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KINETICS OF CYTOCHROME *c* OXIDASE FROM YEAST

MEMBRANE-FACILITATED ELECTROSTATIC BINDING OF CYTOCHROME *c* SHOWING A SPECIFIC INTERACTION WITH CYTOCHROME *c* OXIDASE AND INHIBITION BY ATP

With an appendix on the occurrence of two-dimensional diffusion of cytochrome *c* on the membrane surface

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SUMMARY

The steady-state kinetics of purified yeast cytochrome *c* oxidase were investigated at low ionic strength where the electrostatic interaction with cytochrome *c* is maximized. In 10 mM cacodylate/Tris (pH 6.5) the oxidation kinetics of yeast *iso*-1-cytochrome *c* were sigmoidal with a Hill coefficient of 2.35, suggesting cooperative binding. The half-saturation point was 1.14 μM. Horse cytochrome *c* exhibited Michaelis-Menten kinetics with a higher affinity ($K_m = 0.35$ μM) and a 100 % higher maximal velocity.

In 67 mM phosphate the Hill coefficient for yeast cytochrome *c* decreased to 1.42, and the species differences in Hill coefficients were lessened. Under the latter conditions, a yeast enzyme preparation partially depleted of phospholipids was activated on addition of diphosphatidylglycerol liposomes. When the enzyme was incorporated into sonicated yeast promitochondrial particles the apparent K_m for horse cytochrome *c* was considerably lower than the value for the isolated enzyme.

ATP was found to inhibit both the isolated oxidase and the membrane-bound enzyme. With the isolated enzyme in 10 mM cacodylate/Tris, 3 mM ATP increased the half-saturation point with yeast cytochrome *c* 3-fold, without altering the maximal velocity or the Hill coefficient. 67 mM phosphate abolished the inhibition of the isolated oxidase by ATP.

The increase in affinity for cytochrome *c* produced by binding the oxidase to the membrane was not observed in the presence of 3 mM ATP, with the result that the

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Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-1,4-phenylenediamine; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

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membrane-bound enzyme was more sensitive to inhibition by ATP. ADP was a less effective inhibitor than ATP, and did not prevent the inhibition by ATP.

It is proposed that non-specific electrostatic binding of cytochrome *c* to phospholipid membranes, followed by rapid lateral diffusion, is responsible for the dependence of the affinity on the amount and nature of the phospholipids and on the ionic strength.

ATP may interfere with the membrane-facilitated binding of cytochrome *c* by a specific electrostatic interaction with the membrane or by binding to cytochrome *c*.

INTRODUCTION

Cytochrome *c* is a basic, positively charged protein at neutral pH, with an isoionic point at 10.04 [1]. Its binding to cytochrome *c* oxidase is weakened by high ionic strengths [2] and is therefore electrostatic in nature. Similarly, electrostatic forces seem to be involved in the binding of cytochrome *c* to the mitochondrial inner membrane [3, 4], and to phospholipid vesicles [5]. The surface of many biological membranes carries a negative electrostatic potential (e.g. refs. 6 and 7) owing to the ionized phosphate groups of the phospholipids, and its effects on the kinetics of membrane-bound enzymes has been the subject of considerable study [8–11]. The activity of some purified cytochrome *c* oxidase preparations is enhanced by the addition of phospholipids [12–15], which raises the question of the role of phospholipid membranes in the mechanism of this enzyme.

At neutral pH the overall charge of cytochrome *c* varies considerably from one species to another [1] despite the similarity of the isoionic points. For example, compared with horse cytochrome *c*, yeast *iso*-1-cytochrome *c* has about 0.5 unit more positive charge overall [16], and an extra lysine residue in position 11 on the basic side of the molecule which is likely to be the side which interacts with the oxidase [17, 18]. Although the catalytic activity of cytochrome *c* oxidase is apparently identical towards all species of cytochrome *c* [19], chemical modification of certain amino acid residues leads to changes in the catalytic or binding properties [20–24]. Therefore cytochrome *c* oxidase may be sensitive to the different net charges and charge distributions of cytochrome *c* from different species, if the ionic strength is lowered to increase the electrostatic interactions.

Here we describe studies of the kinetics of yeast cytochrome *c* oxidase which show differences between cytochrome *c* from horse and yeast. The effects of ionic strength and of the addition of phospholipid membranes indicate that the binding of cytochrome *c* is facilitated by an electrostatic interaction with the membrane. Adenine nucleotides were found to influence the kinetics of cytochrome *c* oxidase both bound to the membrane and isolated as a lipoprotein. Experiments are described which show that ATP may affect the rate of respiration by interfering in the electrostatic binding of cytochrome *c* to the membrane-bound oxidase.

The results corroborate a recent report that beef heart cytochrome *c* oxidase also exhibits differences between various species of cytochrome *c* and is inhibited by ATP [25], but suggest that the yeast and beef oxidases are kinetically different. A preliminary account of this work has been presented elsewhere [26].

MATERIALS AND METHODS

Materials

Horse heart cytochrome *c*, ATP, ADP, FCCP and Precinorm 'S' were obtained from Boehringer Mannheim GmbH, phosphatidylethanolamine (synthetic), and diphosphatidylglycerol (bovine heart) from Koch-Light Laboratories Ltd., (Colnbrook, U.K.), phosphatidylcholine (egg) from Nutritional Biochemicals Corp. (Cleveland, Ohio), and bovine serum albumin from Behringwerke AG (Marburg). Yeast extract was supplied by Difco laboratories (Detroit), oligomycin, Tween 20 and Tween 80 by Serva (Heidelberg), TMPD by Sigma Chemie GmbH (München), and hydroxydimethylarsineoxide (cacodylic acid) by J. T. Baker (Groß Gerau, Germany). Sephadex was from Pharmacia (Uppsala). Highly purified nitrogen ($\leqslant$ 5 ppm O_2 by volume) was supplied by Messer-Griesheim. All other salts and reagents were purchased from E. Merck AG (Darmstadt), and were of "analytical" or "biochemical purposes" grade.

Cholic acid was recrystallized before use [27]. ADP and ATP were made up as 22.5 or 30 mM solutions neutralized to pH 6.5 with KOH. Tetrasodium pyrophosphate (30 mM) was neutralized to pH 6.5 with HCl and therefore contained 33.8 mM NaCl. FCCP was added as a 0.2 mM solution in water containing 5.4 mg/ml Tween 20. Oligomycin was dissolved in ethanol to a concentration of 6.6 mg/ml.

Methods

(*a*) *Yeast culture.* For most of this work, *Saccharomyces cerevisiae* strain YF was used. It was grown in a medium containing KH_2PO_4 (7.4 mM), $(NH_4)_2SO_4$ (9.1 mM), $MgSO_4$ (2.8 mM), NaCl (8.5 mM), $CaCl_2$ (3.6 mM), $FeCl_3$ (0.03 mM) and Difco Yeast Extract (1.25 g/l) at pH 4.5, with 2 % (v/v) ethanol as carbon source. For the preparation of cytochrome *c* oxidase, commercial bakers' yeast cake was sometimes used, after derepression [28].

