The rate-limiting step and nonhyperbolic kinetics in the oxidation of ferrocytochrome *c* catalyzed by cytochrome *c* oxidase

Peter Brzezinski, Per-Eric Thörnström and Bo G. Malmström*

Department of Biochemistry and Biophysics, University of Göteborg and Chalmers University of Technology, S-412 96 Göteborg, Sweden

Received 29 September 1985

The level of reduction of cytochrome *a* and Cu_A during the oxidation of ferrocytochrome *c* has been determined in stopped-flow experiments. Both components are partially reduced but become progressively more oxidized as the reaction proceeds. When all cytochrome *c* has been oxidized, Cu_A is also completely oxidized, whereas cytochrome *a* is still partially reduced. These results can be simulated on the basis of a model which requires that the intramolecular electron transfer from cytochrome *a* and Cu_A to cytochrome a_3-Cu_B is a two-electron process and, in addition, that the binding of oxidized cytochrome *c* to the electron- transfer site decreases the rate constants for intramolecular electron transfer from cytochrome *a*. The first requirement is related to the function of the oxidase as a proton pump. Product dissociation is not by itself rate-limiting, making it less likely that the source of the nonhyperbolic substrate kinetics is an effect on this step from electrostatic interaction with ferricytochrome *c* bound to a second site. It is pointed out that nonhyperbolic kinetics is, in fact, an intrinsic property of ion pumps.

Cytochrome c oxidase *Steady state* *Nonhyperbolic kinetics* *Rate-limiting step* *Proton pump*

1. INTRODUCTION

It has long been known that cytochrome *c* oxidase displays nonhyperbolic kinetics if a wide enough range of substrate concentration is used [1–3]. Various explanations for this phenomenon have been offered. Nicholls [1,4] suggested the existence of two active sites with different kinetic parameters (k_{cat}, K_m), and his proposal has received wide acceptance. Recently a model involving a single catalytic site has, however, been formulated [5,6]. According to this, the rate-limiting step in cytochrome oxidase turnover in the steady state is the dissociation of the product, ferricytochrome *c*, from the catalytic site. At high concentration of cytochrome *c* this step is assumed to be speeded up by electrostatic interaction with cytochrome *c* bound to a non-productive binding site, thus accounting for the biphasic dependence of the catalytic rate on the substrate concentration.

A recent analysis [7] of the steady-state rate equation for cytochrome oxidase, based on a minimal kinetic scheme, suggested that it is unlikely that product dissociation is rate-limiting under all conditions. In fact, for such a complex mechanism as that of cytochrome oxidase, with at least 13 elementary reaction steps, the concept of a rate-limiting step becomes rather meaningless. The reason for this is, of course, that even steps with relatively large rate constants limit the rate to some extent, when there are very many of them.

To illuminate further which steps are likely to contribute to the rate limitation, we have numeri-

This paper is dedicated to Professor S.P. Datta

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

cally integrated the rate equations for our kinetic scheme. This procedure has been used to simulate the kinetic behaviour of cytochrome *a* and Cu_A in turnover experiments in which ferrocytochrome *c* was the only reducing substrate and dioxygen was present in excess. A few such experiments have been reported earlier [8,9], but mostly with cytochrome *c* concentrations allowing a single turnover only, whereas we have used a much larger excess of cytochrome *c* over cytochrome *a*.

In agreement with earlier results [8,9] we find that cytochrome *a* and Cu_A are partially reduced in a rapid burst. This is followed by a progressive reoxidation of both chromophores as the ferrocytochrome *c* is consumed. The oxidation of Cu_A parallels that of cytochrome *c*, whereas the oxidation of cytochrome *a* is slower. In fact, even after the complete exhaustion of the substrate, cytochrome *a* is still not fully oxidized. This slow oxidation of cytochrome *a* by dioxygen in the partially reduced enzyme has also been noted previously [9,10].

