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IONIC STRENGTH EFFECTS ON CYTOCHROME aa_3 KINETICS

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Summary

1. The occurrence of an optimal ionic strength for the steady-state activity of isolated cytochrome aa_3 can be attributed to two opposite effects: upon lowering of the ionic strength the affinity between cytochrome *c* and cytochrome aa_3 increases, whereas in the lower ionic strength region the formation of a less active cytochrome *c*-aa_3 complex limits the ferrocytochrome *c* association to the low affinity site.

2. At low ionic strength, the reduction of cytochrome *c*-aa_3 complex by ferrocytochrome c_1 proceeds via non-complex-bound cytochrome *c*. Under these conditions the positively charged cytochrome *c* provides the electron transfer between the negatively charged cytochromes c_1 and aa_3.

3. Polylysine is found to stimulate the release of tightly bound cytochrome *c* from the cytochrome *c*-aa_3 complex. This property points to the existence of negative cooperativity between the two binding sites. We suggest that the stimulation is not restricted to polylysine, but also occurs with cytochrome *c*.

4. Dissociation rates of both high and low affinity sites on cytochrome aa_3 were determined indirectly. The dissociation constants, calculated on the basis of pre-steady-state reaction rates at an ionic strength of 8.8 mM, were estimated to be 0.6 nM and 20 μM for the high and low affinity site, respectively.

Introduction

The mechanism of electron transfer in the terminal part of the respiratory chain has been the subject of a large number of studies [1—7]. The earlier mod-

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

els for the steady-state oxidation of cytochrome *c* catalyzed by cytochrome aa_3 [6—10] as well as the interpretation of the pre-steady-state reaction between both cytochromes [11—16] were based on the assumption of a single cytochrome *c* reaction site on cytochrome aa_3. More recently evidence has accumulated for the existence of various, heterogeneous, cytochrome *c*-binding sites [17—20]. Binding studies [4—6], steady-state kinetics [17,18,21] and pre-steady-state kinetics [20,22] point to the presence of at least two types of sites with different affinities towards cytochrome *c*.

The dissociation constants of both sites are still subject to discussion. Margoliash and coworkers [5,17,23] calculated dissociation constants of 30 nM and 200 nM from binding studies and polarographic steady-state kinetics in a low ionic strength medium. Other authors [6,18,24—26] give estimates that are considerably higher.

In a previous paper [20] we reported about some of the properties of the pre-steady-state reaction of ferrocytochrome *c* with both types of sites. The rate of electron transfer was found to depend on the accessibility of the reacting sites, i.e. whether or not the high affinity site is occupied by complex-bound (porphyrin) cytochrome *c*.

In this paper we report about the ionic strength dependence of the pre-steady-state reactions in correlation with the steady-state activity, which is known to have an ionic strength optimum [27—31]. In a recent paper by our group [31] we showed that this optimum can be completely ascribed to the effect of ionic strength on the apparent K_m, in this paper we propose an explanation for the occurrence of the ionic strength optimum of the steady-state activity. Furthermore, we have obtained substantial evidence for a negatively cooperative regulation of the affinity of the cytochrome *c*-binding sites on cytochrome aa_3.

Materials and Methods

Beef-heart cytochrome aa_3 was prepared according to the method of Fowler et al. [32] as modified in our laboratory [33,34]. Cytochrome *c* was prepared from horse heart as described by Margoliash and Walasek [35], ferrocytochrome *c* was obtained by gel filtration after incubation with ascorbate. Beef-heart cytochrome c_1 was isolated in the reduced state by the method of König et al. [36]. Absorbance coefficients (reduced-oxidized) used for cytochrome aa_3, cytochrome *c* and cytochrome c_1 were 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [37], 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm [38] and 19.2 $mM^{-1} \cdot cm^{-1}$ at 552.5 nm [39], respectively.

The cytochrome *c*-aa_3 complex was isolated according to Orii et al. [40] as described before [20]. Chromatography (cf. Fig. 1B) was performed at 4°C using thermostatted LKB columns (Ultrogel AcA54, 30 × 0.9 cm, 6 ml/h) and fraction collector (Colora/Isco model 328). The absorbance of the eluate was monitored at 410 nm using a Zeiss PM2A spectrophotometer. Spectra of the eluate fractions were recorded on a Cary-17R spectrophotometer.

