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Ferricytochrome *c* Induces Monophasic Kinetics of Ferrocytochrome *c* Oxidation in Cytochrome *c* Oxidase

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The kinetics of ferrocytochrome *c* oxidation by reconstituted cytochrome *c* oxidase (COX) from bovine heart was followed by a spectrophotometric method, using on-line data collection and subsequent calculation of reaction rates from a function fitted to the progress curve. When reaction rates were calculated at increasing reaction times, the multiphasic kinetics of ferrocytochrome *c* oxidation gradually changed into monophasic Michaelis–Menten kinetics. The same phenomenon was observed when ferrocytochrome *c* oxidation was followed in the presence of increasing amounts of ferricytochrome *c*. From these results we conclude that ferricytochrome *c* shifts the multiphasic kinetics of ferrocytochrome *c* oxidation by COX into monophasic kinetics, comparable to high ionic strength conditions. Furthermore, we show that ferricytochrome *c* inhibits the "high affinity phase" of ferrocytochrome *c* oxidation in an apparently competitive way, while inhibition of the "low affinity phase" is noncompetitive. These findings are consistent with a "regulatory site model" where both the catalytic and the regulatory site bind ferro- as well as ferricytochrome *c*.

KEY WORDS: Cytochrome *c* oxidase; multiphasic kinetics; Michaelis–Menten kinetics; ferricytochrome *c*; regulatory site model.

INTRODUCTION

The kinetics of cytochrome *c* oxidation by cytochrome *c* oxidase (COX)[3] is complex and variable, depending on the method of assay, i.e., the spectrophotometric or the polarographic method (Ferguson-Miller *et al.*, 1976, 1978; Smith *et al.*, 1979), on buffer composition (Wainio *et al.*, 1960; Wilms *et al.*, 1981; Brooks and Nicholls, 1982; Büge and Kadenbach, 1986; Reimann *et al.*, 1988; Kossekova *et al.*, 1989), and on the state of the enzyme complex, either soluble or membrane-bound (reconstituted) (Carroll and Racker, 1977). The kinetics of COX do not show a Michaelis–Menten behavior, but exhibit biphasic or multiphasic saturation curves (Smith and Conrad, 1956; Nicholls, 1965; Ferguson-Miller *et al.*, 1976). In order to explain the multiphasic behavior, different models have been proposed, assuming one (Antalis and Palmer, 1982) or two binding sites for cytochrome *c* (Errede *et al.*, 1976; Errede and Kamen, 1978; Ferguson-Miller *et al.*, 1976; Michel and Bosshard, 1989), negative cooperativity in a dimeric enzyme complex (Nalecz *et al.*, 1985), or conformational transitions (Brzezinski *et al.*, 1986). A critical discussion of the various models was recently given by Cooper (1990).

With most assays the substrate cytochrome *c* is kept fully reduced, i.e., in the polarographic assay with ascorbate/TMPD, or when initial rates are measured by the stopped-flow method. In the standard spectrophotometric assay, however, reaction rates are not obtained until a few seconds after start of the reaction, i.e., when considerable amounts of the product ferricytochrome *c* have already accumulated. In order to overcome graphical analysis and

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[3] Abbreviations: COX, cytochrome *c* oxidase (EC 1.9.3.1); CCCP, carbonylcyanide-*m*-chlorophenylhydrazone.

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to obtain initial reaction rates with the standard spectrophotometric assay, we investigated the kinetics of ferro-cytochrome *c* oxidation by reconstituted COX from bovine heart by using on-line data collection and subsequent calculation of the rates from fitted nonlinear functions. This allowed us to calculate rates at different times after start of the reaction and thus to determine actual velocities at various concentrations of the product ferricytochrome *c*. The multiphasic character of the kinetics strongly depended on the concentration of ferricytochrome *c*, leading to monophasic kinetics at higher ferricytochrome *c* concentrations. A model is suggested to explain the changes of the kinetics by ferricytochrome *c*.

