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THE CONCEPT OF HIGH- AND LOW-AFFINITY REACTIONS IN BOVINE CYTOCHROME *c* OXIDASE STEADY-STATE KINETICS

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(1) Analysis of the data from steady-state kinetic studies shows that two reactions between cytochrome *c* and cytochrome *c* oxidase sufficed to describe the concave Eadie-Hofstee plots ($K_m \simeq 1 \cdot 10^{-8}$ M and $K_m \simeq 2 \cdot 10^{-5}$ M). It is not necessary to postulate a third reaction of $K_m \simeq 10^{-6}$ M. (2) Change of temperature, type of detergent and type of cytochrome *c* affected both reactions to the same extent. The presence of only a single catalytic cytochrome *c* interaction site on the oxidase could explain the kinetic data. (3) Our experiments support the notion that, at least under our conditions (pH 7.8, low-ionic strength), the dissociation of ferricytochrome *c* from cytochrome *c* oxidase is the rate-limiting step in the steady-state kinetics. (4) A series of models, proposed to describe the observed steady-state kinetics, is discussed.

Introduction

Cytochrome *c* oxidase (EC 1.9.3.1) is a large multi-subunit membrane enzyme that catalyses the oxidation of cytochrome *c* by molecular oxygen in the mitochondrial respiratory chain. It is a Y-shaped molecule with the one-beam end protruding for 5.5 nm into the intermembrane space of the mitochondrion [1]. This part of the enzyme interacts with cytochrome *c*.

It is known that the interaction domain for cytochrome *c* oxidase and for ubiquinol : cytochrome *c* oxidoreductase on the cytochrome *c* molecule is located near the exposed haem edge (for a review, see Ref. 2). This domain is surrounded by a number of positively charged lysine side chains [3] which have been proposed to interact with complementary carboxyl groups on subunit II of cytochrome *c* oxidase [4]. The other, forked, side of the cytochrome *c* oxidase molecule is largely buried in the mitochondrial inner membrane.

The steady-state kinetics of the reaction between cytochrome *c* and cytochrome *c* oxidase have been studied extensively, either polarographically in the presence of ascorbate and TMPD or spectrophotometrically in the absence of additional reagents. The difference in approach and results between the two systems was discussed previously [5,6].

When studied over an extended range of substrate concentrations, the steady-state reaction rate was shown not to be first-degree in substrate. As pointed out by Errede and coworkers [7,8], a simple Michaelis-Menten analysis cannot be applied.

Ferguson-Miller et al. [5,9] proposed three different reactions between cytochrome *c* and cytochrome *c* oxidase. The so-called low-affinity reaction ($K_m \simeq 10^{-6}$ M) and very-low-affinity reaction ($K_m \simeq 10^{-5}$ M) could be observed in both systems

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride.

[10]. The high-affinity reaction ($K_m \simeq 10^{-8}$ M) was only observable in the polarographic system. This has been ascribed to the fact that the reaction could not be followed spectrophotometrically at cytochrome *c* concentrations that are low enough [8]. Observations of high- and low-affinity reactions in pre-steady-state experiments have also been reported [11,12].

The high-affinity reaction has been ascribed to the cytochrome *c* interaction site on subunit II of the cytochrome *c* oxidase molecule, as mentioned above. This high-affinity site was characterised by cross-linking experiments with cytochrome *c* derivatives chemically modified near the haem cleft. The molecules were bound to subunit II of the cytochrome *c* oxidase [13,14]. Furthermore, Capaldi and coworkers [15] showed that chemical modification of carboxyl groups on subunit II with 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide had a strong effect on the K_m value for the high-affinity reaction.

Recently, there have been several reports in which cytochrome *c* oxidase was shown to be a 4 haem, 4 copper dimer under most common experimental conditions [2,10,16–18]. Capaldi et al. [2] proposed a model in which the two high-affinity sites are in the cleft between the two cytoplasmic domains of the monomers. Subunit II of one monomer forms the front and subunit III of the other the back of the cytochrome *c* binding cleft [2,19]. There is no consensus on cytochrome *c* oxidase functioning as a dimer, since two reports appeared in which monomers with full catalytic activity were described [17,20].

Compared to what is known about the high-affinity interaction site, little is known about interaction sites to which the two other reactions can be ascribed. Capaldi and coworkers discussed that the low-affinity reaction site might partly be formed by cardiolipins [14,21].