(*b*) *Isolation of cytochrome c oxidase.* The washed cells were suspended in homogenization buffer (0.65 M sorbitol, 10 mM Tricine, 0.1 mM EDTA and 1 g/l bovine serum albumin, neutralized to pH 6.5 with Tris base) and homogenized by shaking with glass beads (diameter 0.45–0.50 mm) in a cell mill (Edmund Bühler, Tübingen) fitted with a continuous-flow head. The mitochondria were finally sedimented from a 2500 $\times g$ supernatant by centrifugation at 10 000 rev./min for 40 min (Sorvall GSA rotor at 0 °C) and disrupted with a Branson Sonic Power sonifier for 60 s at 0 °C at a protein concentration of 20 mg/ml in homogenization buffer. The submitochondrial particles were sedimented from a 30 000 $\times g$ supernatant by centrifugation at 50 000 rev./min for 30 min (Spinco rotor No. 50Ti at 0 °C).

Except where otherwise indicated, cytochrome *c* oxidase was isolated from the submitochondrial particles by the method of Sekuzu et al. [29] and was obtained as a green-brown oily floating layer at the stage of 25 % saturation of $(NH_4)_2SO_4$. It was washed several times with 0.2 % (w/v) cholate solution (pH 6.5) containing 35 % saturation of $(NH_4)_2SO_4$ to remove traces of cytochrome c_1 [29], and dissolved in 0.25 M sucrose containing 10 mM K_2HPO_4 and 1 mM EDTA. No loss of activity was detected during storage for several months at −20 °C, provided that thawing and refreezing were avoided. Any turbidity (owing to ammonium cholate) could be removed by low-speed centrifugation.

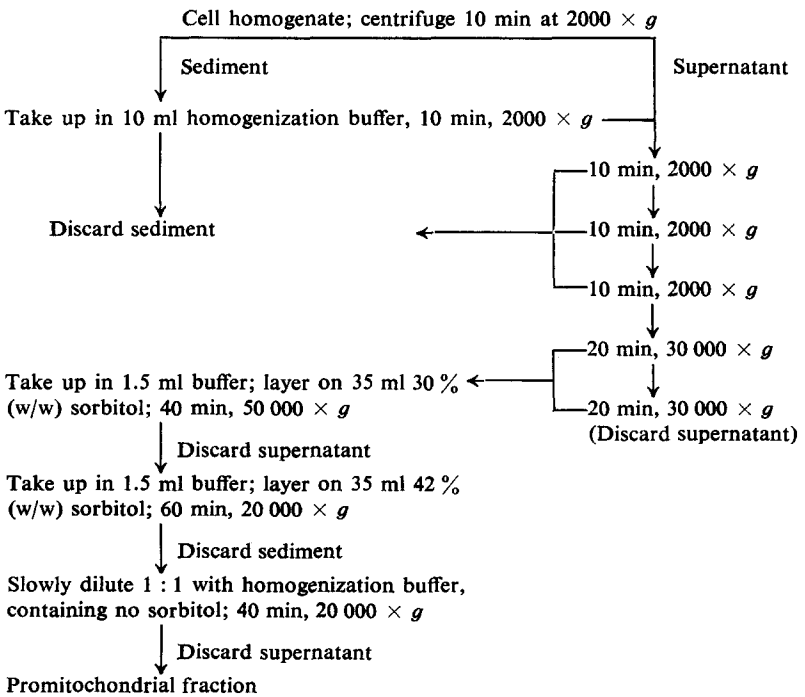
A preparation of cytochrome *c* oxidase containing less phospholipid was made according to the method of Tzagoloff [14] from a crude cytochrome oxidase fraction kindly supplied by Dr. J. Reed.

(*c*) *Isolation of cytochrome c.* Yeast *iso*-1-cytochrome *c* was isolated as described by Sherman et al. [30]. It was dialyzed overnight against 10 mM NH_4HCO_3 solution and then against water, and freeze-dried.

(*d*) *Isolation of promitochondria* [31]. Yeast was cultured anaerobically on the culture medium described above except that the yeast extract concentration was 2.5 g/l, the carbon source was glucose (100 g/l) and Tween 80 (2.5 ml/l) and ergosterin (50 mg/l) were added. At the end of the log phase the cells were harvested under highly purified nitrogen, washed with water, and mechanically disrupted in homogenization buffer as described above. Promitochondria were isolated by differential density centrifugation, which is summarized in Scheme I.

(*e*) *Membrane preparations.* Liposomes were prepared by sonicating suspensions of purified or mixed phospholipids (8 mg/ml) in 10 mM phosphate buffer (pH 6.5) containing 1 mM EDTA, for two 5-min periods at 0 °C or below.

Promitochondrial particles were prepared by sonication of a suspension of promitochondria (20 mg/ml) in 2-(*N*-morpholino)-ethane sulphonate buffer (6 mM, pH 6.5) containing 0.65 M sorbitol, 1 mM EDTA, 1 mg/ml bovine serum albumin and 2 mM $MgCl_2$, for two 30-s periods. After a preliminary centrifugation for 15 min at 15 000 rev./min (Sorvall SS34 rotor) the particles were collected by centrifugation for 30 min at 50 000 rev./min (Spinco rotor No. 50Ti) and were resuspended in buffer.



Scheme I. Centrifugation steps in the isolation of promitochondria from homogenized yeast cells.

For the cytochrome *c* binding studies, the particles were diluted to about 4 mg protein/ml and various concentrations of cytochrome *c* were added. After 15 min at 0 °C, they were recentrifuged as above. The sides of the tube and the surface of the sediment were gently washed with buffer and the sediment was resuspended in buffer. Cytochrome *c* concentrations in the supernatant and particles were determined by a micromethod [32] using 60 mM HCl to give better reproducibility of extraction of cytochrome *c*. Bound cytochrome *c* was extracted with KCl [33], and with cholate+KCl [34] by the published procedures.

Cytochrome *c* oxidase was incorporated into promitochondrial particles by ultrasonically dispersing, for two 30-s periods at 0 °C, the following mixture: promitochondria (40 mg protein), cytochrome *c* oxidase (0.78 mg protein; approx. 4 nmol haem *a*), and phospholipid vesicles (6.6 μmol phosphorus comprising phosphatidylcholine, phosphatidylethanolamine and diphosphatidylglycerol (weight ratio 10 : 10 : 1) previously sonicated together) in 2 ml of the homogenization buffer. The particles were isolated as above for the cytochrome *c* binding experiment and were assayed spectrophotometrically for oxidase activity.

(*f*) *Analytical procedures.* The concentrations of cytochromes aa_3 and *c* were estimated using the reduced-minus-oxidised difference extinction coefficients $\Delta\varepsilon$ (605 nm) = 24 $mM^{-1} \cdot cm^{-1}$ [35] and $\Delta\varepsilon$(550 nm) = 21.1 $mM^{-1} \cdot cm^{-1}$, respectively. Protein concentrations were determined at 620 nm with the Folin-Ciocalteu reagent [36] in the presence of 0.33 % (w/v) deoxycholate, 2 % (w/v) Na_2CO_3 and 0.1 M NaOH ($CuSO_4$ and tartrate were omitted) [31] using Precinorm S as a standard.

