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THE PRE-STEADY STATE REACTION OF FERROCYTOCHROME *c* WITH THE CYTOCHROME *c*-CYTOCHROME *aa*$_3$ COMPLEX

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Summary

1. Using stopped-flow technique we have investigated the electron transfer form cytochrome *c* to cytochrome *aa*$_3$ and to the (porphyrin) cytochrome *c*-cytochrome *aa*$_3$ complex.

2. In a low ionic strength medium, the pre-steady state reaction occurs in a biphasic way with rate constants of at least $2 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ and about 10^7 $M^{-1} \cdot s^{-1}$ (I = 8.8 mM, pH 7.0, 10° C), respectively.

3. A comparison of the rate constants, determined in the presence of an excess of cytochrome *c* with those found in the presence of an excess of cytochrome *aa*$_3$ reveals the existence of two slower reacting sites on the functional unit (2 hemes and 2 coppers) of cytochrome *aa*$_3$. On basis of these results we discuss various models. If no site-site interactions are assumed (non-cooperative model) cytochrome *aa*$_3$ has 2 high and 2 low affinity sites available for the reaction with ferrocytochrome *c*. If negative cooperativity occurs, cytochrome *aa*$_3$ has 2 high affinity sites which change into 2 low affinity sites upon binding of one cytochrome *c* molecule. The latter model is favoured.

Introduction

Cytochrome *aa*$_3$, the terminal part of the mitochondrial respiratory chain, catalyses the electron transfer from reduced cytochrome *c* to dioxygen. The mechanism of the reaction is still not fully understood. Since 1961 the so-called Minnaert IV mechanism involving one catalytic site only has often been

used to describe the steady-state kinetics [1]. Evidence to support the existence of more than one catalytic site was presented by Ferguson-Miller et al. [2] and Errede et al. [3]. The latter have extended the Minnaert IV mechanism [3,4] to include a second catalytic site for cytochrome *c* on cytochrome aa_3. Studies done in our laboratories on the kinetics of the reaction between ferrocytochrome *c* and cytochrome aa_3 have revealed that the pre-steady state kinetics are very dependent on ionic strength [5]; the same was found earlier for the steady-state reaction [cf. Ref. 6]. This indicates that the rate of the electron transfer from ferrocytochrome *c* to cytochrome aa_3 is governed by electrostatic forces.

Until now, pre-steady state reaction measurements had not been used to show the existence of more than one catalytic site for cytochrome *c* on cytochrome aa_3. In this paper we describe some studies we have made on the pre-steady state kinetics of the reaction between ferrocytochrome *c* and cytochrome aa_3 under conditions of low as well as high ionic strength. Under conditions of low ionic strength, a stable complex between ferricytochrome *c* and cytochrome aa_3 can be isolated [7], whereas under conditions of high ionic strength this complex was not found. Using stopped-flow techniques we were able to compare the reaction between ferrocytochrome *c* and cytochrome aa_3 with the reaction between ferrocytochrome *c* and the complex of cytochrome *c*-cytochrome aa_3. From the results presented in this paper it can be concluded that there are at least two distinct types of sites for cytochrome *c* on cytochrome aa_3. Some models are proposed to describe the behaviour of the various sites in the pre-steady state reaction between ferrocytochrome *c* and cytochrome aa_3.

Materials and Methods

Cytochrome aa_3 was isolated from beef heart according to the methods of Fowler et al. [8] and MacLennan and Tzagoloff [9], as modified by Van Buuren [10]. Cytochrome *c* was prepared from horse heart as described by Margoliash and Walasek [11]. 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. [12]. Absorbance coefficients used for cytochrome aa_3 (reduced minus oxidized) were 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [13], for cytochrome *c* (reduced minus oxidized) 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm [14], and for porphyrin cytochrome *c* 13.0 $mM^{-1} \cdot cm^{-1}$ at 504 nm [12].

