Two-electron reduction of cytochrome *c* oxidase triggers a conformational transition

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The slow increase of a cyanide-induced optical change at 437 nm following rapid cyanide inhibition of cytochrome oxidase has been followed as a function of the number of electrons donated from ferrocytochrome *c* to cytochrome *a* and Cu_A. The initial rate of optical change is a parabolic function of this number. The results have been analyzed in terms of a model where addition of electrons causes a conformational transition allowing rapid cyanide binding. The binding is followed by a slow intramolecular reaction responsible for the optical change. The analysis demonstrates that only molecules with both cytochrome *a* and Cu_A reduced can undergo the conformational change, which is suggested to be involved in the proton-pump mechanism of the oxidase.

Cytochrome oxidase *Cyanide binding* *Redox-linked conformation* *Proton pump*

1. INTRODUCTION

Cytochrome *c* oxidase, the terminal component of the mitochondrial respiratory chain, is an electron-transport driven proton pump [1,2]. The operation of such a pump requires redox-linked conformational transitions to provide an alternating access of an acid-base group to the two sides of the membrane and also to ensure an obligatory coupling between electron transfer and proton translocation [3]. Two redox-linked conformational transitions in cytochrome oxidase have been established. The resting oxidase is converted into a more active, pulsed form on reduction and reoxidation with dioxygen [4]. Oxidized cytochrome *c* oxidase is in a 'closed' conformation where cytochrome a_3 binds cyanide very slowly (over a period of hours), but this closed form is converted into an 'open', rapidly reacting conformation on partial reduction [5–8]. The resting-pulsed transition appears too slow [4] to be part of the pump mechanism. Interconversion between open and closed forms, on the other hand, is rapid enough to occur during turnover [7]. This latter transition has consequently been proposed to constitute the mechanical part of the proton pump [8].

In the open, cyanide-inhibited form of the enzyme cytochrome *a* and Cu_A have been reduced by ferrocytochrome *c* whereas internal electron transfer to Cu_B and cytochrome a_3 is blocked [8]. It has, however, not been excluded that reduction of only one of the primary electron acceptors, cytochrome *a* or Cu_A, is sufficient to effect the conformational transition. The purpose of this communication is thus to address the problem of how many primary electron acceptors must be reduced.

Our results indicate that both cytochrome *a* and Cu_A need to be reduced for the closed-open transition to occur. It has previously been shown that, in addition, internal electron transfer does not take place in the one-electron reduced enzyme [9,10].

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Thus, the conformational change triggered by the reduction of cytochrome *a* and Cu_A may also couple electron transfer and proton translocation.

2. MATERIALS AND METHODS

Beef heart cytochrome *c* oxidase was prepared essentially as in [11]. Buffers were 0.05 M Hepes with 0.5% Tween 80, pH 7.4, and 0.167 M K_2SO_4. Cytochrome *c* was purified and reduced as described [12]. Concentrations of stock solutions of oxidase and cytochrome *c* were determined spectrophotometrically [13]. The extinction coefficient difference between partly reduced, cyanide-inhibited oxidase and resting oxidase was $20 \times 10^3\ M^{-1} \cdot cm^{-1}$ from cytochrome *a* at 604 nm, $2.0 \times 10^3\ M^{-1} \cdot cm^{-1}$ from Cu_A at 830 nm, and $3.3 \times 10^4\ M^{-1} \cdot cm^{-1}$ from the cytochrome a_3-cyanide complex at 437 nm, a wavelength which showed isosbestic behavior for cytochrome a^{2+}-a^{3+}. The extinction coefficient difference between cytochrome c^{2+} and c^{3+} at 550 nm is $21.1 \times 10^3\ M^{-1} \cdot cm^{-1}$ [13]. Stopped-flow experiments were carried out on an apparatus described in [14] connected to a Data General Nova minicomputer. Absorbances at 437, 550, 604 and 830 nm were calibrated on the stopped-flow device vs those measured on a standard Beckman Acta MIV spectrophotometer.