To account for this experimental behaviour, two features of our kinetic model are essential. The first one is a requirement that both cytochrome *a* and Cu_A should be reduced for intramolecular electron transfer to the dioxygen-reducing site to occur. The second feature is an effect of bound ferricytochrome *c* on the rate constants for electron transfer from reduced cytochrome *a* to other redox sites. This requirement has the effect that product dissociation does indeed contribute to the rate limitation, though not in the way originally envisioned [5] but rather by limiting the rate of intramolecular electron-transfer steps.

Our analysis makes it unlikely that the electrostatic model [5] provides the true explanation of the biphasic kinetics. The catalytic reaction of cytochrome oxidase drives the translocation of protons across the inner mitochondrial membrane. It can be shown [11] that an intrinsic property of such an ion pump is nonhyperbolic substrate kinetics, even if the pump protein has a single catalytic site.

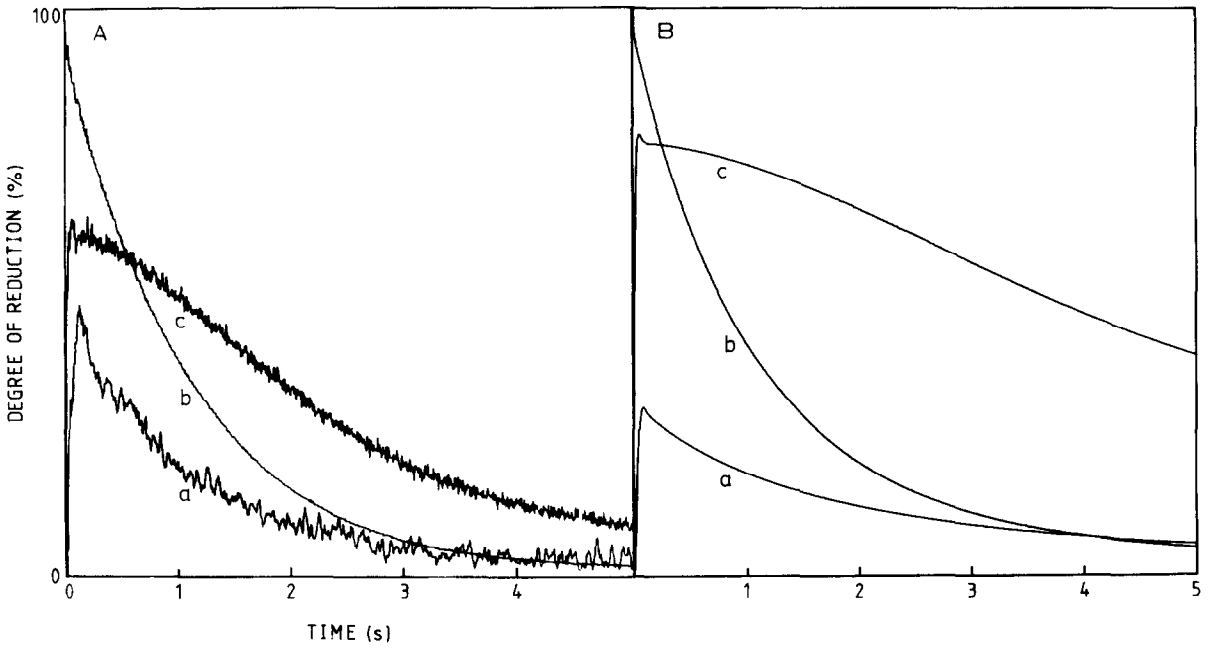

Fig.1. Time course of the degree of reduction of Cu_A (a), cytochrome *c* (b) and cytochrome *a* (c). (A) Traces from a stopped-flow experiment. Conditions: 2.5 μM cytochrome oxidase and 40 μM cytochrome *c* in 0.05 M Hepes, pH 7.4, with 0.167 M K_2SO_4 and 0.5% Tween 80; temperature, 25°C. (B) Traces from a simulation based on the model in fig.2. The following rate constants were used: $k_1 = 2\times10^6\ M^{-1}\cdot s^{-1}$, $k_{-1} = 60\ s^{-1}$, $k_{-1'} = 10\ s^{-1}$, $k_2 = 100\ s^{-1}$, $k_{-2} = 330\ s^{-1}$, $k_3 = 100\ s^{-1}$, $k_{2'} = k_{-2'} = k_{3'} = k_{-3'} = 0$.