The complexes of cytochrome aa_3 with cytochrome *c* and porphyrin cytochrome *c*, respectively, were isolated essentially according to the method of Orii et al. [7] using Ultrogel AcA-54 instead of Sephadex G-75. The complex (200 μM) was stored at 77 K. Immediately before use the complex was diluted 10—100 times. It can be calculated that under low ionic strength conditions (5 mM potassium phosphate, pH = 7.0) dissociation of the diluted complex is less than 1%, using a K_d of 20 nM [2]. All handling of porphyrin cytochrome *c* was performed under conditions that minimize exposure to light.

The pre-steady state kinetics of the reaction between ferrocytochrome *c*

and cytochrome aa_3 or the (porphyrin)-cytochrome *c*-cytochrome aa_3 complex, were measured by use of a modified Durrum stopped-flow apparatus.

The photomultipier output signal was transferred via a log-converter to a Datalab 905 transient recorder as a 1024 points data file and stored in a Hewlett-Packard 2100A computer. All traces referred to in this paper have been subjected to a five-points smoothing procedure [15].

To calculate the rate constant from the initial part of the reaction two methods have been applied, which fit the smoothed traces to an exponential function. With the first method a linear least-squares procedure was applied to the logarithm of the difference between experimental values and (estimated) final level. With the results of the linear regression a theoretical curve was calculated and subtracted from the experimental curve. This residual could be treated subsequently as described above to reveal any second exponential that might be present.

With the second method a non-linear least-squares best-fit to the function:

$$Y(t) = \sum_{i=1}^{n} [P_i + Q_i \{1 - \exp(R_i t)\}]$$

was calculated for $n = 1$. The theoretical and experimental curves were plotted simultaneously with their difference on a graphical terminal to allow visual inspection. At high ferrocytochrome *c* concentration, the traces were obviously biphasic. In this case a fitting procedure to the function $n = 2$ was required in order to obtain the initial reaction rate.

Control experiments revealed that all initial absorption changes observed at 444 nm were accompanied by concomitant absorption changes at 550 nm.

The two fitting methods showed to be consistent within 10% with respect to the calculated k'. The reaction rates, mainly calculated by the second method were the mean values of analyzing 5—15 traces. The standard deviation for k' was found to be 4—12%. An exponential time course can be expected if the initial reaction is essential from the type:

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C$$

when one of the substrates (A) is present in excess. The technical limitations of our stopped-flow apparatus permitted a maximum ratio $[A]_0/[B]_0$ of 12—15 for the rapid reactions we studied.

The minimal ratio to maintain approximately an exponential time course of the reaction was determined using a computer simulation program. The program calculated the time course of a reaction using a second order Kunge-Rutta method [16]. The resulting traces were fitted to exponential functions by the methods described above. For $[A]_0/[B]_0$ ratio greater than 3, the approximation of the initial reaction by an exponential function is still appropriate. Plotting k' vs. $[A]_0$ for these simulated traces reveals that the deviation from the line given by the equation $k_1[A]_0 + k_{-1}$ is less than 10%.

The Guggenheim plots presented in this paper were calculated by means of a weighted least-squares method and yielded values for k_1 and k_{-1} with standard deviations of 3—9% and 7—30%, respectively.

Results

Fig. 1 shows the time course of the reaction of ferrocytochrome *c* with cytochrome aa_3 (trace A), with the (isolated) cytochrome *c*-cytochrome aa_3 complex (trace B) and with a porphyrin cytochrome *c*-cytochrome aa_3 complex (trace C) monitored at 444 nm. We examined the reactions using an excess of ferrocytochrome *c* in a medium of low ionic strength (5 mM potassium phosphate, 1% Tween 20, pH = 7.0). The reactions proceed essentially in the same way as described previously for media of high ionic strength [17,5].

From the traces we calculated the apparent first order rate constant (k') of the reaction. By varying the ferrocytochrome *c* concentration we were able to determine the k' of these reactions as a function of the ferrocytochrome *c* concentration (Fig. 2). From the slope of the straight line the second order rate constant (k_1) can be calculated; the apparent dissociation constant (K_d) is obtained from the intercept of the line with the [ferrocytochrome *c*]-axis.