The polarographic assay of cytochrome *c* oxidase activity was carried out at 25 °C using a Beckman oxygen electrode in a perspex thermostatted chamber containing cytochrome *c*, 667 μM TMPD, 6.67 mM ascorbate, 250 mM sucrose, and buffer (either 10 mM cacodylate, or 2-(*N*-morpholino)-ethane sulphonate (both neutralized to pH 6.5 with Tris base), or 67 mM phosphate (pH 6.5)). The observed rate was corrected for the small background rate of TMPD oxidation in the absence of cytochrome *c*. The output was converted to oxygen concentrations assuming that the buffer was saturated with 240 μM O_2.

The spectrophotometric assay was carried out at 550 nm in 2 ml of phosphate buffer (67 mM, pH 6.5) containing reduced cytochrome *c* [37]. The enzyme was prediluted in 250 mM sucrose containing 10 mM K_2HPO_4, 1 mM EDTA and 4.5 mg/ml Tween 20. At the end of each run, $K_3Fe(CN)_6$ was added to determine the endpoint of the reaction, A_∞, and the pseudo first-order rate constant, *k*, was calculated from the slope of a semilogarithmic plot. The initial velocity, *v*, was calculated from the total cytochrome *c* concentration, c_s, according to $v = k \cdot c_s$ and since $v/c_s = V/K_m - v/K_m$ a plot of *k* against *v* has a slope of $-1/K_m$ and intercept on the abscissa of *V*.

In order to evaluate the kinetic parameters of sigmoidal curves without making any assumptions about the appropriate model, a computer program was used to fit the data by a least squares method [38].

RESULTS

Kinetic studies at low ionic strength

The K_m of cytochrome *c* oxidase for cytochrome *c* decreases with decreasing buffer concentration, and is less than 1 μM at zero ionic strength. The oxygen electrode

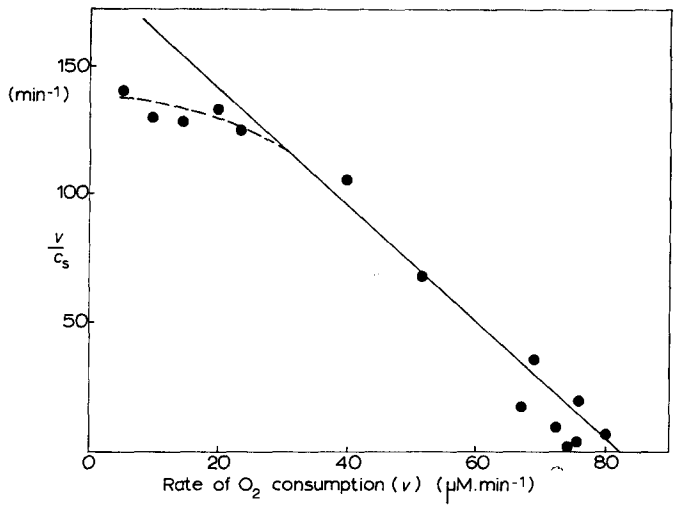


Fig. 1. Eadie-Hofstee plot for horse cytochrome *c*. The rate of oxygen consumption was measured as described under Methods in the presence of purified yeast cytochrome *c* oxidase (final protein concentration 0.114 mg/ml; 26 nM cytochrome aa_3) and 0.038–37.6 μM horse cytochrome.*c* in 10 mM cacodylate/Tris buffer (pH 6.50).

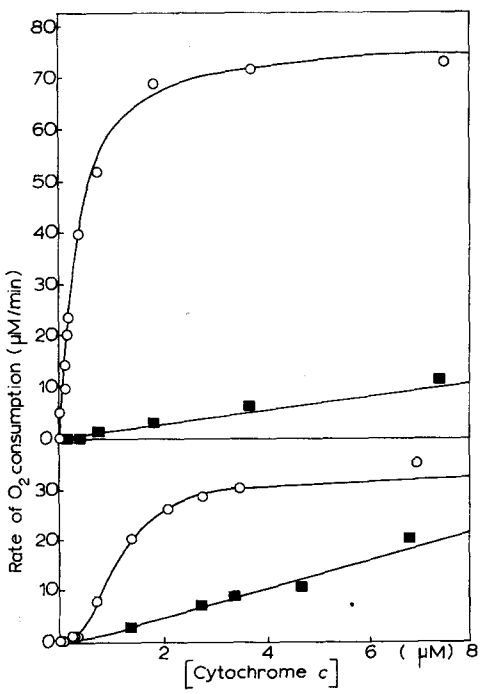


Fig. 2. Comparison of the activity of yeast cytochrome *c* oxidase using (a) horse heart cytochrome *c* and (b) yeast *iso*-1-cytochrome *c*, at low and high ionic strength. Conditions as in Fig. 1 in 10 mM cacodylate/Tris (○) or 67 mM phosphate (■) buffers at pH 6.5. The smooth curves were drawn from the kinetic parameters given in Table I.

TABLE I

KINETIC PARAMETERS FOR THE OXIDATION OF HORSE AND YEAST CYTOCHROME *c* BY YEAST CYTOCHROME *c* OXIDASE

A least-squares procedure was used to fit the data of Fig. 2 by means of the computer program of Wieker et al. [38]. The standard error is given as a percentage in parentheses.

Substrate species	Buffer	Hill coefficient (n_H)	$K_{0.5}$ (μM)	V (μmol/min)
Horse	10 mM cacodylate/Tris	1.22 (2.7)	0.35 (5.1)	75.7 (0.8)
Horse	67 mM phosphate	1.11 (3.0)	56.36 (20.0)	103.3 (19.8)
Yeast	10 mM cacodylate/Tris	2.35 (1.8)	1.14 (2.4)	32.3 (0.5)
Yeast	67 mM phosphate	1.42 (4.0)	10.57 (8.3)	52.2 (7.5)

was found to give the most accurate measurements of the reaction in this concentration range, provided that the activity was always high compared with the background rate in the absence of cytochrome *c*, and yet low enough to allow the cytochrome *c* to remain essentially completely reduced.

In 10 mM cacodylate/Tris buffer (pH 6.5; ionic strength = 4.1 mM) the apparent K_m for horse cytochrome *c* was within the range 0.35–0.57 μM. Fig. 1 is a plot of the results obtained with cytochrome *c* concentrations down to 0.038 μM. The slight convex curvature at low cytochrome *c* concentrations may be due to (a) inaccuracies in the measurement of these low rates, or (b) incomplete reduction of cytochrome *c* in this region where the oxidation is most rapid compared with the first-order reduction, or (c) substrate depletion, since the concentration of cytochrome aa_3 was about 0.02 μM.