MATERIALS AND METHODS

Asolectin (L-α-phoshatidylcholine, type II-s from soybean) and cytochrome *c* (type VI, from horse heart) were purchased from Sigma. Valinomycin and CCCP were obtained from Boehringer. Before use, asolectin was purified by the method of Kagawa and Racker (1971). Cytochrome *c* was reduced with sodium dithionite and separated by gel filtration on a Sephadex G-25 column (1 × 10 cm). When necessary, the commercial cytochrome *c* (6% reduced) was fully oxidized with $K_3(Fe(CN)_6]$ and purified by a similar procedure. The concentration was determined by using the absorption coefficient (reduced minus oxidized) of 21 $mM^{-1} cm^{-1}$ at 550 nm (Van Gelder and Slater, 1962). Mitochondria were prepared from bovine heart according to the method of Smith (1967), and COX was isolated by the Triton X-114/X-100 method as described by Kadenbach *et al.* (1986).

The enzyme was reconstituted in liposomes with asolectin (40 mg/ml) sonicated in 1.5% sodium cholate, 10 mM K-Hepes (pH 7.4), and 40 mM KCl to a final concentration of 3 μM (Hüther and Kadenbach, 1986). The detergent was removed by adsorption to purified Amberlite XAD-2 (Serva) (Shechter and Bloch, 1971), by incubation at 4°C overnight with the polymeric adsorbent at a batch concentration of 50 mg/ml. The orientation of the COX in the vesicles was calculated from the reduced spectrum obtained by impermeant (ascorbate and cytochrome *c*) and permeant (TMPD) reducing agents in the presence of cyanide as described by Casey *et al.* (1982). The average orientation was 65% cytochrome *c* binding site outside. The respiratory control ratio was measured polarographically in 40 mM KCl, 10 mM K-Hepes, pH 7.4, 30 μM EDTA, 25 mM K-ascorbate, 50 μM ferrocytochrome *c*, and 20 nM cytochrome aa_3 in the absence or presence of 1 μg/ml valinomycin and 3 μM CCCP. The proteoliposomes had a respiratory control ratio of 5 ± 1.

The polarographic measurements of COX activity were performed with a Clark-type electrode according to Ferguson-Miller *et al.* (1978) in 10 mM K-Hepes, pH 7.4, 40 mM KCl, 30 μM EDTA, 25 mM ascorbate, 20 nM reconstituted COX, and 0.02–50 μM cytochrome *c* in the presence of 1 μg/ml valinomycin and 3 μM CCCP at 25°C.

The spectrophotometric assay was performed according to Yonetani (1967) at 25°C and at a wavelength of 550 nm using an Uvikon 810 spectrophotometer (Kontron) interfaced to a computer. The assay was performed in 10 mM K-Hepes, pH 7.4, 40 mM KCl, and 0.3–80 μM ferrocytochrome *c* in the presence or absence of 1 μg/ml valinomycin and 3 μM CCCP. The reaction was started by addition of the reconstituted enzyme to a final concentration of 2.5–2.8 nM. For each run, 70 absorption values were sampled at 0.35 s intervals and stored in digital form. The function fitted to these data allowed extrapolation to zero reaction time and calculation of velocities at different times after start of the reaction, taking into account the corresponding ferrocytochrome *c* concentrations.

Reaction rates are presented as turnover number (TN = mol ferrocytochrome *c* oxidized × mol heme $aa_3^{-1} \times s^{-1}$). For the evaluation of K_M and TN_{max} values a single or a double Michaelis–Menten equation was fitted to the rate data as described by Michel and Bosshard (1989).