The k_1 values of the reactions calculated from Fig. 2 are presented in Table I, which shows that under conditions of low ionic strength the reactions of ferrocytochrome *c* with cytochrome aa_3 and with its complexes proceed in a very similar way. Although ferricytochrome *c* or porphyrin cytochrome *c* are tightly bound to cytochrome aa_3, the kinetic parameters are not affected. The

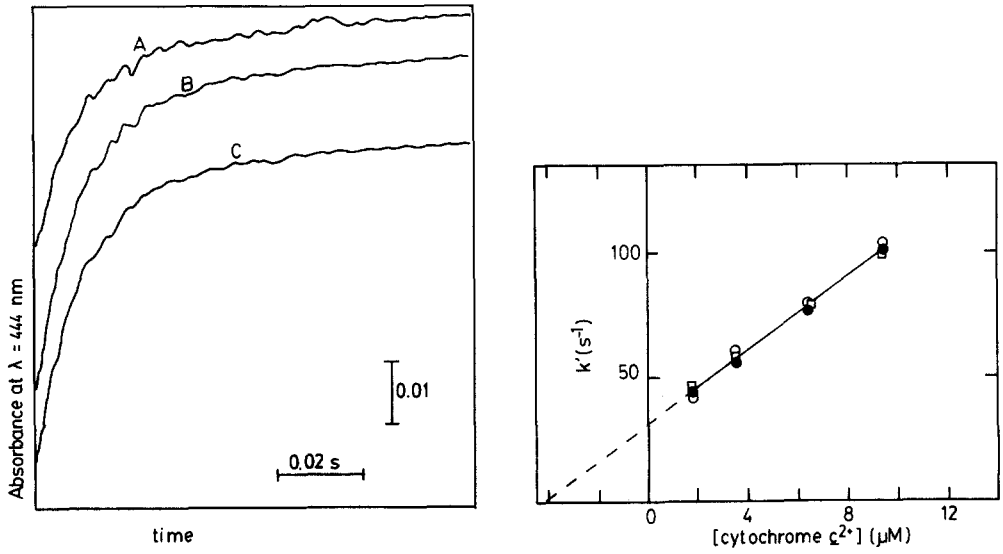


Fig. 1. Time course of the reaction of ferrocytochrome *c* (in excess) with cytochrome aa_3. The experiments were carried out at 10°C in a medium of low ionic strength (5 mM potassium phosphate, 1% Tween 20, pH 7.0). Absorbance changes were followed at 444 nm. Ferrocytochrome *c*, 10 μM. A, with 0.7 μM cytochrome aa_3; B, with 0.7 μM of the 1 : 1 complex of cytochrome *c*-cytochrome aa_3; C, with 0.7 μM of the 1 : 1 complex of porphyrin cytochrome *c*-cytochrome aa_3.

Fig. 2. Rate of cytochrome aa_3 reduction as a function of the ferrocytochrome *c* concentration. Conditions were as described in Fig. 1. ○———○, cytochrome aa_3; ●———●, cytochrome *c*-cytochrome aa_3 complex; □———□, porphyrin cytochrome *c*-cytochrome aa_3 complex.

TABLE I

RATE CONSTANTS AT LOW AND HIGH IONIC STRENGTH OF THE REACTION BETWEEN CYTOCHROME *c* AND CYTOCHROME *aa*$_3$

The reactions were carried out at 10°C in potassium phosphate buffer, pH 7.0. *aa*$_3$, cytochrome *aa*$_3$; c^{2+}, ferrocytochrome *c*; *c-aa*$_3$, 1 : 1 complex of cytochrome *aa*$_3$ with cytochrome *c*; por.*c-aa*$_3$, 1 : 1 complex of cytochrome *aa*$_3$ with porphyrin cytochrome *c*.