Resting, oxidized oxidase was mixed with a solution containing reductant and cyanide. Following mixing, the concentration of oxidase was typically 5 or 2.5 μM, KCN was 10 mM, and varying concentrations of cytochrome *c* up to 40 μM were used. The temperature was 22°C. Several experiments were performed with 10 mM ascorbate/50 μM TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) in the cytochrome *c* solution. A series of experiments was performed with micromolar amounts of NADH and 1 μM PMS (phenazine methosulfate) instead of cytochrome *c* as reductant. To avoid unwanted slow consumption of cytochrome c^{2+} or NADH, experiments were performed in all but a few instances under anaerobic conditions brought on by multiple pump-flush cycles with ultrapure nitrogen.

3. RESULTS

With or without cyanide there is a rapid initial burst of cytochrome *c* oxidation. With the ionic strength used here, this burst lasts less than 1 s. Then cyanide markedly inhibits further reduction of cytochrome *c*, electron transfer to a_3-Cu_B, and O_2 reduction. Fig.1a shows the effect of cyanide on cytochrome *c* consumption when O_2 is available. Inhibition occurs within 1 s, yet spectroscopic evidence for a cytochrome a_3-cyanide complex only appears [15–17] over a period of several hundred seconds, as shown in fig.1b.

Although the cytochrome a_3-cyanide signal arises after inhibition has occurred, this signal

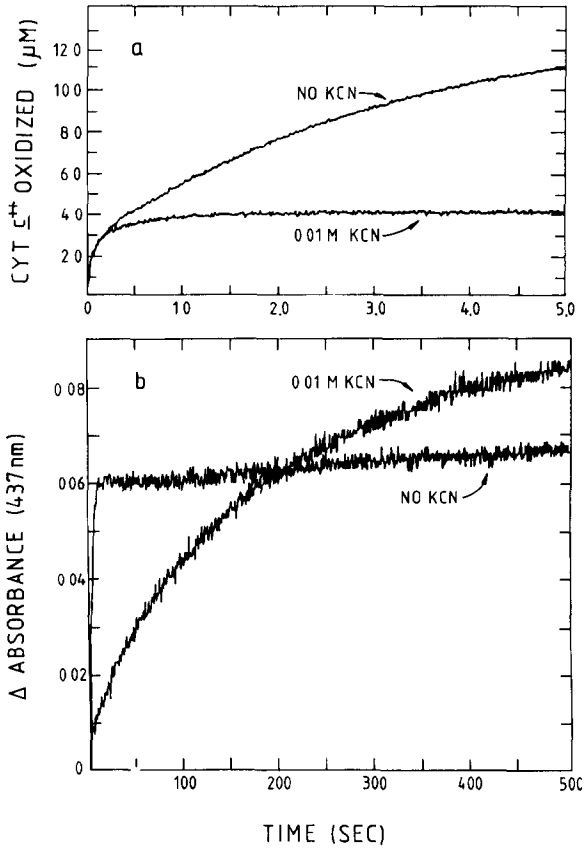

Fig.1. (a) The rapid onset of cyanide inhibition as shown by the cessation of cytochrome c^{2+} oxidation following the initial burst. The cytochrome c^{2+} consumption was estimated from the absorbance change at 550 nm. Cytochrome c^{2+} concentration was initially 20 μM, cytochrome oxidase 2.5 μM, and the buffer was air-saturated. (b) The slow build-up of the absorbance from the cytochrome a_3-cyanide complex in the cyanide-inhibited sample. A rapid initial change in absorbance in the noninhibited sample is due to the rapid noninhibited consumption of cytochrome *c*.

nevertheless does indicate that inhibition has occurred. Its rate of increase, $v = dA_{437}/dt$, determined from the slope of absorbance vs time 20 s after mixing, was measured as a function of the number of electrons taken up per oxidase molecule. The time of 20 s was chosen because it is considerably less than that needed for complete build-up of the cytochrome a_3-cyanide signal but well after initial electron transfer. Values of v measured at wavelengths within a few nanometers of 437 nm were all very similar. The number of electrons donated per oxidase molecule, N, was estimated from the consumption of cytochrome c^{2+}. The uptake of electrons from cytochrome c oxidation correlated within experimental error with the sum of increases in reduced cytochrome a^{2+} and Cu_A^+, which were approximately equal to each other [18].