RESULTS

The oxidation of ferrocytochrome *c* by reconstituted COX from bovine heart was measured by the spectrophotometric method, using a mixed exponential function to fit the digitalized absorption versus time data. The calculation of reaction rates from the slope of fitted nonlinear progress curves has the advantage that rates can be obtained accurately at any point of the curve. As shown in Fig. 1, the absorption vs. time data were closely spaced and slightly curved. The applied mixed exponential function yielded excellent fits in comparison with the analogous recorded curves during the first 30 s of the reaction. For evaluation only data in that time range have

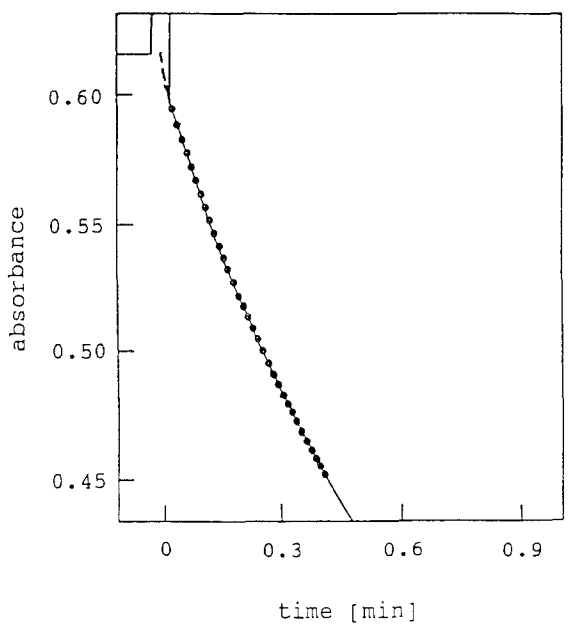

Fig. 1. Correspondence between recorded and computer-fitted curves of ferrocytochrome *c* absorbance during oxidation by reconstituted COX. The absorbance decrease of 20 μM ferrocytochrome *c* at 550 nm during oxidation by 2.5 nM reconstituted bovine heart COX in 10 mM K-Hepes, pH 7.4, 40 mM KCl in the presence of 1 μg/ml valinomycin and 3 μM CCCP was simultaneously monitored with a recorder and by digitalization. The points were calculated by fitting a mixed exponential function to the stored data. In the figure every second point is shown. The curved fit was calculated for the time range of 2.45–19.6 s and extrapolated to zero reaction time.

been used. True initial rates were calculated by numerical extrapolation of the functions to zero time.

In Fig. 2 reaction rates at various ferrocytochrome *c* concentrations are presented in an inverse Eadie–Hofstee plot. The rates were calculated from the fitted curves at 0, 2.5, and 12.5 s reaction times. At 0 s multiphasic kinetics of ferrocytochrome *c* oxidation is evident (Fig. 2A), but with increasing reaction time the fitted curve, calculated by use of a double Michaelis–Menten equation (Michel and Bosshard, 1989), approaches a straight line (Fig. 2C). A high-affinity and two low-affinity phases of ferrocytochrome *c* oxidation have already been distinguished by Garber and Margoliash (1990) for the polarographic assay. Sinjorgo *et al.* (1984) also mentioned three different K_M values for the spectrophotometric assay. It should be pointed out, however, that the high-affinity phase of the reaction could not be accurately assayed by the applied spectrophotometric method.