In most publications concerning the steady-state kinetics, the kinetic parameters were determined by fitting straight lines to the apparently linear parts of the upward-curved Eadie-Hofstee plots. This results in the three reactions mentioned above. In this paper we show that the assumption of only two reactions suffices to explain the curved Eadie-Hofstee plots: a 'high-' ($K_m \simeq 1 \cdot 10^{-8}$ M) and a 'low-affinity' reaction ($K_m \simeq 2 \cdot 10^{-5}$ M). In order to determine if there were indications that these two reactions result from different interaction sites on the cytochrome *c* oxidase molecule, we investigated the kinetics under a series of conditions.

Materials and Methods

Bovine heart cytochrome *c* oxidase was purified according to the method of Fowler et al. [22] as modified in our laboratory [23]. For determination of the concentration of cytochrome *c* oxidase a molar absorption coefficient (reduced minus oxidised) of 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm was used [24].

Horse-heart cytochrome *c* was prepared by the method of Margoliash and Walasek [25]. Ferrocytochrome *c* was obtained by incubating cytochrome *c* with ascorbate followed by gel filtration on Sephadex G-50 superfine (Pharmacia) in 25 mM acetate-Tris/1 mM EDTA (pH 7.8). Concentrations were determined using an absorption coefficient (reduced minus oxidised) at 550 nm of 21.2 $mM^{-1} \cdot cm^{-1}$ [26].

Keilin-Hartree submitochondrial particles were prepared from bovine and human heart by the method of King [27] as modified by Ferguson et al. [9], with the exception that the washed and cytochrome *c*-free mince was not sand-ground in a mortar but homogenized in a Wharing blender in 30 mM K_2HPO_4. The submitochondrial particles were precipitated by centrifugation at pH 5.55 (30 min at $2000 \times g$). The pellet was dissolved in 0.66 M sucrose/50 mM Tris-sulphate (pH 8.0), and stored in liquid nitrogen.

Protein was measured by the biuret procedure after solubilising the particles with deoxycholate [28].

Determination of cytochrome c oxidase concentration in Keilin-Hartree particles

In order to be able to compare steady-state kinetic studies performed with Keilin-Hartree preparations, a good method for determining oxidase concentration in the particles is necessary. Therefore, the three following methods were compared.

(1) Spectrophotometric measurement with an Aminco DW-2 spectrophotometer. The particles were dissolved in 25 mM acetate-Tris/0.25 M sucrose/1 mM EDTA (pH 7.8). The oxidase con-

centration was determined using the equations for the calculation of concentrations of cytochromes in a mixture as described by Tervoort et al. [29]. For the four wavelength pairs in these equations we determined the absorption of the chromophores, which were reduced by dithionite and oxidised by ferricyanide. Measurements were done using the dual wavelength mode of the spectrophotometer. Each time, the wavelength giving the lowest absorption was chosen as the reference.

(2) Spectrophotometric measurement after selective solubilization of the oxidase. To a 5 ml sample of the Keilin-Hartree particle preparation (30 mg/ml), 0.5 ml 10% (w/w) deoxycholate and 400 mg KCl were added. After centrifugation (10 min at 200000 × g) the supernatant contained no oxidase. The pellet was dissolved to 4.5 ml in 0.66 M sucrose/50 mM Tris-sulphate; subsequently 1 ml of deoxycholate plus 400 mg KCl were added. After centrifugation the supernatant contained all of the oxidase and very little of the other cytochromes. Cytochrome c oxidase concentration was determined as in a pure sample.

(3) Copper determination by EPR. The particles were concentrated by centrifugation to about 100 mg/ml protein and an EPR copper spectrum (50 K, non-saturating microwave power) was made. The copper concentration was determined by fitting the experimental spectrum to a simulated copper spectrum [30] and comparing the resulting normalised integral value [31] to one of a copper standard. The oxidase concentration was calculated assuming that 40% of the copper can be detected in this way [32].

The three methods resulted in the same oxidase concentration (within 10%). Mostly the spectrophotometric determination was used since the method is simple and gives a good estimation of the cytochrome c oxidase concentration in Keilin-Hartree particles.