In contrast with the hyperbolic kinetics for horse cytochrome *c* the kinetics with yeast *iso*-1-cytochrome *c* are sigmoidal (Fig. 2). The results of a computer fit of the data are given in Table I, which shows that the oxidation kinetics of yeast cytochrome *c* are cooperative, and have a 3-fold higher half-saturation point and a 50 % lower maximal velocity compared with horse cytochrome *c*. The Hill coefficent of 2.35 suggests that there are cooperative interactions between binding sites.

TABLE II

pH DEPENDENCE OF THE KINETICS OF CYTOCHROME *c* OXIDASE

Maximal velocities and K_m values for horse cytochrome *c* were determined from measurements of the rate of oxygen consumption in the presence of 0.114 mg/ml yeast cytochrome *c* oxidase, and 0.1–4.91 μM cytochrome *c* in a buffer formed from appropriate volumes of 10 mM Tris base and 10 mM 2-(*N*-morpholino)-ethane sulphonic acid. The values given are the mean $\pm$S.E. from linear regression analyses of Eadie-Hofstee plots with the indicated number of data points.

pH	K_m (μM)	V (μM/min)	No. of points
6.0	0.435$\pm$0.055	42.00$\pm$1.26	11
6.5	0.560$\pm$0.045	79.49$\pm$1.61	12
6.8	0.554$\pm$0.061	87.60$\pm$2.49	8
7.67	0.544$\pm$0.040	88.68$\pm$1.26	6

The kinetics do not vary significantly with pH within experimental error, between pH 6.5 and 7.67 (Table II). The maximal velocity begins to decrease below pH 6.5. The slightly lower K_m value at pH 6.0 (Table II) may be owing to the lower ionic strength of the buffer system, which decreases from 4.1 to 3.0 mM between pH 6.5 and 6.0.

The enzyme is strongly affected by the ionic strength. In 67 mM phosphate, which has an ionic strength of about 88 mM at pH 6.5, the maximal velocity is up to 60 % greater than under conditions of low ionic strength (Table I). This observation is in agreement with a previous finding that the activity is highest in 67 mM phosphate buffer at pH 6.5 [29]. However, under these conditions the K_m for yeast cytochrome *c* is an order of magnitude higher, and the effect on the K_m for horse cytochrome *c* is even larger, resulting in a decrease in the difference between them (Fig. 2). These conditions also decrease the sigmoidal character of the kinetics with yeast cytochrome *c*, as indicated by the decrease of the Hill coefficient to 1.42.

Effect of phospholipid membranes on kinetics at high ionic strength

It was found that the kinetics in 67 mM phosphate are dependent on the amount of phospholipid present. Cytochrome *c* oxidase with a lower phospholipid content (15–65 nmol phosphorous per mg protein) prepared according to Tzagoloff [14], showed a low affinity for cytochrome *c*. Two separate batches gave K_m values between 60 and 70 μM. As reported by Tzagoloff [14], the enzyme is activated by phospholipids. When diphosphatidylglycerol liposomes were added to the buffer used for predilution of the enzyme (Fig. 3, upper set of points) the apparent K_m was about 15 μM. This suggests that the affinity of the enzyme for cytochrome *c* increased up to

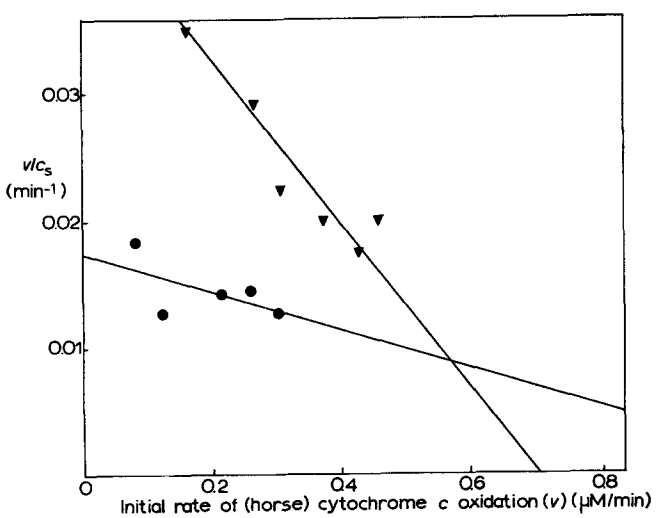


Fig. 3. Effect of diphosphatidylglycerol liposomes on cytochrome *c* oxidase kinetics in 67 mM phosphate buffer. Cytochrome *c* oxidase isolated by the method of Tzagoloff [14] was diluted to 81.8 μg/ml in 0.25 M sucrose containing 10 mM K_2HPO_4, 1 mM EDTA, 121 mM KCl and 4.5 mg/ml Tween 20 in the absence (●) or presence (▼) of diphosphatidylglycerol liposomes (see Methods) (0.1 mg phospholipid/mg protein). After 15 min at 0 °C, 10-μl aliquots were assayed spectrophotometrically in the presence of 5–24 μM cytochrome *c*.

TABLE III

THE EFFECT OF PHOSPHOLIPIDS ON THE ACTIVITY OF CYTOCHROME *c* OXIDASE

Cytochrome *c* oxidase (0.134 μg/ml) isolated according to Tzagoloff [14] was assayed spectrophotometrically as described under Methods in the presence of 24 μM horse cytochrome *c*. Where indicated, phospholipid micelles (see Methods) were added to the cuvette prior to the enzyme, to a final concentration of 20 mg phospholipid phosphorus per mg protein.

Phospholipid	Initial rate (μM/min)
None	0.67
Phosphatidylcholine	0.57
Phosphatidylethanolamine	0.50
Diphosphatidylglycerol	1.50

4-fold, although it is not excluded that the maximal velocity increased. Table III shows that the enzyme is activated by diphosphatidylglycerol liposomes, but not by neutral or zwitterionic lipids like phosphatidylcholine or phosphatidylethanolamine.

These results indicate that the activity of cytochrome *c* oxidase is dependent on the nature and extent of the phospholipid membranes present in the oxidase complex. This effect is further illustrated in studies of the interaction of cytochrome *c* and cytochrome *c* oxidase with promitochondrial membranes, which were taken as a model for