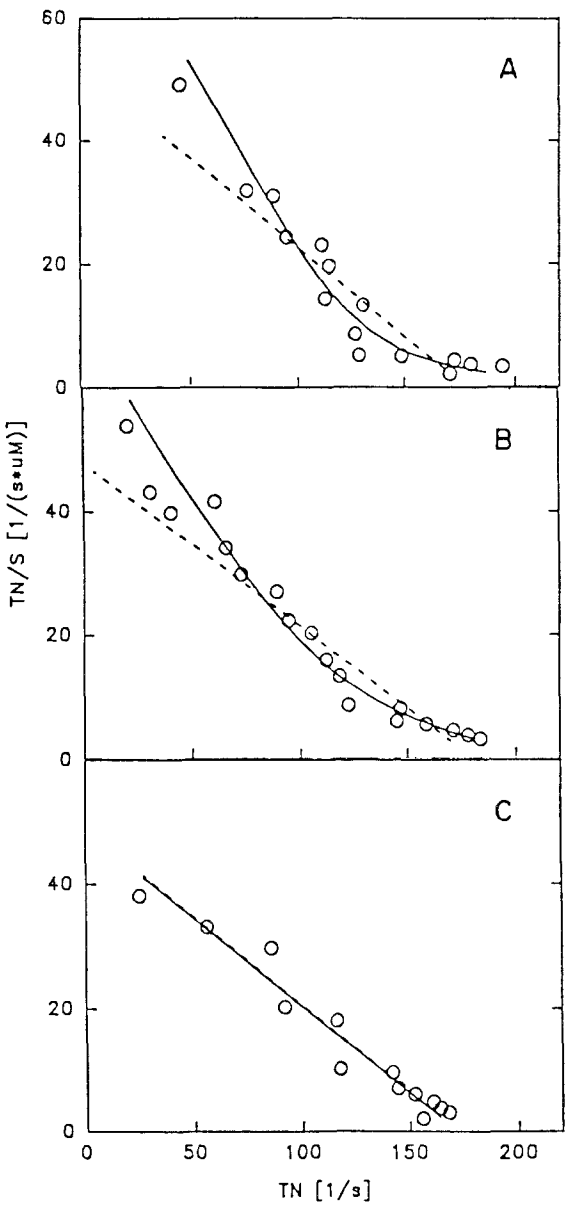

Fig. 2. Reversed Eadie–Hofstee plot of the kinetics of ferrocytochrome *c* oxidation by reconstituted COX calculated for various reaction times. The activity of 2.8 nM reconstituted COX was measured spectrophotometrically in 10 mM K-Hepes, pH 7.4, 40 mM KCl at 25°C in the presence of 1 μg/ml valinomycin and 3 μM CCCP with 1–60 μM ferrocytochrome *c*. The rate of oxidation was calculated for 0 s (A), 2.5 s (B), and 12.5 s (C) after reaction start from the derivatives of the fitted functions at the indicated times and from the actual ferrocytochrome *c* concentrations at that time. The curves were calculated by the computer from the individual points using either a double (solid line) or a single Michaelis–Menten equation (dashed line).

Table I. Kinetic Constants of Ferrocytochrome *c* Oxidation by Reconstituted COX Calculated for Various Reaction Times[a]

Michaelis–Menten equation	Time after reaction start (s)	K_{M1} (μM)	TN_{max1} (s^{-1})	K_{M2} (μM)	TN_{max2} (s^{-1})
Single	0.5	3.5	178		
	2.5	3.8	181		
	12.5	3.5	172		
Double	0.5	1.5	125	75.8	234
	2.5	1.6	104	25.0	217
	12.5	—	—	3.6	172

[a]The original data from Fig. 2 were used to calculate the kinetic constants using either a single or a double Michaelis–Menten equation.

In order to evaluate the K_M and V_{max} values, the data of the Eadie–Hostee plots were fitted to curves by use of a single as well as a double Michaelis–Menten equation. Clearly, at 0 s the best fit is obtained with the double Michaelis–Menten equation (Fig. 2A). while with increasing reaction time the inverse Eadie–Hofstee plot approaches a straight line (Fig. 2C). This linearization of reaction kinetics with increasing reaction time is also evident from K_M values calculated from fits of the double Michaelis–Menten equation (Table I).

In order to demonstrate that the linearization of the kinetics with increasing reaction time is due to increasing concentrations of ferricytochrome *c*, we have recalculated the data at different percentages of accumulated ferricytochrome *c*, and calculated K_M