Spectrophotometric measurement of cytochrome c oxidase activity

The rate of oxidation of ferrocytochrome c at 25 °C was measured at 550 nm using a Cary-17 spectrophotometer. A buffer of low ionic strength [25 mM acetate-Tris/1 mM EDTA/0.25 M sucrose/1% Tween-80 (v/v) (pH 7.8) (I = 24 mM)] was used for both purified cytochrome c oxidase and Keilin-Hartree preparation kinetics. The use of the detergent in the latter case resulted in increased activity of the oxidase and a linear v versus e relationship without solubilising any of the enzyme.

Reduced cytochrome c was added to a final concentration of 0.1–50 μM (below 1 μM a cuvette with 10 cm light path was used). The final concentration of cytochrome c oxidase, either purified or in Keilin-Hartree particles was 1–2 nM. The time course of cytochrome c oxidation was first-order in cytochrome c under all conditions [33]. Turnover numbers (TN) were calculated from the apparent first-order rate constants obtained by plotting the log (ferrocytochrome c) versus time.

Computer analysis of the steady-state kinetics

The following equation was fitted to the steady-state kinetic data by means of the least squares method:

$$TN = \frac{TN_{\max 1} \cdot s}{K_{m1} + s} + \frac{TN_{\max 2} \cdot s}{K_{m2} + s}$$

For this purpose a grid search routine [34], modified to prevent negative values for any of the four parameters, was performed by computer.

Results

Spectrophotometric measurement of the reaction of isolated bovine cytochrome c oxidase in a medium of low ionic strength (I = 24 mM) resulted in a concave Eadie-Hofstee plot (Fig. 1) indicating a steady-state rate equation of a higher degree. Computer analysis showed that the data could be simulated with the sum of two first-degree functions as described in Materials and Methods. This resulted in one reaction with $K_m \simeq 2 \cdot 10^{-8}$ M and $TN_{max} = 15\ s^{-1}$ and a second with $K_m = 1.8 \cdot 10^{-5}$ M and $TN_{max} = 60\ s^{-1}$. These two reactions correspond to the 'high-' and 'very-low'-affinity reactions described by Ferguson et al. [5,9]. It should be noted that the low turnover numbers found are inherent to the reaction conditions chosen. The low ionic strength and high pH buffer were used in order to compare the results with polarographic steady-state experiments from the literature. Since all our kinetic experiments

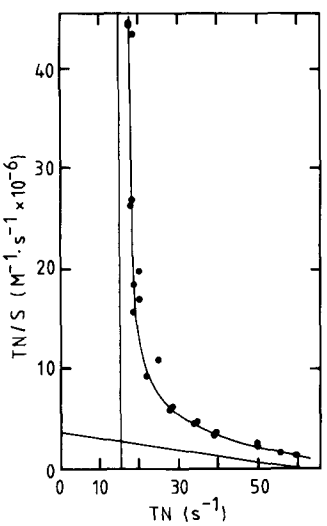

Fig. 1. Eadie-Hofstee plot of steady-state oxidation of horse ferrocytochrome *c* by isolated bovine heart cytochrome *c* oxidase, monitored spectrophotometrically. The curved line is computer simulated as described in Materials and Methods. The two straight lines represent the two first-order kinetic reactions of which the curve is composed.

resulted in curves that could be fitted with the equation chosen, there is no requirement for postulation of an additional third reaction. The often described low-affinity reaction with an intermediate K_m ($\simeq 10^{-6}$ M) can be explained as the result of the measured sum of ferrocytochrome *c* oxidation by the other two reactions, and would therefore be an artefact.

Fig. 2 gives the result of spectrophotometric measurement of the reaction of bovine Keilin-Hartree particles with horse ferrocytochrome *c*. The same two reactions as with purified cytochrome *c* oxidase were found. TN_{max} values were somewhat lower (7 and 46 s^{-1}), probably due to inaccessibility of part of the cytochrome *c* oxidase in particles.