Steady-state activity of cytochrome aa_3 was determined spectrophotometrically [10] and polarographically [41]. Pre-steady-state reactions were studied by means of a Durrum stopped-flow apparatus with 2 cm optical pathlength of

the reaction chamber. Signal handling and reaction-rate calculation have been described previously [20,42]. Ionic strength of potassium phosphate buffers was calculated as described in Ref. 31.

Polylysine (average molecular weight 20 000) was purchased from Sigma.

Results

Fig. 1A shows the ionic strength dependence of the steady-state activity of cytochrome aa_3, determined spectrophotometrically [10] at a single cytochrome *c* concentration. As has frequently been reported [27—31] an optimum was observed for the enzymic activity. Under the conditions of Fig. 1A the optimum was found at 43 mM potassium phosphate (I = 76 mM). Incubation experiments showed that the substantial loss of enzymic activity at relatively low or high phosphate concentrations is reversible and not caused by irreversible denaturation. The enzymic activity was also not affected when part of the potassium phosphate was replaced by potassium chloride without changing the ionic strength. Hence, the results can be ascribed to a general ionic strength effect on the activity and are not necessarily a specific phosphate effect [5,21, 43].

Recently, we reported [31] that the turnover number of cytochrome aa_3, extrapolated to infinite cytochrome *c* concentration, is not affected by the ionic strength of the medium. This phenomenon was found to be valid for the complete ionic strength range referred to in this paper, both in the spectrophotometric and in the polarographic assay. The occurrence of an ionic strength optimum as shown in Fig. 1A will, therefore, originate from the dependence of the apparent K_m on the ionic strength, i.e. of the affinity between both cytochromes.

Orii et al. [40] and King and coworkers [4,24,44] have reported that at low ionic strength a 1 : 1 complex between cytochrome *c* and cytochrome aa_3 is stable and can be isolated chromatographically, which is indicative of a very small dissociation constant [45]. In order to obtain information about the ionic strength dependence of the stability of the cytochrome *c*-aa_3 complex, the complex was isolated at low ionic strength and rechromatographed on six identical columns which were equilibrated with different concentrations of potassium phosphate. From each column the ratio:

$$R = ([\text{cytochrome } c] + [\text{cytochrome } c\text{-}aa_3])/([\text{cytochrome } aa_3] + [\text{cytochrome } c\text{-}aa_3])$$

in the eluate fractions was determined. Fig. 1B shows that the value of the ratio R in the heme *a*-containing fraction decreases upon increasing ionic strength, in accordance with the results of King et al. [44]. Under our experimental conditions the cytochrome *c*-aa_3 complex is almost completely dissociated at I = 75 mM.

The ionic strength dependence of the pre-steady-state reactivity of ferrocytochrome *c* towards cytochrome aa_3 (or towards the cytochrome *c*-aa_3 complex) is shown in Fig. 1C, where the second-order association rate constant k_1 is plotted versus the ionic strength of the medium. Upon lowering of the ionic strength from 260 to 60 mM the value of k_1 increases gradually from $9 \cdot 10^5$ to