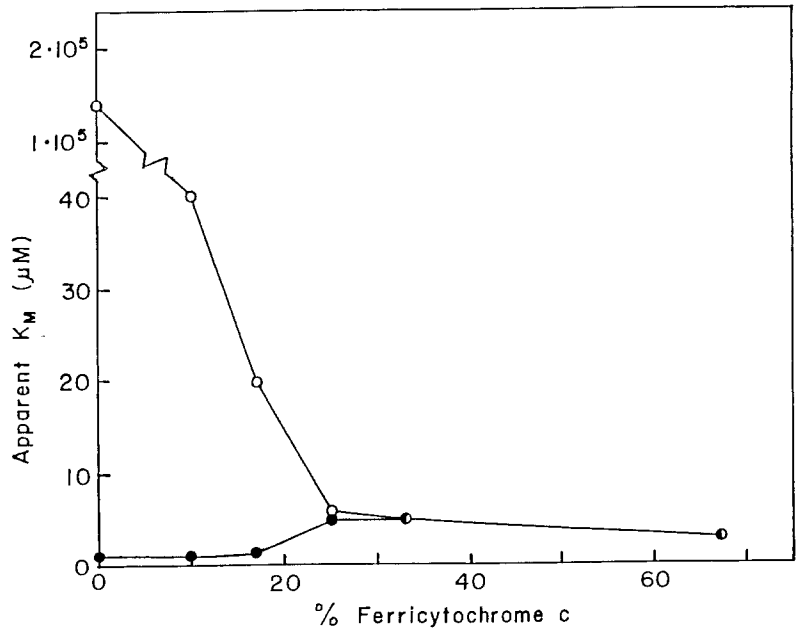

Fig. 3. Change of multiphasic into monophasic kinetics of ferrocytochrome *c* oxidation by reconstituted COX with increasing amounts of ferricytochrome *c*. The data of Fig. 2 were used to calculate rates at various ferrocytochrome *c* concentrations but at defined ferricytochrome *c*/ferrocytochrome *c* ratios (taken at various times after start of the reaction). For each ratio an Eadie–Hofstee plot was produced and the K_M values were calculated for the "low 1" (filled circles) and "low 2" phase (open circles) of ferrocytochrome *c* oxidation using the double Michaelis–Menten equation. The single K_M value at a ferri-/ferrocytochrome *c* ratio of 2/1 (67% added ferricytochrome *c*) was calculated from independent measurements performed in the presence of the corresponding amount of added ferricytochrome *c*.

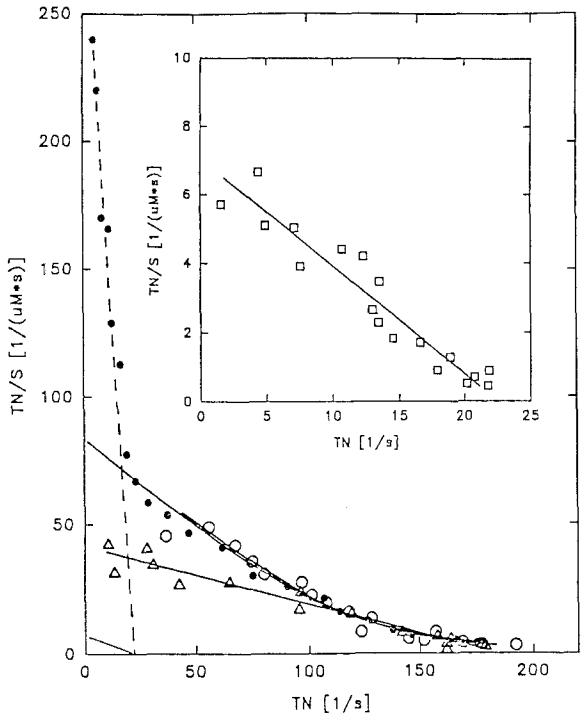

Fig. 4. Inhibition of ferrocytochrome *c* oxidation by ferricytochrome *c*. The Eadie–Hofstee plot of the inset shows data measured as described in the legend to Fig. 2, at a constant ferri-/ferrocytochrome *c* ratio = 2/1 (67% added ferricytochrome *c*), and calculated at 0 s reaction time. The data of the inset are also shown in the lower left corner of the figure (solid line without data points) and compared with data calculated for 1.5 s (open circles) and 7.5 s (triangles) after start of the reaction. Data points for the high-affinity phase of the reaction were obtained from a polarographic assay without TMPD (cloded circles) (see Materials and Methods). The curves were fitted by a double (main panel) or a single Michaelis–Menten equation (inset). The dashed line was drawn by hand.

values according to the double Michaelis–Menten equation. As shown in Fig. 3, the two different K_M values, corresponding to the "low 1" and "low 2 phase" in the paper of Garber and Margoliash (1990), approach a single value with increasing concentrations of ferricytochrome *c*. This is supported by the single K_M value obtained from independent measurements performed in the presence of 67% added ferricytochrome *c* and calculated at 0 s reaction time (see Figs. 3 and 4).