Reaction of c^{2+} with	Reactant in excess *	*I* (mM)	$k_1 \times 10^7$ ($M^{-1} \cdot s^{-1}$)	Site affinity
aa$_3$	c^{2+}	8.8	0.7	Low
aa$_3$	*aa*$_3$	8.8	>20	High
c-aa$_3$	c^{2+}	8.8	0.7	Low
c-aa$_3$	*c-aa*$_3$	8.8	1.3 **	Low
por.*c-aa*$_3$	c^{2+}	8.8	0.7 ***	Low
por.*c-aa*$_3$	por.*c-aa*$_3$	8.8	1.4 **,***	Low
aa$_3$	c^{2+}	132	0.85	High
aa$_3$	*aa*$_3$	132	1.7 **	High

* Reactant was present in 3—10 fold excess.

** The rate constant was calculated on the basis of the concentration of the functional unit.

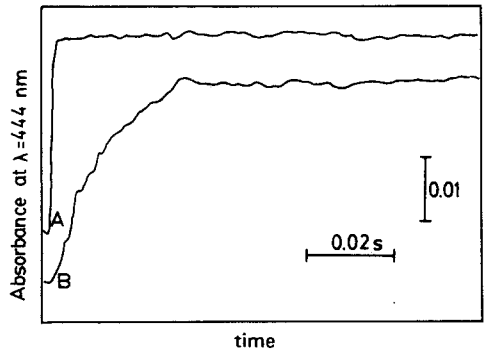
*** Not shown in results.

resemblance between the reactions of ferrocytochrome *c* with cytochrome *aa*$_3$ and with the cytochrome *c*-cytochrome *aa*$_3$ complex can be attributed to a very rapid formation of a complex between cytochrome *c* and cytochrome *aa*$_3$. Once the complex is formed it reacts as fast as the *isolated* (porphyrin)-cytochrome *c*-cytochrome *aa*$_3$ complexes with the remaining ferrocytochrome *c*.

We tested this hypothesis by studying the reaction of ferrocytochrome *c* with an excess of cytochrome *aa*$_3$ or with an excess of the cytochrome *c*-cytochrome *aa*$_3$ complex in a low ionic strength medium. These conditions diminish the contributions of slower reactions of ferrocytochrome *c* with cytochrome *aa*$_3$ and of the turnover activity of the enzyme. Fig. 3 shows the absorbance changes at 444 nm when 2.7 μM cytochrome *aa*$_3$ was mixed with 0.9 μM ferrocytochrome *c* (trace A). We observed a very fast increase in absorbance, which was completed within the mixing time (2 ms); this points to the occurrence of a very fast reduction of cytochrome *aa*$_3$ by ferrocytochrome *c* ($t_{1/2} < 1$ ms). The absorbance change was of the same magnitude as that measured when the reaction was studied in a high ionic strength medium. This indicates that complex formation is accompanied by electron transfer between the cytochromes.

Fig. 3 also shows the absorbance changes at 444 nm when 2.7 μM of the cytochrome *c*-cytochrome *aa*$_3$ complex was mixed with 0.9 μM ferrocytochrome *c*. This reaction proceeds much more slowly than the reaction with cytochrome *aa*$_3$ (Fig. 3, A) but is comparable with the time-course of the reactions shown in Fig. 1. These results are in agreement with the conclusions of Ferguson-Miller et al. [2,18] and Errede et al. [3,4] and also point to the existence of two distinct kinetically active cytochrome *c* binding sites on cytochrome *aa*$_3$.

This view is further supported by the results of experiments carried out in a low ionic strength medium, in which cytochrome *aa*$_3$ was incubated with