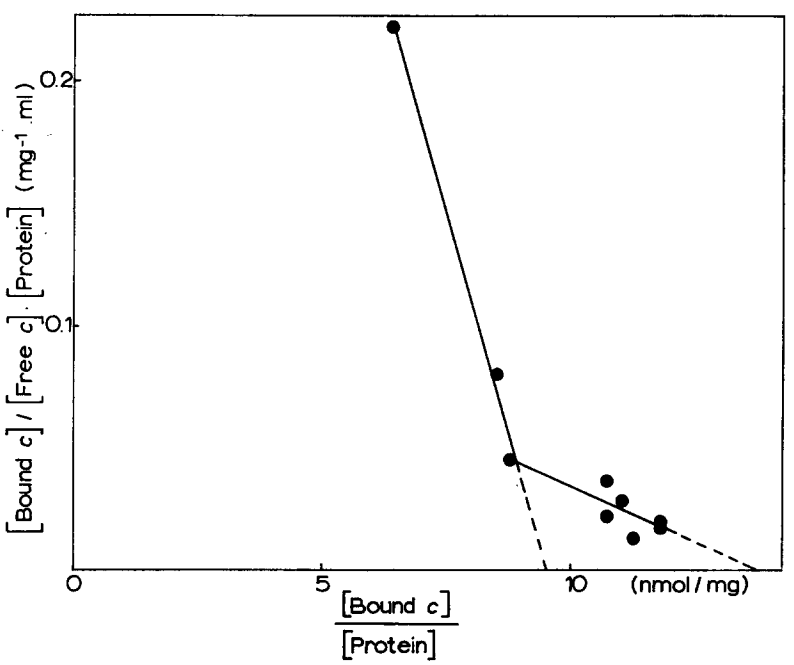


Fig. 4. Scatchard plot of the binding of cytochrome *c* to promitochondria. Sonicated promitochondrial particles were titrated with horse cytochrome *c* (total concentration 0.1–1.0 mM) as described under Methods, and the sedimented particles and supernatant were analyzed for cytochrome *c*.

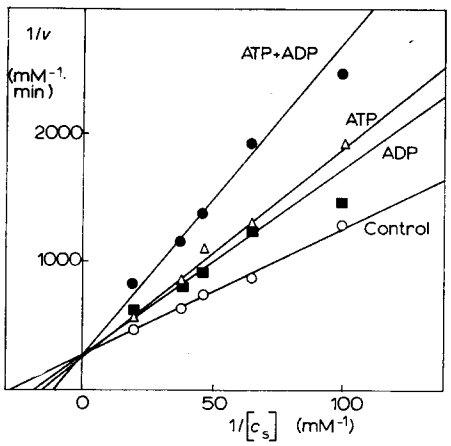


Fig. 5. The effects of ATP and ADP on the kinetics of cytochrome *c* oxidase incorporated into promitochondrial particles. The particles were assayed spectrophotometrically in 67 mM phosphate buffer (pH 6.5) in the presence of reduced horse heart cytochrome *c* (concentrations c_s = 10–50 μM) and the following additions: ○, none (control); △, 3 mM ATP; ■, 3 mM ADP; ●, 3 mM ATP+3 mM ADP.

the mitochondrial inner membrane, as they have a comparable lipid composition [39]. Cytochrome *c* was found to bind quite firmly to sonicated promitochondrial particles in amounts far in excess of that occurring in aerobic yeast mitochondria [40, 41]. A Scatchard plot (Fig. 4) shows that there are 9.5 nmol of binding sites per mg protein, with a dissociation constant of 13 μM, plus some weaker sites, perhaps owing to free cytochrome *c* trapped in the sediment. When the experiment was modified by adding cytochrome *c* before sonicating the promitochondria, the binding of cytochrome *c* was not increased, suggesting that closed vesicles were not formed in either of the experiments, or if vesicles were formed, cytochrome *c* bound only to the outside of them. Extraction with KCl [33] reduced the maximal amount of cytochrome *c* bound to the same value (about 0.5 nmol/mg) in both experiments. This would not be the case if the non-extractable cytochrome *c* was trapped inside closed vesicles only during sonication in the second experiment. Most of the remaining cytochrome *c* could be removed by the cholate-KCl treatment [34]. Similarly, vesicles impermeable to cytochrome *c* were not observed when promitochondria were subjected to a published procedure for the production of particles with cytochrome *c* either inside or outside [42].

An indication that non-specific binding to membranes may be important in the association of cytochrome *c* with the oxidase was obtained when the oxidase was incorporated into promitochondrial particles, as described under Methods. The yield of cytochrome *c* oxidase activity in such particles is about 30 % of the total activity present in the reconstitution mixture. (In the absence of added cytochrome *c* oxidase, the particles have only very slight activity). The apparent K_m for horse cytochrome *c* (Fig. 5, control experiment; K_m = 36 μM) was significantly less than the value for the isolated oxidase (Fig. 7, control experiment; K_m = 50 μM), suggesting that the promitochondrial membrane increases the affinity of the oxidase for cytochrome *c*.

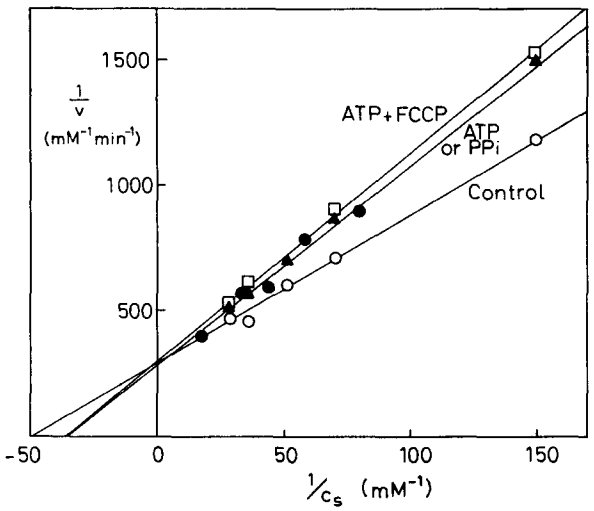


Fig. 6. The effects of pyrophosphate, ATP and uncoupler on cytochrome *c* oxidase activity of reconstituted particles. The assay conditions were as in Fig. 5 with the following additions: ○, none (control); ●, 3 mM pyrophosphate; ▲, 3 mM ATP; □, 3 mM ATP+1 μM FCCP.

Effect of ATP on cytochrome c oxidase kinetics

The kinetics of cytochrome *c* oxidase bound to promitochondrial particles were investigated in order to determine whether the enzyme interacted with the endogeneous ATPase. The oxidation of added reduced cytochrome *c* was competitively inhibited by ATP. 3 mM ATP approximately doubled the apparent K_m (from 36 to 63 μM) (Fig. 5). The inhibition by ATP was not significantly affected by the uncoupler FCCP (Fig. 6), nor by oligomycin at a concentration of 33 μg/ml which produced 85 % inhibition of the ATPase activity of the particles.

The effects of other polyvalent anions were also tested. Fig. 5 shows that ADP decreased the activity, but to a smaller extent than with the same concentration of ATP. When ATP and ADP were added together their effects were roughly additive. Pyrophosphate inhibited as effectively as ATP (Fig. 6). The inhibition by these phosphate compounds is related to the size of their negative charges, which are about 2.5, 2.5, and 3.5, for ADP, pyrophosphate and ATP, respectively, at pH 6.5 (calculated from the published dissociation constants [67, 68]). The greater inhibition by pyrophosphate than by ADP, despite their similar charges, is probably owing to the added ionic strength of the chloride with which it was neutralized (see Methods).

Fig. 7 shows that under the same conditions 3 mM ATP did not significantly affect the activity of the isolated enzyme. The same was found for ADP. Figs. 5 and 7 represent the same oxidase sample incorporated into promitochondrial particles and in the isolated state, respectively. A comparison of the lines shows that, on incorporating the oxidase into promitochondria, the K_m determined in the absence of ATP decreased from 50 to 36 μM. When assayed in the presence of 3 mM ATP, the K_m increased to 63 μM, suggesting that ATP prevents the increase in affinity of the enzyme caused by the presence of the promitochondrial membrane.