While the above data were measured with reconstituted COX in the presence of uncoupler (CCCP and valinomycin), we have also measured the kinetics in the absence of uncoupler and found the same change of the multiphasic into monophasic kinetics with increasing reaction time (not shown).

The assumption that ferricytochrome *c* accumulation is responsible for the change of multiphasic into monophasic kinetics is further verified by the separate experiment shown in the inset of Fig. 4. Here the kinetics of ferrocytochrome *c* oxidation was measured at a constant initial ratio of ferro-/ferricytochrome *c* = 1/2, with 67% added ferricytochrome *c* from the beginning. The curve of the inset in Fig. 4 is also shown in the lower left corner in Fig. 4 (without data points) and compared with data measured nearly in the absence of ferricytochrome *c*. Since our spectrophotometric method did not allow us to accurately determine the high-affinity phase of reaction, data from a polarographic assay, performed without TMPD in the presence of 25 mM ascorbate under otherwise identical conditions, were included in the plot (Fig. 4, filled circles). The spectrophotometric data were calculated for reaction times at 1.5 s (open circles), which correspond to about 5% ferricytochrome, also present in the polarographic assay (Ferguson-Miller *et al.*, 1978), and for reaction times at 7.5 s (triangles). From Fig. 4 three effects of ferricytochrome *c* on the oxidation of ferrocytochrome *c* by reconstituted COX can be seen: (i) Change of the multiphasic into monophasic kinetics; (ii) Competitive inhibition by ferricytochrome *c* in the high-affinity phase of ferrocytochrome *c* oxidation. The V_{max} of the reaction in the presence of excess ferricytochrome *c* equals the V_{max} of the high-affinity phase of ferrocytochrome *c* oxidation in the absence of ferricytochrome *c*, while the apparent K_M is increased. (iii) Predominantly noncompetitive inhibition of the low-affinity phase of ferrocytochrome *c* oxidation by ferricytochrome *c* with significantly decreasing V_{max}.

DISCUSSION

The kinetics of COX is usually measured in the absence of the reaction product ferricytochrome *c*. This holds for both the polarographic assay under steady-state conditions, where cytochrome *c* is largely reduced (95% in our assay) (Van Buuren *et al.*, 1971; Ferguson-Miller *et al.*, 1978) and for the spectrophotometric assay using the stopped-flow method, where true initial rates are measured (Veerman *et al.*, 1980; Sinjorgo *et al.*, 1984; Brunori *et al.*, 1986; Brzezinski *et al.*, 1986). Under these conditions multiphasic kinetics of ferrocytochrome *c* oxidation are observed under low ionic strength conditions.

The computer-aided collection and processing of absorption vs. time data allowed us to determine the rates of ferrocytochrome *c* oxidation at any time from reaction start up to about 30 s with high accuracy. When apparent K_M values at the low 1 and low 2 phase were calculated for different times after start of the reaction, a continuous change into monophasic kinetics was found with increasing reaction time. These values correlated well with data obtained at 0 s reaction time in the presence of a defined concentration of the product ferricytochrome *c* (Fig. 3). This result explains previous data, where monophasic kinetics of ferrocytochrome oxidation by reconstituted COX were found with the standard spectrophotometric assay (Hüther and Kadenbach, 1986, 1987, 1988; Hüther *et al.*, 1988). In these studies calculations could only be done a few seconds after start of the reaction.

Concerning the high-affinity phase of ferrocytochrome *c* oxidation, the inhibition by ferricytochrome *c* is predominantly competitive, i.e., the maximal turnover numbers TN_{max} are comparable in the near absence and with increasing concentrations of the inhibitor (Fig. 4). Competitive inhibition of ferrocytochrome *c* oxidation by ferricytochrome *c* has been described by Minnaert (1961) and Yonetani and Ray (1965). At that time, however, the multiphasic kinetics of COX, discovered in 1976 (Errede *et al.*, 1976; Ferguson-Miller *et al.*, 1976), was unknown.