2. MATERIALS AND METHODS

Beef heart cytochrome oxidase was prepared essentially as described in [12]. Buffers used were 0.05 M Hepes with 0.5% Tween 80, pH 7.4, with or without 0.167 M K_2SO_4. Cytochrome *c* was purified as described in [13]. Concentrations of cytochrome oxidase and cytochrome *c* were determined according to [14].

Stopped-flow experiments were carried out in an apparatus described in [15], connected to a Data General Nova minicomputer for storage and processing of the kinetic traces. The absorbance curves at both 605 and 830 nm were corrected for the contributions from cytochrome *c*. For the calculation of the degree of reduction, the 605-nm contribution of cytochrome *a* to the total absorbance change on full reduction was taken as 80%.

Simulations were made on a computer (Data General Nova) by solving the kinetic differential equations using the Runge-Kutta method with automatic choice of the integration step.

3. RESULTS

The concentration of reduced cytochrome *c*, cytochrome *a* and Cu_A as a function of time on mixing resting cytochrome oxidase with a 16-fold excess of ferrocytochrome *c* under aerobic conditions and at high ionic strength is shown in fig.1A. Also at low ionic strength, cytochrome *a* and Cu_A are partially reduced during turnover but the level of reduction of cytochrome *a* is slightly larger and that of Cu_A smaller than at high ionic strength.

The reaction scheme in fig.2 has been used to simulate the kinetics. We have simplified the scheme in [7] by assuming that the entire turnover involves two 2-electron cycles. We have, however, also added reactions in which intramolecular electron transfer is allowed with product bound to the enzyme (primed symbols). The dioxygen reactions have been assumed to be much more rapid than all other steps.

One of our simulations is included in fig.1B. This agrees with essential features of the experimental behaviour, but Cu_A is less reduced than in fig.1A. We could achieve a larger degree of reduction by changing the rate constants for step 2, but then other features were less well reproduced. If the rate constants for the corresponding primed and unprimed steps are made equal, it is not possible to simulate the results.

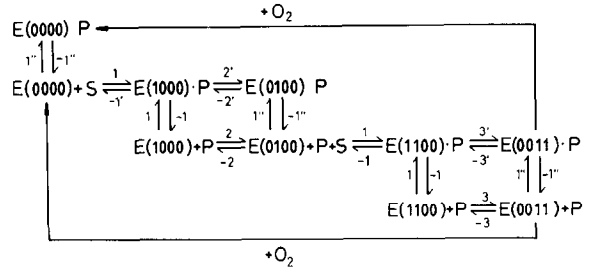

Fig.2. Kinetic scheme for the cytochrome oxidase reaction (modified from [7]). E(0000) represents the fully oxidized enzyme, with the zeros designating oxidized cytochrome *a*, Cu_A, Cu_B and cytochrome a_3 in that order; the numeral 1 is used for a one-electron reduced redox centre. S and P represent ferro- and ferricytochrome *c*, respectively.