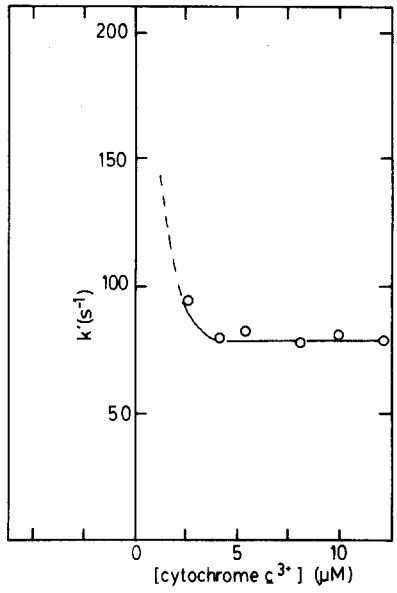


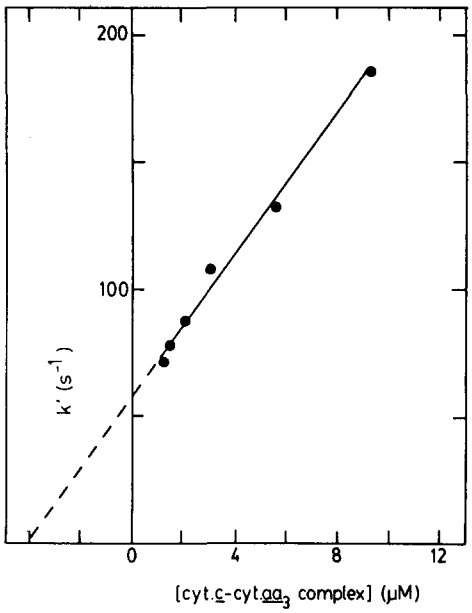
Fig. 3. Time course of the reaction of ferrocytochrome *c* with cytochrome aa_3 (in excess). Conditions were as described in Fig. 1. Ferrocytochrome *c*, 0.9 μM. A, with 2.7 μM cytochrome aa_3; B, with 2.7 μM of the 1 : 1 complex of cytochrome *c*-cytochrome aa_3.

Fig. 4. Effect of ferricytochrome *c* on the rate of reaction between ferrocytochrome *c* and cytochrome aa_3. Ferrocytochrome *c*, 0.9 μM; cytochrome aa_3, 2.7 μM. Other conditions were as described in Fig. 1. k' in the absence of ferricytochrome *c* was greater than 500 s^{-1}.

various concentrations of ferricytochrome *c*.

From Fig. 4 it is clear that the very high reaction rate observed in the absence of ferricytochrome *c* decreases upon addition of ferricytochrome *c*. At a cytochrome *c*/cytochrome aa_3 ratio of approximately one, the reaction rate is equal to the rate measured in the reaction with the isolated complex (Fig.3,B). In our opinion, the added ferricytochrome *c* occupies a site on cytochrome aa_3, which reacts very fast with ferrocytochrome *c*. When the cytochrome *c*/cytochrome aa_3 ratio increases from 1 to 5 the reaction rate remains constant. This suggests that ferricytochrome *c* does not interfere with the reaction of ferrocytochrome *c* and cytochrome aa_3, at the low affinity site. The same result was obtained when porphyrin cytochrome *c* was used instead of ferricytochrome *c* (not shown).

A relatively slow reacting site is seen if a cytochrome *c*-cytochrome aa_3 complex has been formed in the first few milliseconds (Fig. 1,A) or of this complex is present a priori (Fig. 1,B, 3,B). By varying the complex-concentration (present in excess towards ferrocytochrome *c*) the second order rate constant (k_1) of the reaction between ferrocytochrome *c* and this slow reacting site on cytochrome aa_3 can be determined (Fig. 5). The value of this rate constant is apparently twice the value obtained from experiments in which the complex reacted with an excess of ferrocytochrome *c* (cf. Table I). The implications of this apparent discrepancy are treated in more detail in the Discussion.



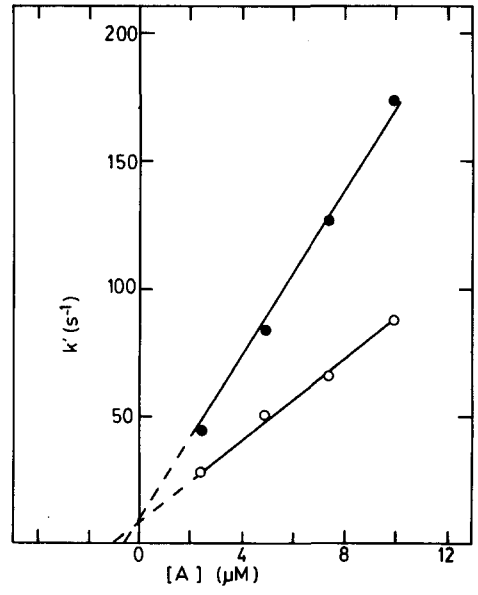


Fig. 5. Rate of cytochrome aa_3 reduction as a function of the concentration of complex of cytochrome *c*-cytochrome aa_3. Ferrocytochrome *c*, 0.7 μM. The experiments were performed at 10°C in a medium containing 5 mM potassium phosphate, 1% Tween 20, pH 7.0.