Our results are in accordance with the hypothesis of two different binding sites for cytochrome *c* on the enzyme complex, a catalytic and a regulatory one (see Garber and Margoliash, 1990). We suggest that both can bind ferro- as well as ferricytochrome *c*, however, with different relative affinities (see the model in Fig. 5). We assume that the catalytic binding site has a high affinity to both reduced and oxidized cytochrome *c*. This is supported by the competitive inhibition of ferrocytochrome *c* oxidation (Fig. 4). Binding of a second molecule of ferrocytochrome *c* to COX at the regulatory site may induce, either via a conformational change or by charge repulsion, a decreased affinity of the catalytic site for reduced cytochrome *c*, leading to an increased rate of dissociation of substrate and product. Such an interaction could explain the increase of both K_M and TN_{max} with increasing ferrocytochrome *c* concentrations. At higher concentrations, ferricytochrome *c* may be capable of efficiently competing with reduced cytochrome *c* at the regulatory site. This would counteract the allosteric effect of ferrocytochrome *c* on the catalytic site and thus abolish multiphasic behavior. As actually observed (Fig. 4), such an effect of ferricytochrome *c* would result in a noncompetitive type of inhibition of ferricytochrome *c* under these conditions, besides the well-established competitive inhibition by ferricytochrome *c* at the catalytic site.

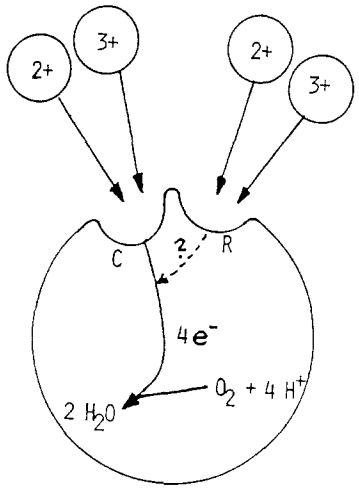

Fig. 5. Model of the interaction of COX with cytochrome *c*. The model suggests two binding sites for cytochrome *c* at COX, a catalytic (c) and a regulatory (r) site. Both sites are assumed to bind ferro- and ferricytochrome *c*, but the affinity for both forms is higher at the catalytic site. The effect of bound cytochrome *c* at the regulatory site on the kinetics of ferrocytochrome *c* oxidation at the catalytic site could occur either via electrostatic repulsion or attraction, or via conformational change by influencing the subsequent electron transfer steps (dotted arrow with question mark).

Our model could also explain the monophasic kinetics of COX observed at high ionic strength (Ferguson-Miller *et al.*, 1976; Brooks and Nicholls, 1982; Sinjorgo *et al.*, 1986). High salt concentrations will compete with the ionic interaction of the relatively weakly bound ferrocytochrome *c* at the regulatory binding site of COX, thus eliminating the effects of ferricytochrome *c* on the K_M for ferrocytochrome *c* with respect to the catalytic site.

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REFERENCES

Antalis, T. M., and Palmer, G. (1982). *J. Biol. Chem.* **257**, 6194–6206.