The high-affinity cytochrome *c* interaction domain on the oxidase has been characterised [4,12–15], but little is known about an interaction site corresponding to the reaction of low K_m ($2 \cdot 10^{-5}$ M). In order to determine whether this reaction results from a site spatially distinct from the

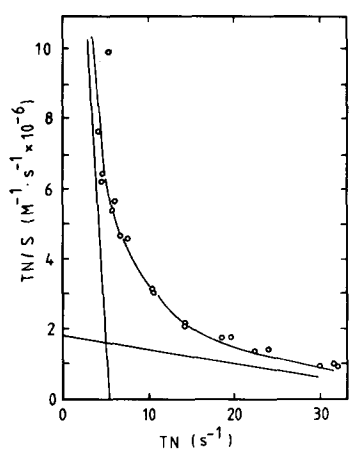

Fig. 2. Eadie-Hofstee plot of steady-state oxidation of horse ferrocytochrome *c* by a Keilin-Hartree particle preparation from bovine heart, monitored spectrophotometrically. The curved line is computer simulated as described in Materials and Methods. The two straight lines represent the two first-order kinetic reactions of which the curve is composed.

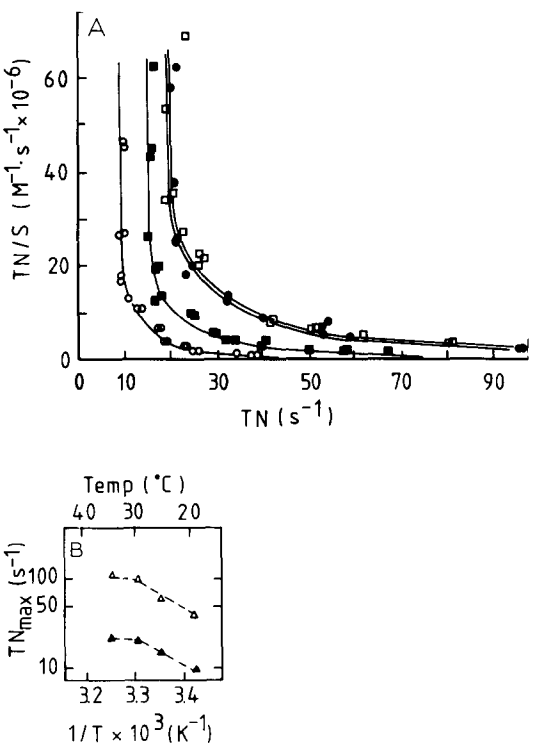

Fig. 3. (a) Eadie-Hofstee representation of steady-state oxidation of horse ferrocytochrome *c* by purified bovine heart cytochrome *c* oxidase, monitored spectrophotometrically at four different temperatures, and computer simulated as described in Materials and Methods. ○———○, 19.5 °C; ■———■, 25.0 °C, ●———●, 31.5 °C, □———□, 35.5 °C. (B) Arrhenius plot of maximal molecular activities of high (▲———▲)- and low (△———△)-affinity reactions.

TABLE I

PARAMETERS DESCRIBING CYTOCHROME *c* OXIDASE STEADY-STATE KINETICS UNDER VARIOUS CONDITIONS

The values were obtained in 25 mM acetate-Tris/250 mM sucrose (pH 7.8) (I = 24 mM). Temperature, detergent and the type of cytochrome *c* and oxidase are specified. Detergent concentrations used: 1% (v/v) Tween-80/0.02% (w/v) cholate/0.05% (w/v) laurylmaltoside. The kinetic parameters were determined by computer analysis as described in Materials and Methods. Abbreviations: ox, cytochrome *c* oxidase; Cyt. *c*, cytochrome *c*; bo, bovine; ho, horse; hu, human.

Temperature (°C)	Detergent	ox	Cyt.*c*	$K_{m1} \cdot 10^{-8}$ (M)	TN_{max1} (s^{-1})	$K_{m_2} \cdot 10^{5}$ (M)	TN_{max2} (s^{-1})
25.0	Tween-80	bo	ho	≃ 2	15	1.8	60
35.5	Tween-80	bo	ho	≃ 1	20	1.7	105
31.5	Tween-80	bo	ho	≃ 1	19	1.7	95
19.5	Tween-80	bo	ho	≃ 3	11	1.7	40
25.0	Cholate	bo	ho	≃ 1	5	2.0	16
25.0	Laurylmalt--oside	bo	ho	≃ 1	22	1.5	85
25.0	Tween-80	bo [a]	ho	< 30 [b]	7	2.5	46
25.0	Tween-80	bo [a]	hu	< 30 [b]	1	2.0	9
25.0	Tween-80	hu [a]	ho	< 30 [b]	10	1.7	65
25.0	Tween-80	hu [a]	hu	< 30 [b]	15	1.8	115

[a] In Keilin-Hartree particles.