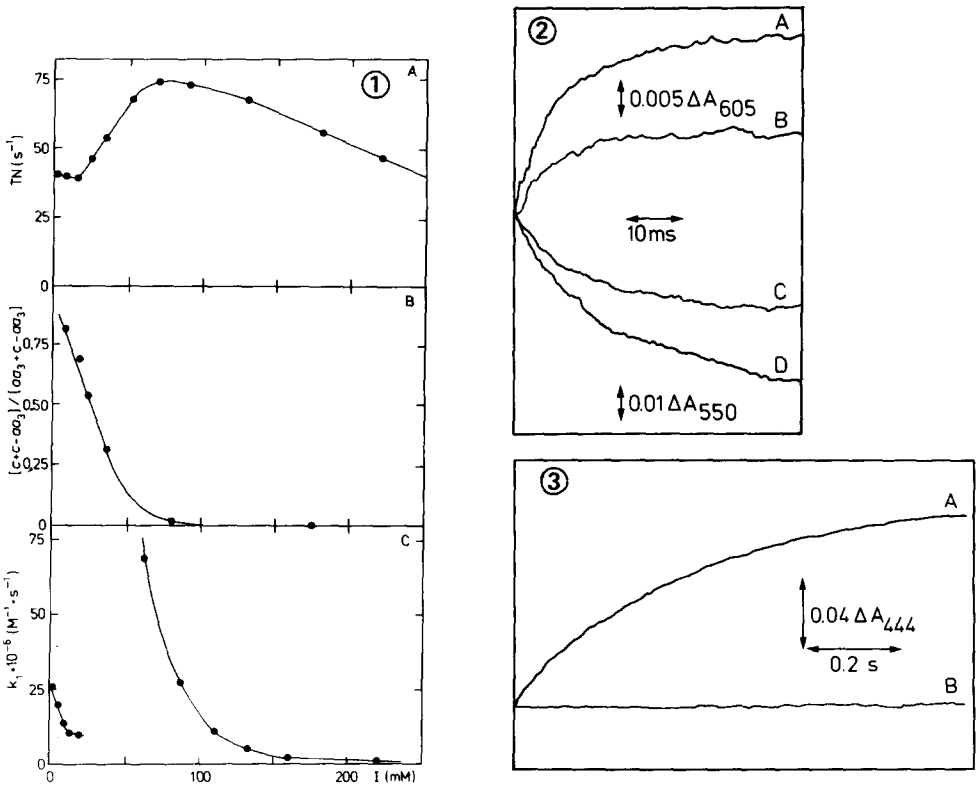

Fig. 1. (A) Ionic strength dependence of the steady state oxidation of ferrocytochrome *c* by molecular oxygen, catalyzed by cytochrome aa_3. Conditions: 20 μM cytochrome *c*, 20 nM cytochrome aa_3, 2—150 mM potassium phosphate, pH 7.0, 1% Tween 80, 10° C. (B) Ionic strength dependence of the stability of the cytochrome *c*-aa_3 after rechromatography on Ultrogel AcA 54. Columns were equilibrated with 5—100 mM potassium phosphate (pH 7.0) and 1% Tween 80 at 4° C and eluted with the same medium as equilibrated. The *R* value was determined spectrophotometrically using the formula [13]:

$$R = (\Delta A_{550}^{\text{red-ox}} + 0.08\Delta A_{605}^{\text{red-ox}})/(0.044\Delta A_{550}^{\text{red-ox}} + 0.88\Delta A_{605}^{\text{red-ox}})$$

For each column over 95% of the constituents of the applied amount of isolated cytochrome *c*-aa_3 complex (*R* value 0.96; isolated in 5 mM potassium phosphate, pH 7.0) was recovered in the red-brown fraction of the eluate. (C) Ionic strength dependence of the pre-steady-state reaction of ferrocytochrome *c* with cytochrome aa_3. Conditions: 0.8 μM cytochrome aa_3; 2.7—8.1 μM ferrocytochrome *c*; 1—125 mM potassium phosphate, pH 7.0, 1% Tween 20, 10° C.

Fig. 2. Time course of the reaction of ferrocytochrome *c* with cytochrome *c*-aa_3 complex. Absorbance changes were monitored at 605 nm (trace A, B) and at 550 nm (trace C, D). Conditions 5 mM potassium phosphate, pH 7.0, 1% Tween 20, 10° C. Concentrations after mixing: A, D: 1 μM cytochrome *c*-aa_3, 5 μM ferrocytochrome *c*. B, C: 5 μM cytochrome *c*-aa_3, 1 μM ferrocytochrome *c*.

Fig. 3. Reactions of ferrocytochrome c_1 with cytochrome aa_3 and the cytochrome *c*-aa_3 complex. Absorbance changes were followed at 444 nm. Conditions: 5 mM potassium phosphate, pH 7.0, 1% Tween 20, 10° C. Concentrations after mixing: 7.5 μM ferrocytochrome c_1 and: A: 1 μM cytochrome *c*-aa_3 complex; B: 1 μM cytochrome aa_3.

$7 \cdot 10^7\ M^{-1} \cdot s^{-1}$. Further lowering of the ionic strength enhances the pre-steady-state reaction to be completed within the mixing time of our stopped-flow apparatus ($k_1 > 2 \cdot 10^8\ M^{-1} \cdot s^{-1}$). A slower heme *a* reduction can be observed at an ionic strength of 30 mM and below I = 15 mM the time course of this

reaction can be approximated satisfactorily by an exponent with an apparent first-order rate constant proportional to the initial concentration of ferrocytochrome *c*. The second-order rate constant of the slower reaction increases from $1 \cdot 10^7$ to $2.5 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ when the ionic strength is lowered from 13 to 1.8 mM.