Fig. 6. Rate of cytochrome aa_3 reduction as a function of the concentration of either cytochrome aa_3 or ferrocytochrome *c*. The experiments were carried out at 10°C in a medium of high ionic strength (75 mM potassium phosphate, 1% Tween 20, pH 7.0). o———o, with variable concentrations of cytochrome *c* and 0.7 μM cytochrome aa_3; •———•, with variable concentrations of cytochrome aa_3 and 0.7 μM cytochrome *c*.

In order to investigate the fast reacting site on cytochrome aa_3 the use of an excess of cytochrome aa_3 with respect to ferrocytochrome *c* is required to prevent the reaction of ferrocytochrome *c* with the slow reacting site. At low ionic strength the pre-steady state reaction is completed within 2 ms. This indicates that ferrocytochrome *c* reacts with the fast reacting site with a second order rate constant greater than $2 \cdot 10^8\ M^{-1} \cdot s^{-1}$ (I = 8.8 mM, pH = 7.0, 10°C). Due to experimental limitations no further information could be obtained about the fast reacting site under these conditions (i.e. low ionic strength, [cytochrome aa_3] > [ferrocytochrome *c*]).

From preliminary experiments it appears that the rate of the reaction of ferrocytochrome *c* with both the fast and the slow reacting site on cytochrome aa_3 decreases with increasing ionic strength. Therefore, we have examined the reaction between ferrocytochrome *c* and cytochrome aa_3 at a higher ionic strength; in this way difficulties concerning the time-resolution of our stopped-flow apparatus could be avoided (Fig. 6).

The calculated second-order rate constant of this reaction ($k_1 = 1.7 \cdot 10^7\ M^{-1} \cdot s^{-1}$, I = 132 mM, pH = 7.0, 10°C) is much smaller than the estimated rate at low ionic strength ($k_1 > 2 \cdot 10^8\ M^{-1} \cdot s^{-1}$, I = 8.8 mM, pH = 7.0, 10°C). However, the reaction at high ionic strength is even faster than the reaction of ferrocytochrome *c* with the slow reacting site on cytochrome aa_3 at low ionic

strength (cf. Fig. 3,B). This indicates that the measured reaction of ferrocytochrome *c* with cytochrome aa_3 at high ionic strength occurs via the fast reacting site. Again the rate constant differs apparently by a factor 2, depending on which cytochrome was present in excess. The same phenomenon was observed for the reaction of the cytochrome *c*-cytochrome aa_3 complex with ferrocytochrome *c* at low ionic strength (Fig. 2, Fig. 5). Both findings will be discussed below.

No information could be obtained about the behaviour of the slow reacting site at high ionic strength, because this slow reaction interferes with the steady state oxidation of cytochrome *c* by molecular oxygen.

Discussion

The mechanism of the pre-steady state reaction of ferrocytochrome c with cytochrome aa_3

In our experiments the reduction of cytochrome aa_3 could be approximated by an exponential increase of the absorption at 444 nm. This indicates a (pseudo) first-order process, of which the rate constant (k') can be calculated. Plotting k' versus the concentration of the reactant present in excess (ferrocytochrome *c*, cytochrome aa_3 or their complex) reveals a linear relationship. This points to a reaction which is first order with respect to ferrocytochrome *c*, cytochrome aa_3 or to their complex.

The inhibitory action of ferricytochrome *c* in steady-state kinetics is reflected in the decrease of the observed rate constants of the pre-steady state reaction between ferrocytochrome *c* and cytochrome aa_3. The observation that free ferricytochrome *c* up to concentrations of 15 μM does not affect the reaction at the low-affinity site. This implies that the K_d for the low-affinity site must be at least 30 μM, which is considerably higher than the values referred to in the mechanisms proposed for the steady-state kinetics [1,3,4].