[b] In experiments where Keilin-Hartree particles were used, no cytochrome *c* concentrations below 0.3 μM were tested. Therefore, K_{m1} values cannot be given more accurately.

high-affinity reaction site, the effects of varying experimental conditions on both reactions were compared. Fig. 3 shows the temperature dependence of the two reactions. The K_m values did not measurably change with temperature, in contrast to the maximal turnover numbers (Table I). The effect of temperature on the velocity of the two reactions describing the Eadie-Hofstee plots is shown in an Arrhenius plot (insert to Fig. 3). The high- and the low-affinity reactions show the same segmented temperature dependence: parallel Arrhenius plots with a break at 30 °C. If in the lower temperature range the reaction velocity would be dominated by a single rate-limiting step, the enthalpy of activation ($\Delta H^{\neq}$) for this reaction step is 60 kJ $\cdot mol^{-1}$. Nonlinearity in Arrhenius plots is common for reactions of cytochrome *c* oxidase [12] and other membrane proteins.

The effect of the detergents cholate and laurylmaltoside on the activity of the two reactions is demonstrated in Fig. 4. Cholate, a well-known inhibitor of cytochrome *c* oxidase activity, affected both reactions to the same extent. There is hardly any effect on K_m values, whereas the maximal turnover numbers decreased in the presence of this detergent (Table I), indicating a non-competitive type of inhibition. This has been observed before in steady-state experiments in which inhibitory

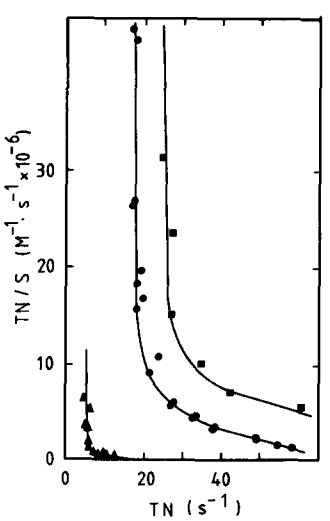

Fig. 4. Eadie-Hofstee representation of steady-state oxidation of horse ferrocytochrome *c* by purified bovine heart cytochrome *c* oxidase monitored in the presence of different detergents. ●———●, 1% Tween-80 (v/v); ▲———▲, 0.02% cholate (w/v); ■———■, 0.05% laurylmaltoside (w/v).

effects of cholate were studied at only one cytochrome *c* concentration [35]. The detergent laurylmaltoside, known for its stimulation of the cytochrome oxidase activity, increased turnover numbers of both reactions without changing the K_m values (Table I). Either with the inhibitory or with the stimulatory detergent, the effect on the high-affinity reaction is like the effect on the low-affinity reaction.

In recent polarographic studies of Osheroff and coworkers [36] it was shown that human cytochrome *c* is not as active in the high-affinity reaction with bovine heart cytochrome *c* oxidase as is the horse cytochrome *c*. This was ascribed to the formation of too tight an enzyme-product complex resulting in a slower reduction of the bound ferricytochrome *c* by TMPD. We studied the reactions with human cytochrome *c* in the spectrophotometric system, where possible artefacts due to TMPD are excluded. As purified human cytochrome *c* oxidase, required for control experiments, was not available we used Keilin-Hartree particles. The kinetics of the reactions of human Keilin-Hartree particles with horse and human cytochrome *c* are shown in Fig. 5B. High maximal velocities for both high- and low-affinity reactions were measured with either of the cytochrome *c* species, the human cytochrome *c* oxidase being more active with its own cytochrome *c* (Table I). The activities of bovine Keilin-Hartree particles with human and horse cytochrome *c* show large differences between the two cytochrome *c* proteins (Fig. 5). With human cytochrome *c* both high- and low-affinity reactions have very low maximal turnover numbers, and again both reactions are affected to the same extent (Table I).