The discontinuity in the plot of k_1 versus the ionic strength (Fig. 1C) corresponds rather well with the optimum in the steady-state activity versus ionic strength plot (Fig. 1A). The occurrence of this optimum can be explained by the existence of an increasing fraction of cytochrome *c-aa*$_3$ complex in media with lower ionic strength (Fig. 1B), since it has been demonstrated [20] that the cytochrome *c-aa*$_3$ complex has a lower affinity towards cytochrome *c* than free cytochrome *aa*$_3$ under the same ionic strength conditions.

Some of the kinetic properties of the cytochrome *c-aa*$_3$ complex have been studied under conditions of low ionic strength (5 mM potassium phosphate, pH 7.0). Fig. 2 shows the time course of the pre-steady-state reaction of excess ferrocytochrome *c* with the cytochrome *c-aa*$_3$ complex (traces A and D) and between excess of the cytochrome *c-aa*$_3$ complex and ferrocytochrome *c* (traces B and C). The reactions were followed by monitoring absorbance changes of cytochrome *aa*$_3$ at 605 nm (traces A and B) and of cytochrome *c* at 550 nm (traces C and D). When the concentrations of the reactions are interchanged the apparent rate constants for the burst phase differ about a factor 1.7, somewhat less than reported in a previous paper [20]. The amount of heme *a* reduced in the initial phase of the reaction with an excess of ferrocytochrome *c* is twice that when the cytochrome *c-aa*$_3$ complex is present in excess. Since the data obtained at 550 nm are compatible (traces C and D), the observed differences cannot be explained by spectral interactions between the chromophores in cytochrome *aa*$_3$ (cf. Ref. 46) but will originate from the nature of the low-affinity site, where ferrocytochrome *c* reacts with the cytochrome *c-aa*$_3$ complex.

The role of the high-affinity site of the cytochrome *c-aa*$_3$ complex in reactions at low ionic strength was studied by measuring its dissociation rate indirectly. Since no spectral changes occur upon dissociation of the cytochrome *c-aa*$_3$ complex [44], the difference in reactivity of complex-bound and free ferricytochrome *c* towards isolated ferrocytochrome c_1 was used to monitor the dissociation of the cytochrome *c-aa*$_3$ complex.

Fig. 3, trace A shows that the reaction of ferrocytochrome c_1 with the cytochrome *c-aa*$_3$ complex proceeds with a rate of 2.5 s^{-1}. Increasing the concentration of ferrocytochrome c_1 did not affect the reduction rate of heme *a* in the cytochrome *c-aa*$_3$ complex, indicating that the electron transfer is governed by a zeroth order reaction in ferrocytochrome c_1. The direct reduction of cytochrome *aa*$_3$ by ferrocytochrome c_1 (trace B) is very slow, the rate of electron transfer between both negatively charged proteins was found to be 10^3 $M^{-1} \cdot s^{-1}$ (cf. [47]). Both the reduction of free ferricytochrome *c* by ferrocytochrome c_1 (cf. Refs. 21, 36 and 48) and the reduction of the cytochrome *c-aa*$_3$ complex or cytochrome *aa*$_3$ by ferrocytochrome *c* (Fig. 2, trace B) are very fast [20] and will therefore not act as a rate-limiting step in the electron transfer process from ferrocytochrome c_1 to heme *a*. Direct reduction of the cytochrome *c-aa*$_3$ complex via bound cytochrome *c* is very unlikely [6,49,50] and would, more-

over, not be zeroth order in ferrocytochrome c_1. Thus, we suggest that the rate of 2.5 s^{-1} observed for the reaction between ferrocytochrome c_1 and the cytochrome *c-aa*$_3$ complex will be limited by the dissociation rate of the complex.

The effect of cytochrome *c* on the dissociation rate of the cytochrome *c-aa*$_3$ complex can be imitated by polylysine, a positively charged polypeptide that inhibits the steady-state activity of cytochrome *aa*$_3$ competitively towards cytochrome *c* [51,52]. Since polylysine aggregates with the negatively charged cytochrome c_1, we used the reduction of cytochrome *c* by TMPD (reduced by excess ascorbate) to monitor the release of cytochrome *c* from the cytochrome *c-aa*$_3$ complex. The time course of the reaction can be followed at 416 nm. At this wavelength the reduction of cytochrome *c* causes an increase of the absorbance and the reduction of heme *a* a decrease. Fig. 4 shows the absorbance changes at 416 nm when ascorbate/TMPD was rapidly mixed with ferricytochrome *c* (trace A), cytochrome *aa*$_3$ (trace B) and with cytochrome *c-aa*$_3$ complex (trace C), respectively. Comparison of traces A and B shows that the reduction of ferricytochrome *c* by TMPD proceeds at a rate about 50 times faster than the reduction of cytochrome *aa*$_3$ alone. The biphasic absorbance change in trace C is composed of the initial reduction of heme *a* via cytochrome *c* and the subsequent reduction of cytochrome *c* (at the same rate observed in trace A), as was confirmed at appropriate wavelengths (444 and 550 nm, not shown).