Brooks, S. P. J., and Nicholls, P. (1982). *Biochim. Biophys. Acta* **680**, 33–43.
Brunori, M., Sarti, P., Antonini, G., and Malatesta, F. (1986). *Bioelectrochem. Bioenerg.* **16**, 159–165.
Brzezinski, P., Thörnström, P.-E., and Malmström, B. (1986). *FEBS Lett.* **194**, 1–5.
Büge, U., and Kadenbach, B. (1986). *Eur. J. Biochem.* **161**, 383–390.
Carroll, R. C., and Racker, E. (1977). *J. Biol. Chem.* **252**, 6981–6990.
Casey, R. P., Ariano, B. H., and Azzi, A. (1982). *Eur. J. Biochem.* **122**, 313–318.
Cooper, C. E. (1990). *Biochim. Biophys. Acta* **1017**, 187–203.
Errede, B., and Kamen, M. D. (1978). *Biochemistry* **17**, 1015–1027.
Errede, B., Haight, G. P., and Kamen, M. D. (1976). *Proc. Natl. Acad. Sci. USA* **73**, 113–117.
Ferguson-Miller, S., Brautigan, D. L., and Margoliash, E. (1976). *J. Biol. Chem.* **251**, 1104–1115.
Ferguson-Miller, S., Brautigan, D. L., and Margoliash, E. (1978). *J. Biol. Chem.* **253**, 149–159.
Garber, E. A. E., and Margoliash, E. (1990). *Biochim. Biophys. Acta* **1015**, 279–287.
Hüther, F.-J., and Kadenbach, B. (1986). *FEBS Lett.* **207**, 89–94.
Hüther, F.-J., and Kadenbach, B. (1987). *Biochem. Biophys. Res. Commun.* **147**, 1268–1275.
Hüther, F.-J., and Kadenbach, B. (1988). *Biochem. Biophys. Res. Commun.* **153**, 525–534.
Hüther, F.-J., Berden, J., and Kadenbach, B. (1988). *J. Bioenerg. Biomembr.* **20**, 503–516.
Kadenbach, B., Stroh, A., Ungibauer, M., Kuhn-Nentwig, L., Büge, U., and Jarausch, J. (1986). *Methods Enzymol.* **126**, 32–45.
Kagawa, Y., and Racker, E. (1971). *J. Biol. Chem.* **256**, 5477–5487.
Kossekova, G., Atanasov, B., Bolli, R., and Azzi, A. (1989). *Biochem. J.* **262**, 591–596.
Michel, B., and Bosshard, H. R. (1989). *Biochemistry* **28**, 244–252.
Minnaert, K. (1961). *Biochim. Biophys. Acta* **50**, 23–34.
Nalecz, K. A., Bolli, R., Ludwig, B., and Azzi, A. (1985). *Biochim. Biophys. Acta* **808**, 259–272.
Nicholls, P. (1965). In *Oxidases and Related Redox Systems*, Vol. IV (King, T. E., Mason, H. S., and Morrison, M., eds.), Wiley, New York, pp. 764–777.
Reimann, A., Hüther, F. J., Berden, J. A., and Kadenbach, B. (1988). *Biochem. J.* **254**, 723–730.
Schechter, J., and Bloch, K. (1971). *J. Biol. Chem.* **246**, 7690–7696.
Sinjorgo, K. M. C., Meijling, J. H., and Muijsers, A. O. (1984). *Biochim. Biophys. Acta* **7676**, 48–56.
Sinjorgo, K. M. C., Steinbach, O. M., Dekker, H. L., and Muijsers, A. O. (1986). *Biochim. Biophys. Acta* **850**, 108–115.
Smith, A. L. (1967). *Methods Enzymol.* **10**, 81–86.
Smith, L., and Conrad, H. (1956). *Arch. Biochem. Biophys.* **63**, 403–413.
Smith, L., Davies, H. C., and Nava, M. E. (1979). *Biochemistry* **18**, 3140–3146.
Van Buuren, K. H. J., Van Gelder, B. F., and Eggelte, T. A. (1971). *Biochim. Biophys. Acta* **234**, 468–480.
Van Gelder, B. F., and Slater, E. C. (1962). *Biochim. Biophys. Acta* **58**, 593–595.
Veerman, E. C. I., Wilms, J., Casteleijn, G., and Van Gelder, B. (1980). *Biochim. Biophys. Acta* **590**, 117–127.
Wainio, W. W., Eichel, B., and Gould, A. (1960). *J. Biol. Chem.* **235**, 1521–1525.
Wilms, J., Veerman, E. C. I., König, B. W., Dekker, H. L., and Van Gelder, B. F. (1981). *Biochim. Biophys. Acta* **635**, 13–24.
Yonetani, T. (1967). *Methods Enzymol.* **10**, 332–335.
Yonetani, T., and Ray, G. S. (1965). *J. Biol. Chem.* **240**, 3392–3398.