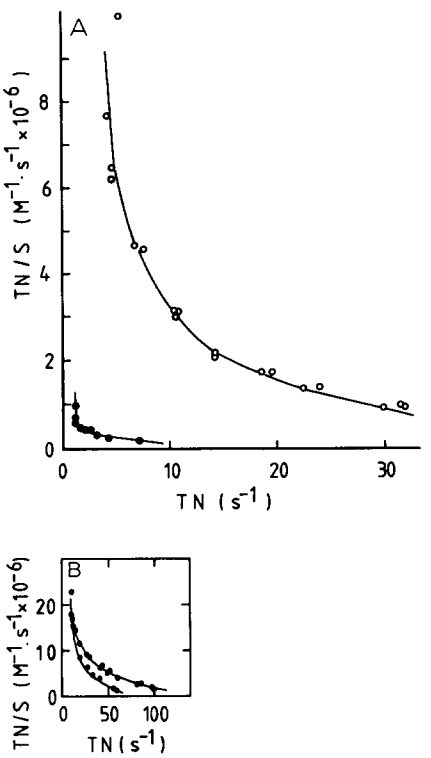

Fig. 5. (A) Eadie-Hofstee plot of steady-state oxidation of horse (○———○) and human (●———●) cytochrome *c* by bovine heart Keilin-Hartree particles monitored spectrophotometrically. The curves are computer simulated as described in Materials and Methods. (B) Inset: oxidation of horse (○———○) and human (●———●) cytochrome *c* by human heart Keilin-Hartree particles.

Discussion

Our analysis of the steady-state kinetics of the reaction of purified cytochrome *c* oxidase and cytochrome *c* shows that all data fit an equation composed of the sum of two Michaelis-Menten rate equations with K_m of approx. 10^{-8} M and of $2 \cdot 10^{-5}$ M. Since the reaction that was always referred to as the low-affinity reaction ($K_m \simeq 10^{-6}$ M) now proves to be an interpretational artefact, we propose to call the reaction with K_m of $2 \cdot 10^{-5}$ M the low-affinity reaction.

In our opinion there are three basically different ways to interpret the concave plots (Fig. 6):

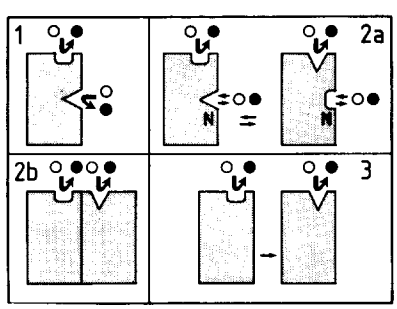

Fig. 6. Schematic representation of four models, fitting the observed steady-state kinetics. The numbers correspond to the numbers in the discussion. —⌣—, high-affinity cytochrome *c* interaction site; —⌣—, low-affinity cytochrome *c* interaction site; N, noncatalytic site; ○, ferrocytochrome *c*, ●, ferricytochrome *c*.

(1) The cytochrome *c* oxidase molecule has two or more different, catalytically active cytochrome *c* interaction sites per monomer [5,9].

(2) The cytochrome *c* oxidase monomer has only one catalytically active interaction site; the nonlinear Eadie-Hofstee plots result from cooperativity: (a) there is an additional non-catalytic cytochrome *c* binding site in each molecule [37]; (b) the cytochrome *c* oxidase acts as a dimer, resulting in cooperativity between two equivalent sites [16].

(3) The cytochrome *c* oxidase monomer has only one catalytically active cytochrome *c* interaction site. The non-linearity is the result of at least two kinetically relevant association steps of cytochrome *c* with the oxidase [38]. This is inherent to the need for four electrons in the oxidase for reduction of dioxygen to water.

If the concave Eadie-Hofstee plots do result from kinetically different electron transfer reactions (possibilities (1) and (2), which are kinetically indistinguishable), K_m and TN_{max} values should be determined by resolving the plots of two simultaneous reactions as described in this paper. The 'dependent site' and 'independent site' mechanisms proposed by Errede and Kamen [8] which are extensions of Minnaert's mechanism IV [33] correspond to our possibility (1). Possibility (2a) was recently proposed by Speck, Dye and Margoliash [37], while possibility (3) has been suggested by Antalis and Palmer [38]. If the third possibility is correct, kinetics of consecutive reactions must be applied. In that case branched reaction schemes lead to rate equations containing higher degree terms in substrate.