The presence of polylysine affected neither the rate nor the extent of the reactions of ascorbate-reduced TMPD with cytochrome *c* or with cytochrome *aa*$_3$. Trace D shows the absorbance increase at 416 nm when an ascorbate/

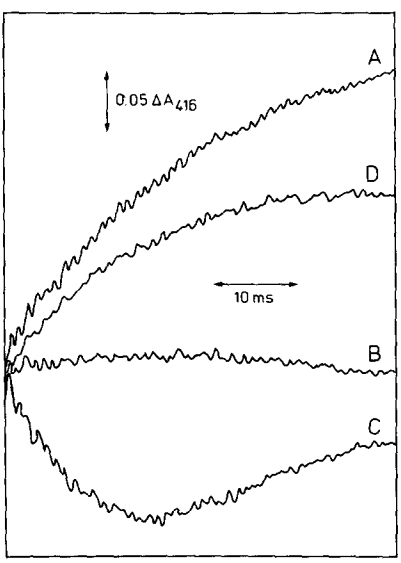

Fig. 4. Effects of polylysine on the reduction of cytochrome *c*, cytochrome *aa*$_3$, and of their complex by ascorbate/TMPD. Absorbance changes were followed at 416 nm. Conditions: 5 mM potassium phosphate, pH 7.0, 1% Tween 20, 10°C. Concentrations after mixing: 5 mM TMPD, 15 mM ascorbate and: A: 3 μM ferricytochrome *c*; B: 3 μM cytochrome *aa*$_3$; C: 3 μM cytochrome *c-aa*$_3$ complex; D: 3 μM cytochrome *c-aa*$_3$ complex, 40 μM polylysine.

TMPD/polylysine mixture was rapidly mixed with the cytochrome *c-aa*$_3$ complex. The observed reaction has the same rate as the reaction in the absence of polylysine. Monitoring the time course at 444 nm (not shown) shows that within the initial 50 ms of the reaction only little reduction of heme *a* occurs, indicating that the electron transfer from cytochrome *c* (reduced by ascorbate/TMPD) to heme *a* is blocked by the competitive inhibitor polylysine.

The rate constants of the reactions shown in traces A and D are linearly dependent on the concentration TMPD in a range from 0.1 to 5 mM. At the highest TMPD concentration a first-order rate constant of 50 s^{-1} was calculated.

Discussion

The occurrence of an optimal ionic strength for the steady-state activity of cytochrome *aa*$_3$ is caused by the effect on only the apparent K_m since the turnover number extrapolated to infinite cytochrome *c* concentration is found to be unaffected by ionic strength [31]. The apparent K_m, which is composed of the ionic strength dependent contributions of both high and low affinity sites, is minimal at $I = 43$ mM.

The rechromatography experiment (Fig. 1B) shows that lowering of the ionic strength induces a shift from cytochrome *aa*$_3$ to the cytochrome *c-aa*$_3$ complex as the species present in the solution and hence a shift from the high to the low affinity site acting as the electron acceptor for ferrocytochrome *c* [20]. This shift has an effect on the enzymic activity opposite to the primary salt effect. The latter causes an increment in the electrostatic attraction between both cytochromes when lowering the ionic strength.

The position of the optimum for the steady-state activity of cytochrome *aa*$_3$ will be dependent on the ratio [cytochrome *aa*$_3$]/[cytochrome *c-aa*$_3$] in the assay and thus on the concentrations of cytochrome *c* and cytochrome *aa*$_3$ used.

