction was observed and, consequently, kinetic parameters could not be determined.

Fig. 2 shows the steady-state reaction of cytochrome *c* oxidase at pH 7.8 at different ionic strengths. The high-affinity and low-affinity kinetic parameters calculated from the different curves are shown in Table II. Maximal turnover numbers of both the high-affinity and the low-affinity reactions increased when the ionic strength was raised. The apparent K_m of the high-affinity reaction was

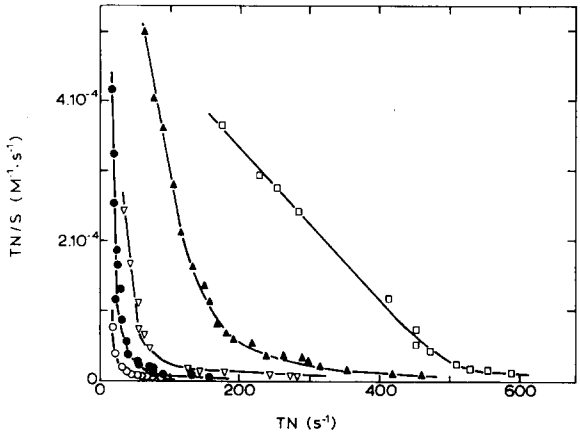

Fig. 1. Eadie-Hofstee representation of steady-state oxidation of horse ferrocytochrome *c* by purified bovine heart cytochrome *c* oxidase, monitored spectrophotometrically in Tris-Mops buffer ($I = 25$ mM). The curves were simulated by computer as described in Materials and Methods. ○——○, pH 8.6; ●——●, pH 7.8; ▽——▽, pH 7.0; ▲——▲, pH 6.2; □——□, pH 5.4.

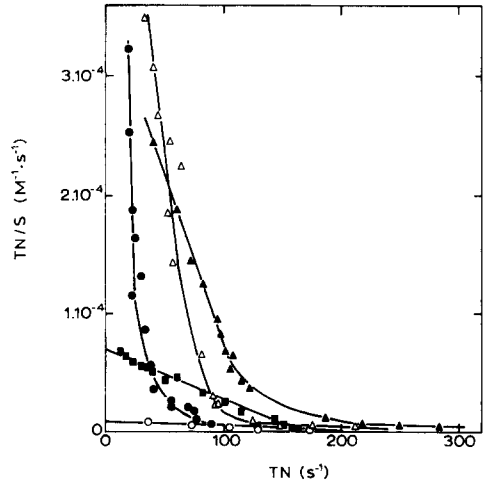

Fig. 2. Eadie-Hofstee plot of steady-state oxidation of horse-heart ferrocytochrome *c* by purified bovine heart cytochrome *c* oxidase, measured spectrophotometrically in Tris-Mops buffers (pH 7.8). The curves were simulated by computer as described in Materials and Methods. ●——●, $I = 25$ mM; △——△, $I = 35$ mM; ▲——▲, $I = 50$ mM; ■——■, $I = 100$ mM; ○——○, $I = 200$ mM.

TABLE I

THE pH DEPENDENCE OF HIGH- AND LOW-AFFINITY KINETIC PARAMETERS OF CYTOCHROME *c* OXIDASE STEADY-STATE REACTIONS

The values were obtained at 25°C in a buffer containing 25 mM Tris-Mops/1 mM EDTA/0.05% laurylmaltoside ($I = 25$ mM). The cytochrome *c* concentration was between 0.05 and 100 μM, the cytochrome *c* oxidase was 1 nM. The kinetic parameters were determined by computer analysis as described in Materials and Methods. n.d., not determined.

pH	Low affinity		High affinity	
	K_m (μM)	TN_{max} (s^{-1})	K_m (μM)	TN_{max} (s^{-1})
5.4	n.d.	n.d.	0.85	270
6.2	30	560	0.2	106
7.0	30	410	0.12	60
7.8	40	230	0.04	33
8.6	12	110	n.d.	n.d.