We studied the steady-state kinetics under a series of conditions, one of which being the substitution of human cytochrome *c* for horse cytochrome *c* (Table I), in order to investigate possibility (1): a separate interaction domain with a K_m of $2 \cdot 10^{-5}$ M on the cytochrome *c* oxidase molecule. Compared to the three control experiments bovine oxidase showed very low turnover numbers with human cytochrome *c* over a wide cytochrome *c* concentration range. These low TN_{max} values we observed represent one or more rate-limiting steps in the reaction between the cytochromes and oxygen. The electron transfer of cytochrome *c* oxidase to oxygen has never been found to be rate-limiting under steady-state conditions. It is unlikely that the internal electron transfer in the oxidase is the slowest step, since the same cytochrome *c* oxidase shows different TN_{max} values with different types of cytochrome *c*. Under TN_{max} conditions association of the cytochrome *c* is not rate-limiting, also the rate of electron transfer between the two cytochromes has been determined to be very fast for all types of cytochrome *c* used [36]. So it seems that under our reaction conditions the rate-limiting step in the steady-state reaction studied by spectrophotometry is the dissociation of the (ferricytochrome *c*)-(cytochrome *c*-oxidase) complex. This is in accordance with the results of Osheroff et al. [36] who showed bovine cytochrome *c* oxidase to have very low K_D values with human cytochrome *c*. Gibson et al. [39] observed that electron transfer between the cytochrome *a* and a_3 part of the oxidase is a slow process. They suggested that the slow dissociation of oxidised cytochrome *c* from the oxidase triggers electron transfer from *a* to a_3.

The fact that both high- and low-affinity reactions of human cytochrome *c* with bovine oxidase show a very low TN_{max} value suggests that, if there are two separate interaction sites for cytochrome *c* on the cytochrome *c* oxidase molecule, they must be very much alike, since they both show a decreased dissociation rate of the ferricytochrome *c*-oxidase complex. The observed correlation between reaction rate and cytochrome *c* types, which is the same for the high- and low-affinity reactions, is in agreement with transient [40] and steady-state kinetics [5] with chemically modified horse cytochromes *c*.

The temperature dependence of the TN_{max} values shows the same activation enthalpy ($\Delta H^{\neq} = 60$ kJ $\cdot$ mol^{-1}) for the high- and the low-affinity reactions, also suggesting the same types of interaction sites. This line of reasoning can also be followed for the experiment in which different detergents were used (Table I). The dissociation velocities of the cytochrome *c*-oxidase complexes, reflected by the turnover numbers, were affected to the same extent.

Since it seems difficult to imagine how two different cytochrome *c* interaction domains on the asymmetrical C-side of the cytochrome *c* oxidase molecule can react so much alike, we propose that there is only one catalytically active cytochrome *c*

interaction domain on the oxidase molecule. Therefore, of the three possibilities to explain the observed kinetics, we favour the second and third to the first. It does seem likely that binding of a strongly positively charged cytochrome *c* molecule to either a non-catalytic site on a monomeric oxidase or to a nearby identical catalytic site on the other monomer of an oxidase dimer, will change the charge and dipole of the cytochrome *c* oxidase. Since electrostatic interactions seem to be very important in the cytochrome *c* oxidase interaction, this would result in increased K_m and TN_{max} values. A one-site electron-transfer reaction would require re-evaluation of the data of Veerman et al. [11] who measured that the reaction rate of a second ferrocytochrome *c* with the (high-affinity 1 : 1 oxidase)-(cytochrome *c*) complex was dependent on the ferrocytochrome *c* concentration, in apparent contradiction to dissociation of an enzyme-product complex as the rate-limiting step.

As yet our data do not allow a discrimination between the remaining possibilities to explain the observed kinetics. The lack of consensus in published work on this subject from other groups also prevents a decision in favour of models 2 or 3 (Fig. 6). Thompson and Ferguson-Miller [20] and Darley-Usmar et al. [41] described largely monomeric oxidase preparations having both a high- and a low-affinity reaction, whereas Nalecz et al. [16] reported loss of the low-affinity reaction upon monomerisation of the oxidase. Vik et al. [21] described loss of the low-affinity reaction when the tightly bound cardiolipin molecules were removed from cytochrome *c* oxidase. The half-site effect described by Bisson et al. [14] (i.e., total loss of catalytic activity upon covalent binding of one cytochrome *c* per two cytochrome *c* oxidase molecules) can only be explained by a form of negative cooperativity of binding of cytochrome *c* to an oxidase dimer.

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