4. DISCUSSION

The kinetic observations in fig.1, as well as other similar results [8,9], exclude the kinetic model of Speck et al. [5] in its original form. A model in which electron transfer from cytochrome *c* to the oxidase occurs at a single catalytic site, with the dissociation of oxidized cytochrome *c* as the rate-limiting step, i.e. much slower than the intramolecular electron-transfer steps, would predict that cytochrome *a* and Cu_A should be fully oxidized during turnover. Instead these redox sites are partially reduced during the entire reaction (fig.1). In addition, the level of reduction of Cu_A is closely linked to the concentration of reduced cytochrome *c* remaining in the reaction mixture, suggesting that internal electron transfer from Cu_A to the dioxygen-reducing site limits the rate. Our results are not dependent on the use of high ionic strength, as partial reduction of cytochrome *a* and Cu_A is also observed at low ionic strength. It has also been shown [16,17] that the limiting rate in the high-activity phase is independent of ionic strength, which only affects the Michaelis constant. Thus, the same steps must limit the rate at high as well as at low ionic strength. It has already been pointed out [7] that this by itself provides strong evidence against the electrostatic model [5], as electrostatic repulsion between two cytochrome *c* molecules should be strongly dependent on ionic strength.

Our kinetic model can adequately describe the main features in the experimental behaviour, as shown in fig.1. A complete fit can, of course, not be achieved, as even the extended model is grossly oversimplified. An essential feature of the model is that electron transfer from cytochrome *a* to the dioxygen-reducing site can only occur if also Cu_A is reduced, as first suggested in 1970 [10]. The lack of intramolecular electron transfer in the one-electron reduced enzyme can be understood if such a transfer can take place only in the 'open' but not in the 'closed' conformation [18], as reduction of cytochrome *a* and Cu_A has been shown [19] to trigger the conformational transition. Such a conformational requirement could also provide an obligatory coupling between electron transfer and proton translocation, which is a basic characteristic of any electron-transport driven pump [20].

To simulate the delayed oxidation of cytochrome *a*, we must assume that when oxidized cytochrome *c* is bound at the electron-transfer site, the reduction potential of cytochrome *a* is increased by some mechanism so that electron transfer from cytochrome *a* is slowed down. This could be the result of an electrostatic interaction between cytochrome *c* and cytochrome *a*. From the known dipole moment of cytochrome *c* [21] we have estimated that such an interaction could decrease the rate more than 10-fold. Consequently, product dissociation really does affect the rate, but it does so by controlling the rate of intramolecular electron-transfer steps. The model thus reconciles a variety of seemingly conflicting observations which have been used to support the alternative views that either product dissociation or intramolecular electron transfer limits the rate of cytochrome *c* oxidation.

The best simulation of our experimental results is achieved if the rate constants for product dissociation and intramolecular electron transfer have comparable values (see section 3), so that none of these steps is rate-limiting by itself. This, together with the previous analysis, makes it desirable to find a new explanation for the non-hyperbolic kinetics of the catalytic reaction. It does not seem to be generally appreciated that such kinetics is also displayed by enzymes with a single substrate-binding site, if this site has different affinities in two states of the protein linked by a slow transition [22]. The existence of such states is an obligatory part of any pump mechanism [20,23], and one would consequently expect the kinetics of the driving reaction to be nonhyperbolic. Such kinetics has indeed been observed with ATP-driven pumps [11,24,25]. In terms of cytochrome oxidase the two states of the protein would be two distinct conformations in which cytochrome *a* has different reduction potentials. The existence of such states is evident from the redox interactions between cytochrome *a* and a_3 [26]. In the resting, oxidized enzyme the reduction potential of cytochrome *a* is 0.285 V, but it is lowered to 0.220 V in states in which cytochrome a_3 is reduced [27]. If the transition between these states is slow, cytochrome oxidase should show biphasic kinetics. We have argued for the change between two conformational states to limit the rate of the intramolecular electron transfer, and this must in turn contribute to the limit of the overall turnover, as cytochrome *a* and Cu_A are partially reduced in the steady state. We would thus like to suggest that the nonhyperbolic kinetics of cytochrome oxidase is a direct consequence of its function as a proton pump.