The reactions of ferrocytochrome c with cytochrome aa_3 and with (porphyrin) cytochrome c-cytochrome aa_3 complexes

The results of our experiments carried out at low ionic strength indicate clearly that two kinetically distinct types of sites for cytochrome *c* are present on cytochrome aa_3. Ferrocytochrome *c* reacts rapidly with a fast reacting site (the so called high-affinity site) on cytochrome aa_3. The short lifetime of the reaction of cytochrome aa_3 indicates that this complexation reaction has a second order rate constant greater than $2 \cdot 10^8\ M^{-1} \cdot s^{-1}$ (10°C, I = 8.8 mM, pH = 7.0).

In the presence of an excess of ferrocytochrome *c* also a second, slower reaction takes place with a second order rate constant of $10^7\ M^{-1} \cdot s^{-1}$ (10°C, I = 8.8 mM, pH = 7.0). This slower reaction cannot be due to a slow dissociation of the initially formed complex followed by a rapid reaction of another ferrocytochrome *c* molecule with the fast reacting site. In that case the observed rate constant should be independent of the ferrocytochrome *c* concentration since the dissociation step is a real first order process.

Porphyrin cytochrome *c*, bound at the high affinity site, does not inhibit the reaction of an excess of ferrocytochrome *c* with cytochrome aa_3 at the slow reacting site (Fig. 2). Because of the great difference in redox potential of

cytochrome *c* and its porphyrin derivative, it is unlikely that the slower reaction is caused by an electron transfer between free cytochrome *c* and its bound oxidation product. We therefore conclude that the slower reaction of cytochrome aa_3 with ferrocytochrome *c* at low ionic strength takes place at a site that is distinct from the high affinity site. So under these conditions the reaction between ferrocytochrome *c* and cytochrome aa_3 can be represented by the following scheme:

$$2\ \text{cyt. } c^{2+} + \text{cyt. } aa_3 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{cyt. } c^{3+} - \text{cyt. } aa_3^- + \text{cyt. } c^{2+} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{cyt. } c^{3+} - \text{cyt. } c^{3+} - \text{cyt. } aa_3^{2-}$$

In the initial step of the reaction, a partially reduced cytochrome *c*-cytochrome aa_3 complex is rapidly formed and subsequently reacts with another ferrocytochrome *c* molecule. The rate constants that were measured in the experiments shown in Figs. 1 and 3, B are those of the second reaction. This rate constant (k_2) is equal to the rate constant of the reaction of ferrocytochrome *c* (excess) with the chromatographically isolated cytochrome *c*-cytochrome aa_3 complex. Therefore we conclude that the redox state of the cytochrome *c*-cytochrome aa_3 complex does not affect the reaction of the complex with ferrocytochrome *c*. The same was concluded by Rosén [19] for the 'resting' and the partially reduced cytochrome aa_3.

The reactions of cytochrome aa_3 complex with ferrocytochrome c

As we mentioned in the Results, the rate constants of the reaction of ferrocytochrome *c* with the cytochrome *c*-cytochrome aa_3 complex differ by a factor 2 depending on which of the cytochromes is present in excess. This apparent difference can be explained by assuming that only half of the cytochrome *c* molecules were reactive or that cytochrome *c* was present as a dimer in which only one of the hemes transfers its electron to cytochrome aa_3. This explanation is very unlikely since cytochrome *c* was freshly prepared and did not react with carbonmonoxide, indicating the absence of dimeric and polymeric cytochrome *c* in the preparation. Since we obtain essentially the same results when Tris-cacodylate is used instead of potassium phosphate, these results cannot be due to an inhibitory effect of phosphate which is known to bind cytochrome *c* [20].