Similar to the 'dependent site' mechanism proposed by Errede and Kamen [18], we suggest that at low ionic strength the reduction of the cytochrome

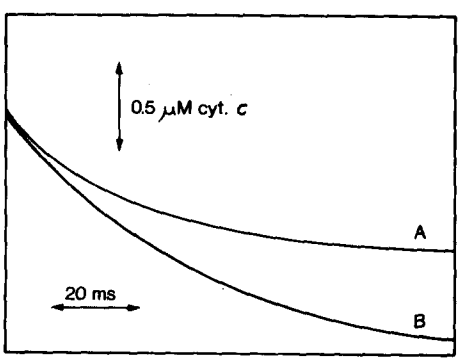

Fig. 5. Computer simulation of time courses according to the reactions of Eqns. 1 and 2 (see text). Parameters used: $k_1 = k_2 = k_3 = k_4 = 1.2 \cdot 10^7\ M^{-1} \cdot s^{-1}$ and $k_{-1} = k_{-2} = k_{-3} = k_{-4} = 250\ s^{-1}$, with initial concentrations for A: cytochrome *c-aa*$_3$, 5 μM; cytochrome c^{2+}, 1 μM and B: cytochrome c^{2+}, 5 μM; cytochrome *c-aa*$_3$, 1 μM.

c-aa_3 complex by ferrocytochrome c is composed of two subsequent processes. The initial reaction (cf. Fig. 2, traces B, C) can be written as:

$$\text{cytochrome } c^{2+} + \text{cytochrome } c\text{-}aa_3 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{cytochrome } c^{3+}\text{-}c\text{-}aa_3^- \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \text{cytochrome } c\text{-}aa_3^- + \text{cytochrome } c^{3+} \quad (1)$$

Analogous to the fully oxidized cytochrome c-aa_3 complex, the partially reduced cytochrome c-aa_3^- complex will react further when an excess of ferrocytochrome c is present:

$$\text{cytochrome } c^{2+} + \text{cytochrome } c\text{-}aa_3^- \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{cytochrome } c^{3+}\text{-}c\text{-}aa_3^{2-} \underset{k_4}{\overset{k_{-4}}{\rightleftharpoons}} \text{cytochrome } c\text{-}aa_3^{2-} + \text{cytochrome } c^{3+} \quad (2)$$

Fig. 5 shows the simulated time course for the pre-steady-state reaction between an excess of cytochrome c-aa_3 complex and ferrocytochrome c (A) and between excess ferrocytochrome c and cytochrome c-aa_3 (B), computed according to Eqns. 1 and 2, using $k_1 = k_{-2} = k_3 = k_{-4} = 1.2 \cdot 10^7\ M^{-1} \cdot s^{-1}$ (cf. Fig. 1C) and $k_{-1} = k_2 = k_{-3} = k_4 = 250\ s^{-1}$ (cf. Fig. 4). The chosen rate constants imply equal affinity of cytochrome aa_3 towards ferro- and ferricytochrome c [17] and that electron transfer from cytochrome c to cytochrome aa_3 does not affect their mutual affinity [6,8,10,18].

The rate constant calculated from Fig. 2 trace B is 1.7 times smaller than that of trace A, the extent of the absorbance change is greater by a factor of two in the case that ferrocytochrome c is in excess. Even in this latter case we observed at 605 nm only 60% of the absorbance change expected from the absorbance changes at 550 nm and from the optical spectrum of reduced-minus-oxidized cytochrome aa_3 (cf. Refs. 11, 13 and 14 but also Refs. 2, 15 and 53).

The high and low affinity sites on cytochrome aa_3 are not necessarily spatially distinct parts on the cytochrome aa_3 molecule. We suggest that they can be interpreted merely as a negatively charged region on cytochrome aa_3. This region is able to bind the first cytochrome c molecule very tightly, thus acting as a high affinity site. The remaining part of the region, now acting as a low affinity site, has still the ability to bind a second cytochrome c molecule. After the sites are occupied, the mutual electrostatic repulsion between both positively charged cytochrome c molecules will stimulate the dissociation of one of the cytochrome c molecules from the intermediately formed 2 : 1 cytochrome c-cytochrome aa_3 complex. This repulsion has been reported for cytochrome c molecules, which interact when present in the solution [54,55]. This model also explains the results of Petersen and Cox [53], who reported that complex dissociation depends on the cytochrome c concentration. When the ionic strength is increased, the repulsion between the cytochrome c molecules decreases and the shielding of the binding region by indifferent ions becomes more effective. Thus the distinction between high and low affinity sites will diminish at higher ionic strength and the binding region on cytochrome aa_3 will finally behave as two sites with equal affinity towards cytochrome c [20].