ACKNOWLEDGEMENTS

This investigation has been supported by the Swedish Natural Science Research Council. We would like to thank Mrs A.-C. Smiderot for preparing the cytochrome *c*. Professor C. Scholes has given us helpful comments on electrostatic interactions.

REFERENCES

[1] Nicholls, P. (1965) in: Oxidases and Related Redox Systems (King, T.E. et al. eds) pp. 764–777, Wiley, New York.
[2] Errede, B., Haight, G.P. and Kamen, M.D. (1976) Proc. Natl. Acad. Sci. USA 73, 113–117.
[3] Ferguson-Miller, S., Brautigan, D.L. and Margoliash, E. (1976) J. Biol. Chem. 251, 1104–1115.
[4] Nicholls, P. (1974) Biochim. Biophys. Acta 346, 261–310.
[5] Speck, S.H., Dye, D. and Margoliash, E. (1984) Proc. Natl. Acad. Sci. USA 81, 347–351.
[6] Sinjorgo, K.M.C., Meijling, J.H. and Muijsers, A.O. (1984) Biochim. Biophys. Acta 767, 48–56.
[7] Malmström, B.G. and Andréasson, L.-E. (1985) J. Inorg. Biochem. 23, 233–242.

[8] Andréasson, L.-E., Malmström, B.G., Strömberg, C. and Vänngård, T. (1972) FEBS Lett. 28, 297-301.
[9] Wilson, M.T., Greenwood, C., Brunori, M. and Antonini, E. (1975) Biochem. J. 147, 145-153.
[10] Antonini, E., Brunori, M., Greenwood, C. and Malmström, B.G. (1970) Nature 228, 936-937.
[11] Reynolds, J.A., Johnson, E.A. and Tanford, C. (1985) Proc. Natl. Acad. Sci. USA 82, 3658-3661.
[12] Van Buuren, K.J. (1972) PhD thesis, University of Amsterdam.
[13] Brautigan, D.L., Ferguson-Miller, S. and Margoliash, E. (1978) Methods Enzymol. 53, 128-164.
[14] Jensen, P., Aasa, R. and Malmström, B.G. (1981) FEBS Lett. 125, 161-164.
[15] Andréasson, L.-E., Brändén, R., Malmström, B.G., Strömberg, C. and Vänngård, T. (1973) in: Oxidases and Related Redox Systems (King, T.E. et al. eds) pp. 87-95, University Park Press, Baltimore.
[16] Wilms, J., Van Rijn, J.L.M.L. and Van Gelder, B.F. (1980) Biochim. Biophys. Acta 593, 17-23.
[17] Thörnström, P.-E., Soussi, B., Arvidsson, L. and Malmström, B.G. (1984) Chem. Scr. 24, 230-235.
[18] Jones, M.G., Bickar, D., Wilson, M.T., Brunori, M., Colosimo, A. and Sarti, P.(1984) Biochem. J. 220, 57-66.
[19] Jensen, P., Wilson, M.T., Aasa, R. and Malmström, B.G. (1984) Biochem. J. 224, 829-837.
[20] Malmström, B.G. (1985) Biochim. Biophys. Acta 811, 1-12.
[21] Koppenol, W.H. and Margoliash, E. (1982) J. Biol. Chem. 257, 4426-4437.
[22] Ainslie, G.R., Shill, J.P. and Neet, K.E. (1972) J. Biol. Chem. 247, 7088-7096.
[23] Tanford, C. (1983) Annu. Rev. Biochem. 52, 379-409.
[24] Skou, J.C. (1974) Biochim. Biophys. Acta 339, 246-257.
[25] Neet, K.E. and Green, N.M. (1977) Arch. Biochem. Biophys. 178, 588-597.
[26] Wikström, M., Krab, K. and Saraste, M. (1981) in: Cytochrome Oxidase, pp. 91-93, Academic Press, London.
[27] Peterson, L.C. and Andréasson, L.-E. (1976) FEBS Lett. 66, 52-57.