Another explanation is that we underestimated by a factor 2 the concentration of reactive sites on the cytochrome *c*-cytochrome aa_3 complex, resulting in an overestimation of the slope of the line in Fig. 5 (i.e. k_1). Since on the complex only slower reacting sites are available for the reaction with ferrocytochrome *c*, it is suggested that there are two 'low affinity' sites on cytochrome aa_3.

As already mentioned, the observed reaction of ferrocytochrome *c* with cytochrome aa_3 at high ionic strength occurs mainly via the high affinity site. Again the rate constant of this reaction is twice the value obtained with cytochrome aa_3 present in excess. This indicates that cytochrome aa_3 also possesses two fast reacting (high affinity) sites for cytochrome *c*.

Summarizing, we suggest that ferrocytochrome *c* reduces cytochrome aa_3 via 2 high and 2 low affinity sites. From the results presented here we cannot conclude definitely whether or not these two types of sites are present at the

same time and consequently we do not know whether low affinity sites are kinetically or spatially distinct from high affinity sites.

We have devised three models for cytochrome aa_3: a non-cooperative (2 + 2 independent sites), a cooperative (differentiation of site activity) and a mixture of these extremes.

The possibility that low and high affinity sites exist simultaneously can be incorporated in a model with 4 independent sites that are spatially distinguishable. Due to the great difference in rate constants between high and low affinity sites on cytochrome aa_3, ferrocytochrome *c* will react preferentially with the high affinity sites. In the presence of an excess of ferrocytochrome *c* with respect to cytochrome aa_3 the reduction of cytochrome aa_3 via its low affinity sites can be measured after the reaction via both high affinity sites has almost completed. However, binding studies at low ionic strength indicate a ratio of 1 : 1 for the tightly binding of cytochrome *c* to the functional unit of cytochrome aa_3 [7], which is in contrast to the 2 : 1 ratio, inherent in the purely non-cooperative model.

A model in which cytochrome aa_3 contains only two cytochrome *c* binding sites involves negative cooperativity. In this model, perferred by us since it also explains the results of binding studies [2], cytochrome aa_3 has two high affinity sites, and in the complex with cytochrome *c* the reactivity of both sites is lowered to the same extent. It is assumed that after the second molecule of cytochrome *c* has reacted with the enzyme, one cytochrome *c* molecule dissociates before the next ferrocytochrome *c* molecule can react with the enzyme. This cooperative model, in which both cytochrome *c* molecules are bound with about the same affinity to cytochrome aa_3 explains the observation that bound cytochrome *c* is able to exchange with free cytochrome *c* [18] in spite of the fact that 1 : 1 complex of cytochrome *c* with its oxidase is stable during chromatography.

We may want to know the molecular basis of the change in reactivity of the sites for cytochrome *c* on cytochrome aa_3 in the 1 : 1 complex. It has been found that the reaction of cytochrome *c* with cytochrome aa_3 is governed by electrostatic interactions between oppositely charged residues on both proteins [5,6,18]. We suggest that the bound cytochrome *c* diminishes the effective charge of a binding site on cytochrome aa_3, thus causing a lowering of the reactivity towards cytochrome *c*. The possibility that a conformational change causes the negative cooperativity still remains. However no spectral changes were detected when cytochrome *c* was bound to cytochrome aa_3, indicating that at least the heme region of the cytochromes does not alter upon binding.

It will be clear that the two models mentioned above represent two extreme possibilities (purely non-cooperativity and negative cooperativity). As an example of the intermediate possibilities a third model is described which involves negative cooperativity between the two high affinity sites but not beween the two low affinity sites. In this model the 1 : 1 ratio of tightly bound cytochrome *c* to cytochrome aa_3 at low ionic strength may be explained by the existence of a 2 : 2 complex consisting of a dimer of cytochrome aa_3 in which two molecules of cytochrome *c* occupy two high affinity sites on the dimer whereas the two other high affinity sites become inaccessible to cytochrome *c*. This model explains the properties of the complex between cyto-

chrome *c* and cytochrome aa_3 such as the inaccessibility of hydrophilic agents to cytochrome *c* bound in the complex with cytochrome aa_3 [21], the stability of the complex during chromatography and the stoichiometry of the complex.

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