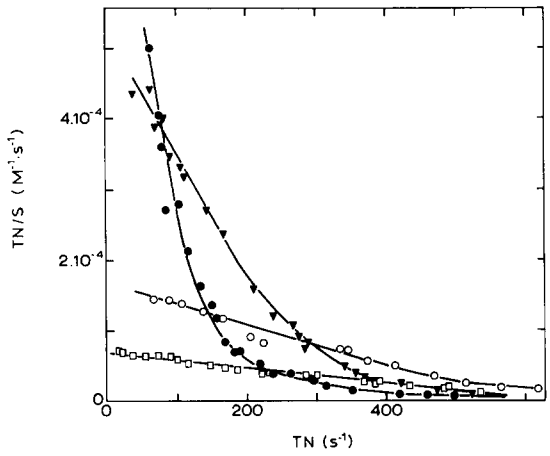

Fig. 3. Eadie-Hofstee representation of the steady-state oxidation of horse ferrocytochrome *c* by purified bovine cytochrome *c* oxidase, measured spectrophotometrically in Tris-Mops buffer (pH 6.2). The curves were simulated by computer as described in Materials and Methods. ●——●, $I = 28.4$ mM; ▼——▼, $I = 40$ mM; ○——○, $I = 70$ mM; □——□, $I = 100$ mM.

also found to increase upon increasing ionic strength. The low-affinity K_m, on the other hand, seemed little dependent on ionic strength. Above $I = 100$ mM, straight lines were obtained in Eadie-Hofstee plots, indicating a steady-state reaction rate that is first-degree in substrate concentration. At pH 7.8 in this experiment, the cytochrome *c* molecule had a positive net charge of +7 [15]. The charge of the cytochrome *c* oxidase molecule is not precisely known, but it is certainly negative. Assuming that 20% of its amino termini will be deprotonated at this pH and that the protonation of its histidyl side chains can be neglected, and taking the charge of the two tightly bound cardiolipin molecules into account, cytochrome *c* oxidase has 13 more negatively charged

TABLE II

THE IONIC-STRENGTH DEPENDENCE OF CYTOCHROME *c* OXIDASE HIGH-AFFINITY AND LOW-AFFINITY STEADY-STATE KINETIC PARAMETERS AT pH 6.2 AND pH 7.8

The values were obtained at 25°C in Tris-Mops buffers containing 1 mM EDTA/0.05% laurylmaltoside. The cytochrome *c* concentration was between 0.05 and 100 μM; the cytochrome *c* oxidase concentration was 1 nM. The kinetic parameters were determined by computer analysis as described in Materials and Methods.

I (mM)	pH 7.8				pH 6.2			
	Low affinity		High affinity		Low affinity		High affinity	
	K_m (μM)	TN_{max} (s^{-1})	K_m (μM)	TN_{max} (s^{-1})	K_m (μM)	TN_{max} (s^{-1})	K_m (μM)	TN_{max} (s^{-1})
25	40	255	0.04	33				
28.4					30	590	0.23	165
35	50	280	0.13	81				
40					35	645	0.55	280
50	60	300	0.3	112				
70					40	700	3.5	535
100	–	–	2.2	160	–	–	8.6	600
200	–	–	22	210				

than positively charged groups [36].

Fig. 3 shows the steady-state reaction of cytochrome *c* oxidase at pH 6.2 at various ionic strengths. At this pH, the net electrical charge of the cytochrome *c* molecule is +8 [15]. Again, the net electrical charge of cytochrome *c* oxidase cannot be determined, but the protonation of many of the 67 histidyl residues will have given the protein a positive charge. This is in agreement with preliminary results of isoelectric focussing experiments performed in our laboratory. Apparent K_m and TN_{max} values of the high-affinity and the low-affinity reactions at this pH are shown in Table II. Although at this pH (6.2) cytochrome *c* oxidase and cytochrome *c* both have a positive net charge, we surprisingly found the same positive correlation between ionic strength and K_m and TN_{max} values as we observed at pH 7.8 where the two proteins had opposite charges. Again, Fig. 3 demonstrates that above 100 mM ionic strength straight lines are found in the Eadie-Hofstee plots, with K_m values in the micromolar range. Since the high-affinity K_m gradually increased from very low values at $I = 25$ mM up to 10–20 μM at high ionic strength, we conclude that it is the high-affinity reaction that is observed at high ionic strength, and that under these conditions (close to the physiological state) the low-affinity reaction does not contribute to catalytic activity.

Discussion

No ionic strength or pH optimum is found in cytochrome c oxidase steady-state kinetics

We determined the effects of pH and ionic strength on cytochrome *c* oxidase steady-state kinetics. Using an extended range of cytochrome *c* concentrations, we could distinguish between effects on the high-affinity reaction and those on the low-affinity reaction. Studies of ionic-strength effects at only one cytochrome *c* concentration always showed an optimum in enzymic activity [6–10]. However, we observed no optimum in K_m nor in TN_{max} values of the two reactions. By inspecting our measurements at one cytochrome *c* concentration in Figs. 2 and 3 (intersection points of the curves with a straight line through the origin), it can be seen that the experiments done at only one cytochrome *c* concentration would lead to this misinterpretation, because a combination of the high-affinity and the low-affinity reactions is being measured.