The effect of ATP on the isolated oxidase was investigated at low ionic strength

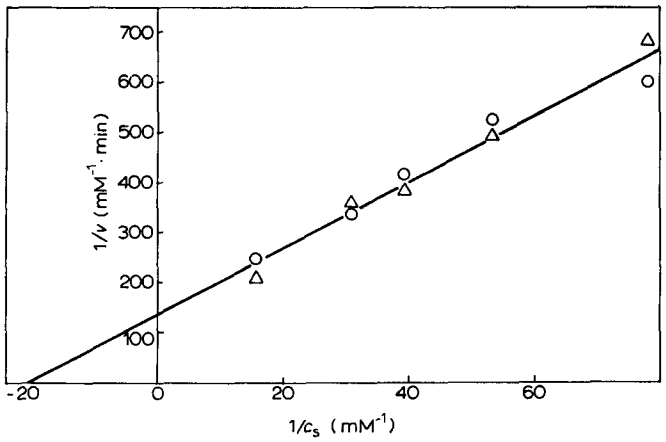


Fig. 7. The effects of ATP on the kinetics of isolated cytochrome *c* oxidase at high phosphate concentration. Cytochrome *c* oxidase isolated by the method of Sekuzu et al. [29] (6.24 μg protein per ml) was assayed as in Fig. 5 in the presence of the following: ○, no additions (control); △, 3 mM ATP.

in 10 mM cacodylate/Tris buffer. Under these conditions the enzyme's affinity for cytochrome *c* was considerably reduced by 3 mM ATP (Fig. 8), as indicated by the shift of the mid-points of the curves to higher cytochrome *c* concentrations (from 0.35 to 7.8 μM for horse cytochrome *c*, and from 1.14 to 3.59 μM for yeast *iso*-1-cytochrome *c*). The least-squares analysis of the data showed that the Hill coefficient was unaffected by 3 mM ATP. n_H was 2.29 (±0.09) and 2.35 (±0.04) (standard error of

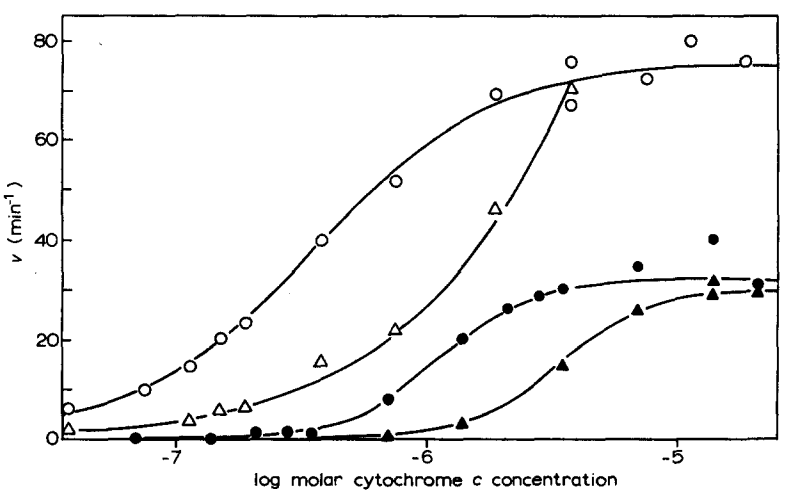


Fig. 8. The effects of ATP on the activity of cytochrome *c* oxidase at low buffer concentration. The assay method and conditions were as for Fig. 1. The smooth curves were drawn from the kinetic parameters determined according to ref. 38. The substrate was cytochrome *c* from horse heart (○, △) or yeast (●, ▲). ATP concentration was zero (○, ●) or 3 mM (△, ▲).

mean) in the presence and absence of ATP, respectively. This observation suggests that ATP specifically decreases the affinity for cytochrome *c* whereas merely increasing the ionic strength by the addition of phosphate decreases the sigmoidal character of the kinetics (Table I).

DISCUSSION

Until recently, any differences in the activity of cytochrome *c* oxidase towards cytochrome *c* from different species was attributed to very tightly bound but diffusible "inhibitory substances" [19] including buffer ions. The high affinity, specific binding site on beef heart cytochrome *c* oxidase reported recently [25] had not been detected previously because either the buffer concentration or the cytochrome *c* concentration, or both, were too high, and it was only when the kinetic measurements were performed at low ionic strength that species differences became apparent [25].

Under similar conditions it can now be shown that there are kinetic differences between the oxidation of horse and yeast cytochrome *c* by yeast cytochrome *c* oxidase. With both the beef heart and the yeast oxidases, the species differences are manifested in the affinity of the oxidase for the various cytochromes *c* (Fig. 2 and ref. 25). As the ionic strength is raised, the affinities and their differences decrease, pointing to the importance of the electrostatic interaction with the oxidase for the specificity. However, the high affinity phase of the kinetics reported for the beef heart oxidase [25] was not seen with the yeast oxidase. On the contrary, the kinetics of the yeast enzyme with yeast cytochrome *c* show an increasing affinity with increasing saturation of the enzyme, and with horse cytochrome *c* the kinetics are monophasic. Therefore species differences may have evolved in both cytochrome *c* and cytochrome *c* oxidase.

The high variability of the amino acid sequence of cytochrome *c* [16, 17] is largely confined to residue positions in the external part of the structure, where nearly all the charged residues are located. The basic residues are mostly grouped together at one side of the molecule, where the edge of the haem is exposed. This may be the pathway of electron transfer into and out of the molecule [43], and the positively charged side of the protein may therefore bind to the neighbouring electron transfer chain components [17].

In yeast cytochrome *c* a lysine is found in place of a valine in position 11 on this face of the molecule. Unexpectedly, this increase in the positive charge does not result in a higher affinity for the oxidase, but, as Table I shows, a 3-fold lower affinity relative to horse cytochrome *c*. A partial explanation of this is that the kinetics are sigmoidal, and the half-saturation point of the curve does not represent a true binding constant.

The sigmoidal kinetics could be accounted for by several mechanisms. (a) Non-productive binding of cytochrome *c* at low concentrations may occur, although it is unlikely that a sufficiently high concentration (about 1 μM), of specifically yeast cytochrome *c*, binds inactively to account for the "lag-phase" in Fig. 2b, where the cytochrome aa_3 concentration was about 0.02 μM. (b) Cytochrome *c* may bind to a second, activating, non-catalytic site [44] (for which there is no evidence, but which cannot be ruled out). (c) Cooperative interactions may occur between several oxidase molecules associated into oligomers [45] undergoing cluster formation (see below). A special case of this mechanism is where the R and T forms correspond to active mono-

mers and less active oligomers, respectively. Cooperative interactions between oxidase molecules may occur directly or via the phospholipids in which they are embedded. Purified oxidase preparations containing large quantities of phospholipids readily form membraneous vesicles in solution [47, 48].