Fig. 6 visualizes the model described above for low and high ionic strength circumstances. Cytochrome c, cytochrome aa_3 and their complexes are sche-

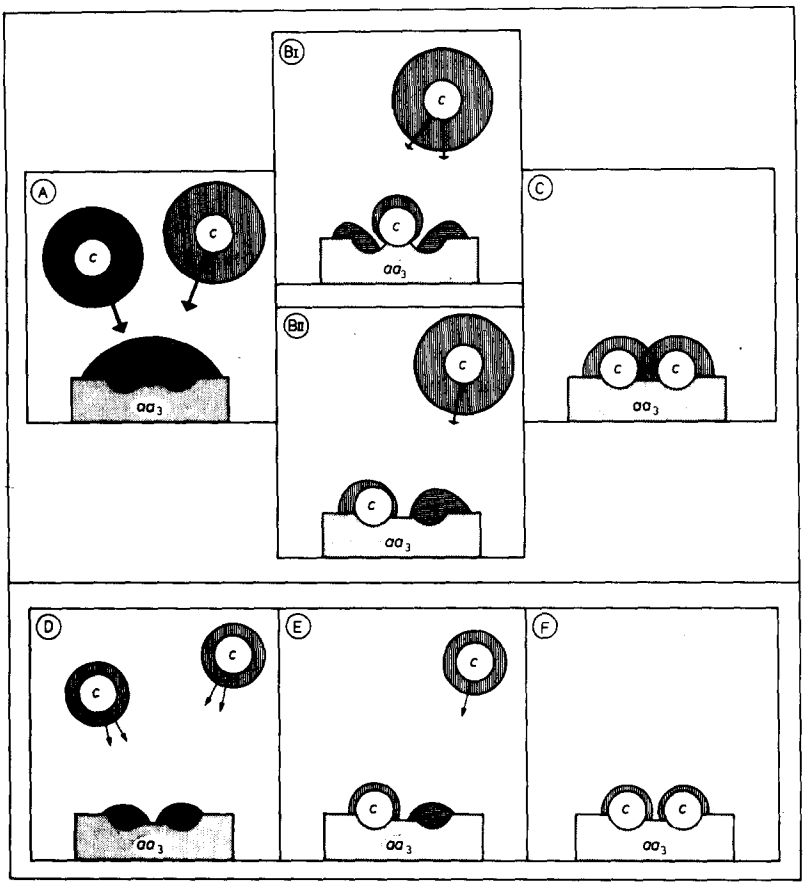

Fig. 6. Schematic representation of the reaction of cytochrome *c* with cytochrome aa_3 at low (A—C) and at high (D—F) ionic strength. Size and form of cytochromes and their electric influence spheres (shaded areas) are artistically drawn. Arrows represent electrostatic forces on the cytochrome *c* molecules.

matically drawn with the extent of the electrical fields relevant for the association/dissociation processes. Insert A represents the binding of cytochrome *c* to cytochrome aa_3 in a low ionic strength medium. The relatively large electrical influence spheres surrounding the two cytochrome *c*-binding sites on cytochrome aa_3 are suggested to overlap considerably and will thus act as the negatively charged region mentioned before, having the joined (high) affinity of the two sites. When one cytochrome *c* molecule is bound to cytochrome aa_3 part of the electrical field around the binding region of the complex is neutralized, causing a lower association rate for free cytochrome *c*. The kinetic properties of the complex were previously described [20] according to a model depicted in insert BI, in which the binding region on cytochrome aa_3 is split into two approximately equal parts. An alternative model, suggested in this paper, is shown in insert BII and involves the association of a cytochrome *c* molecule to one of the binding sites. Consequently, the electrical field around the binding region an thus the affinity towards other cytochrome *c* molecules is reduced (cf. Eqns. 1 and 2 and Fig. 5). Insert C represents an unstable complex in which

two molecules cytochrome *c* have associated to cytochrome aa_3. The mutual repulsion between the electric influence spheres of the cytochrome *c* molecules is suggested to cause an increased dissociation rate of the complex and a lower affinity towards cytochrome *c*.

At high ionic strength counter ions will diminish the extent of the electric fields and no overlap of the directive effects of the two cytochrome *c* binding sites occurs (insert D). Statistically ferrocytochrome *c* has two identical chances to react with one molecule cytochrome aa_3. After one cytochrome *c* is bound to cytochrome aa_3, no change in affinity towards free cytochrome *c* will occur (insert E) due to the limited range of the electrical sphere of bound cytochrome *c*. Although the life time of such a complex is expected to be very short at high ionic strength, under certain conditions ([cytochrome *c*] >> [cytochrome aa_3]) a 2 : 1 complex might be formed (insert F).