The fact that also in studies of pH effects on cytochrome *c* oxidase steady-state kinetics at only one cytochrome *c* concentration an optimum was found [7–9] cannot be explained in this way. Fig. 1 shows that an increase in enzyme activity should be observed at increasing pH, since the different lines only intersect at very low cytochrome *c* concentrations. As was proposed by Wilms et al. [11], we believe that the observation of a pH optimum must be ascribed to the fact that in those experiments the ionic strength of the buffer system was not kept constant.

We have demonstrated that the only reaction between cytochrome *c* and cytochrome *c* oxidase observed at ionic strengths of $I \geqslant 100$ mM is related to the high-affinity reaction at low ionic strength. This is in agreement with the results of Brooks and Nicholls [14]. We would therefore emphasise that, although the K_m value of this reaction is in the micromolar range, it should be named the high-affinity reaction.

Local electrostatic interactions vs. total charge interactions

We studied the ionic-strength dependence of cytochrome *c* oxidase steady-state kinetics to investigate whether the electrostatic interactions between cytochrome *c* and cytochrome *c* oxidase could be described by the sum of electrostatic interactions between total monopoles and total dipoles of the proteins, as was proposed by Koppenol and co-workers [15,17] and Van Leeuwen [37]. Their models are based on the assumption that all charges on the surface of the reacting protein contribute equally to the electrostatic interaction.

We studied the oxidase when it has a positive total charge (pH 6.2) or a negative one (pH 7.8). Under both conditions the same ionic-strength dependence was observed. The turnover numbers of the high-affinity and the low-affinity reactions and the high-affinity K_m increased with ionic strength, which is indicative of a negatively charged cytochrome *c* oxidase reacting with a positively charged cytochrome *c*. Because the reversal in overall charge of the cytochrome *c* oxidase mole-

cule hardly reflected on the ionic-strength dependence of its steady-state kinetics, we conclude that the charges on the surfaces of the reacting proteins do not contribute equally to the electrostatic interactions. We propose that the configuration of charges on the large cytochrome *c* oxidase molecule is too inhomogeneous to validate a description of electrostatic interactions based on the total monopole and total dipole of the protein [37].

Our observations are in line with those of Tollin et al. [38], who found that both horse-heart cytochrome *c* and *P. denitrificans* cytochrome c_2 reacted with flavodoxin as though they were positively charged proteins, even though the cytochrome c_2 carries a net negative charge.

Since total-charge interactions fail to explain the observations, we postulate that the electrostatic interactions between cytochrome *c* and the oxidase can (at least above $I = 25$ mM) be approximated by the electrostatic interactions between the interaction domains on the two proteins.

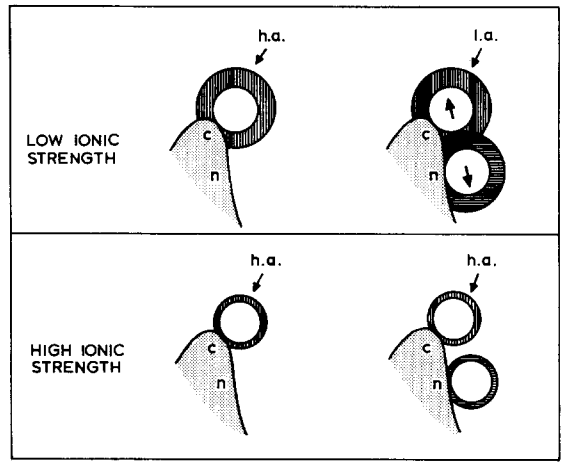

Fig. 4. Schematic representation of the effects of binding of a cytochrome *c* molecule to the non-catalytic site at low and high ionic strength. The shaded circles surrounding the cytochrome *c* molecules represent their electric influence spheres. Arrows show the electrostatic forces between the cytochrome *c* molecules. c, catalytic site; n, non-catalytic site; h.a., high-affinity reaction; l.a., low-affinity reaction.

Our studies support the existence of one catalytic and one allosteric cytochrome c binding site on the oxidase

As demonstrated before [5,32], both high-affinity and low-affinity reactions of cytochrome *c* oxidase may result from only one electron-accepting site on subunit II of cytochrome *c* oxidase. Speck et al. [32] proposed that binding of a cytochrome *c* molecule to a regulatory site on cytochrome *c* oxidase leads to a decrease in affinity of the catalytic site for cytochrome *c* coupled with an increase in the turnover of the cytochrome *c* oxidase reaction.

We will demonstrate in a qualitative fashion that the results of our pH and ionic-strength dependence studies can be explained on the basis of this model, combined with the approximation of electrostatic interactions named above.