The activity of cytochrome *c* oxidase depends on the amount of phospholipid present (Table III and ref. 13). It is known that cytochrome *c* binds to phospholipid membranes [5, 49–51] and that negatively charged phospholipids play an important role in the binding [5] which is dependent on pH and salt concentration [52]. This behaviour closely parallels that of cytochrome *c* oxidase in the presence of phospholipids. While drastic methods of extracting phospholipids have been reported to decrease both K_m and the maximal velocity [53], possibly reflecting fundamental changes in the oxidase structure on removal of phospholipids closely associated with it, in general K_m has been found to decrease on adding phospholipids. Diphosphatidylglycerol is a tightly bound, essential constituent of the enzyme [54], and is a more effective activator than neutral or zwitterionic phospholipids (Table III and refs. 12, 13 and 55). The fact that activation occurred even when the diphosphatidylglycerol was present only in the assay cuvette instead of during predilution of the enzyme, suggests that it is not caused by the phospholipid preventing the aggregation of the oxidase (cf. ref. 56), which should not depend on the charge on the phospholipid and which should affect the maximal velocity. Rather, negatively charged phospholipids may interact with cytochrome *c* to facilitate its binding to the oxidase in the phopholipid membrane.

The effect of the presence of a negatively charged surface on the kinetics of an enzyme bound to it can be described by the following relation [8, 10, 11]:

$$K'_m = K_m \exp(\psi ze/kT)$$

where K'_m and K_m are the Michaelis constants, measured from the bulk concentration and in the local environment of the enzyme, respectively; ψ is the average electrostatic potential prevailing in the neighbourhood of the membrane; z is the number of charges on the substrate molecule; and e is the charge of a proton. The K_m value for the isolated enzyme at high ionic strength ($K_m = 50$ μM approx.; Fig. 7) is the actual value of K_m when the electrostatic perturbations are minimal. Taking z to be 9.5 [16], it can be calculated that the K_m of 0.35 μM for horse cytochrome *c* at low ionic strength corresponds to $\psi = -13$ mV. Therefore the observed increase in affinity under these conditions can be accounted for by a relatively small electrostatic potential on the membrane. The negative electrostatic potential decreases the local pH [11], and since the electrostatic potential decreases as the ionic strength increases, this explains the decrease of the optimal pH observed with increasing ionic strength [57].

The mammalian mitochondrial membrane binds relatively large amounts of cytochrome *c* [58, 59] and the results of Fig. 4 show that yeast promitochondrial particles bind cytochrome *c* with a similar affinity. Our experiment did not cover the tight binding region observed by Erecińska [23], who reported that there were two binding sites per cytochrome aa_3 molecule, with a K_d of 0.02 μM.

Promitochondria also bind cytochrome *c* oxidase. From the turn-over number of the isolated oxidase it was calculated that the specific activity of the oxidase incorporated into promitochondria in Fig. 5 corresponds to about 0.02 nmol of cytochrome aa_3 per mg protein. Fig. 4 indicates that promitochondrial particles bind up to 500 times more cytochrome *c* than this. Under the conditions of the assay, a considerable

proportion of the cytochrome *c* would therefore be bound to the membrane, which should cause an increase in the apparent K_m if membrane-bound cytochrome *c* does not participate in the reaction. Since the K_m is lower than for the isolated oxidase, it is likely that non-specific binding to the membrane can lead to the formation of an active complex with the membrane-bound cytochrome *c* oxidase, and this may increase the affinity.

The occurrence of two-dimensional diffusion of cytochrome *c* on the surface of the membrane is analyzed in Appendix, and, as shown theoretically [60], can increase the association rate constant with the membrane-bound oxidase. This effect is enhanced by increasing the membrane area round each oxidase molecule, which explains the dependence of K_m on the concentration of phospholipids and an optimum area size should be expected.

A membrane-facilitated binding process could produce the observed cooperative kinetics of the isolated oxidase with yeast cytochrome *c*. A sigmoidal binding curve for cytochrome *c* and lecithin-diphosphatidylglycerol "liquid crystals" has already been reported [5]. The interaction of cytochrome *c* with phospholipids causes considerable structural changes [51, 61], which are reflected in altered properties such as the lipid transition temperature [62] and ionic permeability of phospholipid vesicles [63]. If the extra positive charges on yeast cytochrome *c* promoted such changes over a sufficiently large area, resulting in an increased affinity of the membrane for cytochrome *c*, or directly activating several oxidase molecules embedded in the membrane, then sigmoidal kinetics would be observed. A model where the membrane is broken up into relatively rigid plates or patches, within which long-range cooperative changes may occur [64], has been put forward recently [65].

Cytochrome *c* may be visualized as moving rapidly on the membrane surface between cytochrome b–c_1 and cytochrome aa_3 complexes as shown in Appendix. This idea is supported by the observation [66] that cytochrome *c* catalyzes the rapid equilibration of cytochrome aa_3 molecules with one another. This opens the possibility that cytochrome *c* oxidase could be regulated by altering the distribution of cytochrome *c* between the medium and the membrane surface.

The inhibition by ATP of membrane-bound cytochrome *c* oxidase but not of the isolated enzyme at high ionic strength, suggests a mediation by the promitochondrial membrane. That the oligomycin-sensitive ATPase is involved can be ruled out since the inhibitory effect of ATP is oligomycin insensitive, independent of energy coupling (Fig. 6), and not directly dependent on the ratio $[ATP]/[ADP] \cdot [P_i]$ (Fig. 5). The inhibition by anions increases with increasing charge, and polyvalent anions appear to be much stronger inhibitors than phosphate ions, which are essentially monovalent at pH 6.5.

The isolated oxidase is inhibited by ATP only at low ionic strength, showing that high ionic strength masks the effect of ATP, again suggesting that it is an electrostatic effect. ATP does not increase the maximal velocity with yeast cytochrome *c*, and it does not decrease the Hill coefficient, unlike the addition of high phosphate concentrations.

In view of the fact that ATP abolishes the activation of cytochrome *c* oxidase by promitochondrial membranes, it is likely that ATP prevents the attraction or the binding of cytochrome *c* to the membrane. In the isolated oxidase, where the endogeneous phospholipids probably form much less extensive membranes, the effects of

polyvalent anions are seen only at low ionic strength where the membrane electrostatic potential is not masked by the buffer.

Some of the results indicate that the effect of ATP is larger than, and of a different type from that due to Debye-Hückel screening of the negative electrostatic potential on the membrane, an effect which depends on the ionic strength. Ferricytochrome *c* binds anions, including ATP and ADP [69], but there is some evidence that ADP at least, does not bind to ferrocytochrome *c* [70]. There is also no clear evidence that ATP binds to ferrocytochrome *c* (see ref. 69), and NMR studies have shown that ATP and ADP interact only very weakly compared with phosphate [71]. The binding of anions to ferricytochrome *c* is not sufficient to account for the inhibition observed in both the steady-state and initial rate measurements (Figs. 5 and 8). However, the possibility that a cytochrome *c* · ATP complex inhibits the oxidase should be considered and tested in further experiments.