One of the features of this type of model is that it implies almost equal affinity of cytochrome aa_3 towards ferri-, ferro- and even porphyrin-cytochrome *c*, thus giving a basis to the mechanisms of Minnaert [8] and Errede and Kamen [18] and to the results of Yonetani and Ray [7]. The exponential time course in the Smith-Conrad assay [10], as well as the inhibition of the ferrocytochrome *c* oxidation by ferri- [7], porphyrin-cytochrome *c* [55] and by polylysine [51,52], can be explained by a plain competition of positively charged proteins for a strongly negatively charged region on cytochrome aa_3.

The enhancement of the dissociation of the cytochrome *c*-aa_3 complex by polylysine has now become plausible. The positively charged inhibitor is bound to the low affinity site, repulses the tightly bound cytochrome *c* and will then also occupy the remainder of the binding region on cytochrome aa_3. In this way polylysine precludes consecutive reduction of heme *a* by TMPD-reduced cytochrome *c*. Since the rate of reduction by TMPD of 'complex-dissociated' cytochrome *c* is identical with the reduction rate of a priori present 'unbound' cytochrome *c*, the dissociation of the cytochrome *c*-aa_3 complex in the presence of polylysine must occur much faster than this reduction reaction.

Simulations revealed that the dissociation rate of the cytochrome *c*-aa_3 complex must be at least 5-times faster than the consecutive reduction of ferricytochrome *c* by TMPD. This implies that the dissociation rate of the cytochrome *c* — cytochrome aa_3 complex in the presence of polylysine is greater than 250 s^{-1}. This finding is in line with the observation that at relatively high concentrations of ferrocytochrome *c* (40 μM) no deviation is observed from the linear relationship between pseudo-first-order rate constant and ferrocytochrome *c* concentration. A levelling-off of the observed rate is expected if the dissociation rate of the cytochrome *c*-aa_3 or cytochrome *c*-aa_3^- complex would become rate-limiting [56]. The dissociation rate of the cytochrome *c*-aa_3 complex enhanced by the binding of a second cytochrome *c* molecule must, therefore, be greater than 500 s^{-1}, which is consistent with estimations reported previously [57]. This value and that found in the presence of polylysine differ greatly from the dissociation rate constant of the cytochrome *c*-aa_3 complex (2.5 s^{-1}), probably because no induced dissociation by electrostatic repulsion will occur.

The dissociation constant of the low-affinity site at low ionic strength (5 mM potassium phosphate) can be estimated from the results presented in Fig. 1C ($k_{on} = 1.2 \cdot 10^7\ M^{-1} \cdot s^{-1}$) and in Fig. 4 ($k_{off} > 250$—$500\ s^{-1}$) as 20—40 μM. In a

similar way the dissociation constant of the high-affinity site at low ionic strength can be calculated. The dissociation rate constant is obtained from the experiments with ferrocytochrome c_1 (k_{off} = 2.5 s^{-1}). The association rate constant can be estimated from the plot of log k versus $\sqrt{I}$, studied at high ionic strength (cf. Fig. 1C) by linear extrapolation to 8.8 mM. Although there are theoretical objections against any interpretation of especially the slopes of these plots, their linearity over a wide ionic strength range has been reported for several protein-protein reactions [58—60]. Using the extrapolated association rate constant ($4 \cdot 10^9$ $M^{-1} \cdot s^{-1}$) a K_d of about 0.6 nM can be calculated.

The values of both dissociation constants estimated from our pre-steady-state experiments differ considerably from the value obtained from steady-state kinetics, cytochrome *c* reducibility [26] and binding studies. However, the K_d determination from binding studies was carried out in a concentration range not corresponding with the value of the dissociation constant as found by us.

At low ionic strength the mutual repulsion between the two cytochrome *c* molecules of the intermediate 2 : 1 cytochrome *c*-aa_3 complex can be interpreted as a form of negative cooperativity, which predicts non-linear Eady-Hofstee plots as has frequently been reported [2,5,17,18,23]. However, an approximation of the curved line by two straight lines representing the contribution of two independent sites is known to yield non-reproducible values [21, 61] as the values of the kinetic parameters obtained depend highly on the substrate concentration range applied.

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