Fig. 4 schematically shows the electrostatic interactions between cytochrome *c* molecules bound at the catalytic and regulatory sites and of these cytochrome *c* molecules with the oxidase. Since at ionic strengths higher than 25 mM we only expect changes in electrostatics in the vicinity of the catalytic site to affect cytochrome *c* oxidase kinetics, we propose that the non-catalytic cytochrome *c* binding site is located close to the catalytic one.

Characteristic for the model with one catalytic and one non-catalytic site is the fact that both high-affinity and low-affinity turnover numbers and high-affinity K_m values represent interactions of cytochrome *c* with the subunit II catalytic site (dissociation rates and affinity, respectively).

The low-affinity K_m represents the affinity of cytochrome *c* for the allosteric site and should consequently be governed by other electrostatic interactions than the other three kinetic parameters.

A number of carboxyl groups have been postulated as giving the subunit II catalytic site a negative charge even at pH 6.2 [26]. The increase in dissociation rate of the positively charged cytochrome *c* from this negatively charged site (whether or not the cytochrome *c* oxidase molecule has a total net positive charge) would explain the effect of ionic strength on turnover numbers. A concomitant decrease in affinity of this site for cytochrome *c* would explain the effect of I on high-affinity K_m. Ionic-strength effects on the low affinity maximal turnover will be more complicated than on the high-affinity maximal turnover. An increase in ionic strength will, as the

high-affinity TN_{max} shows, enhance the dissociation rate of the positively charged cytochrome *c* at the negatively charged subunit II. But on the other hand, the affinity of the allosteric site for cytochrome *c* (discussed later) and the electrostatic repulsion of the cytochrome *c* bound at the regulatory site will decrease with increasing ionic strength. This would explain that the low-affinity TN_{max} values were found to be less dependent upon ionic strength than the high-affinity values (Table II). Furthermore, since above $I = 100$ mM no low-affinity reaction was observed, we propose that at high ionic strength the influence of the electric field of the regulatory cytochrome *c* on the cytochrome *c* bound at the catalytic site is no longer measurable. This would explain that only one reaction is observed (Fig. 4). Because of the high ionic strength in the mitochondrial intermembrane space the low-affinity reaction probably does not contribute to the catalytic activity of the oxidase in vivo.

The low-affinity K_m was found to be almost independent of ionic strength (Table II). It must be noted, however, that this does not indicate that the binding of cytochrome *c* to the regulatory site is not also electrostatic in nature. Upon binding of a cytochrome *c* molecule to this regulatory site at high cytochrome *c* concentrations, the catalytic site will be occupied, resulting in electrostatic repulsion between the two cytochrome *c* molecules (Fig. 4). This effect will diminish when the ionic strength is raised, with a concomitant increase of the affinity of the noncatalytic site for cytochrome *c*. Since we did not observe a decrease in low-affinity K_m at increasing ionic strength, we propose that this expected effect of ionic strength is cancelled out by another ionic-strength effect. This then leads to the proposal that between pH 6.2 and pH 7.8 the non-catalytic site also has a net negative charge, as should be expected for a cytochrome *c* binding site. This is in line with the proposal [39] that cardiolipin molecules are responsible for the low-affinity cytochrome *c* binding.

Also the results of our pH studies are in agreement with the line of reasoning described above. The high-affinity K_m was found to be pH dependent. This indicates that the subunit II catalytic site contains one or more groups that can be protonated in the pH range tested (probably histidine). Protonation of these groups resulted in a decrease in affinity of the catalytic site for its positively charged substrate.

The low-affinity K_m showed almost no pH dependence. In our opinion, this would indicate that the allosteric site contains no groups with pK_a values in the pH region studied.

The effect of pH on maximal turnover of the cytochrome *c* oxidase reaction is usually discussed in terms of degree of protonation of acid/base groups participating in the catalytic function of the enzyme [11,13]. Since the activation energy ($\Delta G^{\neq}$) of the dissociation reaction depends on electrostatic forces between the two proteins, we think the effect of pH on the electrical charges of the interaction domains of the two proteins should not be overlooked and might explain the observed pH effect on maximal turnover numbers. The observed dependence of high-affinity and low-affinity TN_{max} values on pH indicates the presence of protonable groups on the subunit II catalytic site just as the high-affinity K_m did. Protonation of these residues would result in an increased dissociation rate of the positively charged cytochrome *c* molecule.

Summarising, we conclude that the effects of pH and ionic strength on cytochrome *c* oxidase kinetics are in agreement with the model of Speck et al. [32] and with a qualitative description of electrostatic interaction domains. In order to determine the exact nature of the electrostatic interactions involved, a better quantitative description of electrostatic interactions between large proteins has to be awaited.

Acknowledgements

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