A simultaneous fit to the data in the absence and presence of ATP (Fig. 8) using the model of Monod et al. [45] was performed in collaboration with Dr. M. Markus. The effect of ATP could be described by considering an ATP · ferrocytochrome *c* complex as an allosteric inhibitor. The following results were obtained: $V = 33\ \mu M/min \pm 6\,\%$, $L_0(K_R)^3 = 1.9\ \mu M^3 \pm 30\,\%$, $c_i = 0.3 \pm 20\,\%$, $c = 0$, $n = 3$, where V is the maximal velocity, L_0 is the allosteric constant, K_R is the dissociation constant of the substrate · R protomer complex, c and c_i are the non-exclusive binding coefficients for the substrate and the substrate · ATP complex, respectively, and n is the number of protomers. The correlation between the parameters L_0 and K_R as well as between the dissociation constants of the two substrate · ATP · protomer complexes was so great for the given data, that they could not be determined separately.

Some kind of conformational change or rearrangement of the oxidase may occur when yeast cytochrome *c* binds to it. Reports from this and other laboratories have indicated that cytochrome *c* oxidase can bind up to 2 mol of cytochrome *c* per mol of cytochrome aa_3 [25, 26, 46]. A Hill coefficient of 2.35 requires that more than two sites interact to produce this conformational change. It may be relevant that a good fit of the data was obtained with $n = 3$ or $n = 4$, suggesting that cooperative interactions occur within groups of three of four oxidase molecules.

Evidence is accumulating that membrane proteins may be associated in clusters within the membrane. This is the case for the bacteriorhodopsin of *Halobacterium halobium* [72] and may also be true for complexes of the mitochondrial respiratory chain [82–86]. By lowering the affinity of cytochrome *c* for the membrane, ATP would decrease the average number of cytochrome *c* molecules which are present on the membrane surface and as suggested are undergoing lateral diffusion between the electron transfer complexes.

The cytochrome *c* binding site of the oxidase is located on the outer surface of the mitochondrial inner membrane (for a review, see ref. 87). Therefore the external concentration of ATP could affect the rate of electron transfer between cytochrome c_1 and *a*, as has been previously proposed [25]. The specific interaction between yeast cytochrome *c* and the yeast oxidase may have evolved because of the selective advantage of the sigmoidal kinetics which produce a higher sensitivity to ATP. Although ADP is also an inhibitor of cytochrome *c* oxidase, there are indications that the external concentration of ATP is at least four times that of ADP [88]. A similar inhibition

by ATP has been reported for NADH dehydrogenase [89]. These results raise the possibility that the level of ATP regulates electron transfer at several steps, in addition to controlling the ATPase via the formation of a complex between the ATPase and its natural inhibitor [90].

APPENDIX

On the occurrence of two-dimensional diffusion of cytochrome c on the membrane surface

The observation that there is very little free cytochrome *c* in the cytoplasm [73] implies that it is virtually all bound to the mitochondrial membrane. Thus two-dimensional diffusion on the surface of the membrane is the most likely mechanism by which cytochrome *c* transfers electrons between cytochromes c_1 and *a*. This possibility has been mentioned elsewhere [74].

Cytochrome *c* will be attracted by the negative electrostatic potential on the surface of the phospholipid membrane. During its residence time on the surface, lateral two-dimensional diffusion will occur with a relatively high diffusion coefficient owing to the mobility of the phospholipids in the membrane ($D = 10^{-8}$ cm^2/s [75]). Thus statistically a certain area of the membrane can be covered by the substrate, producing a highly efficient "sink" for substrate molecules round each "target" oxidase molecule. The encounter distance for the reaction between the enzyme and the non-specifically bound substrate may then be represented by the diffusional distance, *r* [76], which is of the order of [60]:

$$r \approx \sqrt{(D_2/k_{\text{diss}})} \tag{1}$$

where D_2 is the two-dimensional diffusion coefficient and k_{diss} the dissociation constant of the non-specifically bound substrate. The average inner membrane area per cytochrome *a* molecule is about 50 000 Å^2 [77], which corresponds to a radius of diffusion of 126 Å. This agrees reasonably well with observations of the inner mitochondrial membrane under the electron microscope [78] which showed that the distance between cytochrome *c* oxidase molecules ($\approx 2r$) is about 400 Å. If D_2 is taken as 10^{-8} cm^2/s, Eqn. 1 gives $k_{\text{diss}} \cong 6 \cdot 10^3$ s^{-1}. A dissociation rate constant greater than 10^3 s^{-1} has been postulated [79], to fit the experimental data in 66 mM phosphate, which will speed up the dissociation. If oxidase molecules occur in clusters, the estimated value of k_{diss} is inversely proportional to the number of molecules per cluster.

The non-specific binding of a ligand to the membrane, followed by rapid two-dimensional diffusion, can enhance the association rate constant, as shown by Richter and Eigen [60], who derived the following relation:

$$k_{\text{on}} = 4\pi D_3 \frac{-b}{\exp(-\pi b/r)-1} \tag{2}$$

where D_3 is the three-dimensional diffusion coefficient and *b* is Bjerrum's length:

$$b = -z_s z_m e^2/4\pi\varepsilon kT \tag{3}$$

z_s and z_m are the number of charges on the substrate and membrane, respectively, *e* is the charge of a proton, and ε is the dielectric constant. The charge on the membrane

is related to the surface electrostatic potential, ψ, according to the Gouy-Chapman theory [80, 81] for a 1 : 1 electrolyte, as follows:

$$\psi = (2kT/e)\sinh^{-1}(\sigma^2 e^2/8\varepsilon nkT)^{\frac{1}{2}} \quad (4)$$

where σ is the surface charge density and n is the number of ions per unit volume of the medium. For 10 mM cacodylate/Tris (5.9 mM monovalent cations and anions), $\psi = -13$ mV (see Discussion above) and from Eqn. 4, $\sigma = -1.4 \cdot 10^{12}$ charges per cm^2. This is of the same order of magnitude as the value for *Escherichia coli* determined from electrophoretic mobilities [6].

If the membrane area per cytochrome *a* molecule is 50 000 $Å^2$ [77], then $z_m = -7.2$. Since z_s is about 9.5 for cytochrome *c* [16], Eqn. 3 gives $b = 490$ Å, in the absence of shielding by the ionic atmosphere. Therefore, if $D_3 \approx 10^{-6}$ cm^2/s, from Eqn. 2 k_{on} is of the order of $4 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ at zero ionic strength. Experimental values for the rate constant of reaction of cytochrome *c* with cytochrome *a* are of the order of 10^7 $M^{-1} \cdot s^{-1}$ [91–94] and the rate constant of complex formation is expected to be more rapid than this. From the calculated values of k_{diss} and k_{on} the equilibrium binding constant may be calculated to be about $2 \cdot 10^{-7}$ M, which is near the experimental value for K_m reported in this paper ($3.5 \cdot 10^{-7}$ M, Fig. 1).

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