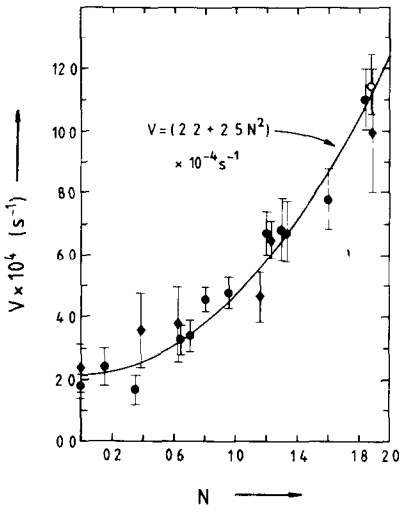

Fig.2. The initial velocity, v, of cyanide-induced absorbance change at 437 nm shown as a function of the number of electrons consumed per oxidase, as measured from the optical change at 550 nm. Most points were taken with 5 μM oxidase (●), while the remainder were taken with 2.5 μM oxidase (◆); the ordinates of these latter points were multiplied by 2 to compensate for the 2-fold concentration difference. Errors in v were estimated from the noise-induced uncertainty in measuring the slope of the absorbance curve. Errors in N were <0.1 unit. The highest point which is shown by an open circle (○) was obtained with 5 μM oxidase mixed with 20 μM cytochrome c in the presence of 10 mM ascorbate/50 μM TMPD so as to ensure continuous, complete reduction of cytochrome c; for this point N was estimated from the sum of increases in a^{2+} and Cu_A^+.

Fig.2 gives v as a function of N. About 15% of oxidase molecules bind cyanide in the absence of reductant. It is obvious that v increases substantially after 1 e^-/oxidase has been consumed. A number of points in fig.2 were obtained with 2.5 rather than 5 μM oxidase to ensure that intermolecular electron transfer does not complicate the results. The normalized values of v at the lower concentration agreed with corresponding measurements on 5 μM oxidase. The maximum value of v was obtained when ascorbate/TMPD was present to keep the cytochrome c reduced and thus to provide a maximum driving force for electron transfer. Finally, when NADH/PMS was used, v increased most markedly after 1 e^-/oxidase had been added and attained its maximum when 2 e^-/oxidase or more were added.

4. DISCUSSION

The results in fig.1 confirm earlier observations [15–17] that the onset of inhibition of cytochrome oxidase by cyanide is much more rapid than the development of an optical change due to a cytochrome a_3-cyanide complex, suggesting that the primary site of inhibition is perhaps Cu_B [16,19]. Our stopped-flow experiments can consequently be interpreted in terms of the following kinetic scheme, in which the addition of n electrons causes the transition from the closed to the open conformation, then the open form binds cyanide without any detectable optical change, and the initial complex is converted to the optically visible cytochrome a_3-cyanide complex in an intramolecular, first-order reaction:

$$\underset{(A)}{\text{Closed forms}} \xrightarrow{ne^-} \underset{(B)}{\text{open forms}} \underset{k_{-2}}{\overset{\text{HCN}\ k_2}{\rightleftharpoons}} \underset{(C)}{\text{inhibited, spectroscopically silent forms}} \xrightarrow{k_3} \underset{(D)}{\text{inhibited, spectroscopically visible forms}}$$

In our work the effective rate for the addition of n electrons in the first step is approx. 1 s^{-1}. From

inhibition studies [7], k_2 and k_{-2} have been indirectly estimated as 10^6 $M^{-1} \cdot s^{-1}$ and 0.01 s^{-1}, respectively. Thus, the rate of cyanide binding with the concentration used in our experiments (10 mM) is 10^4 s^{-1}. We found k_3 to be approx. 0.01 s^{-1}. These rate values mean that the binding species B is formed within approx. 1 s, and then very rapidly converted into the inhibited form C, where the enzyme accumulates. The initial rate of appearance of form D is proportional to the concentration of C which, because of the rapid nature of step 2, is in turn identical to the total concentration of binding forms B. We have used $v = dA_{437}/dt$ as a measure of the initial rate of formation of D. The dependence of this rate on N, i.e. the number of electrons added per oxidase molecule, should yield an insight into the problem of the number of electrons needed to trigger the conversion to the open conformation.

From fig.2 it is clear that v is not linearly dependent on N. The data can be well fitted by least squares to the function: $v = a + bN^2$, in which $a = (2.2 \pm 0.2) \times 10^{-4}$ s^{-1} and $b = (2.5 \pm 0.2) \times 10^{-4}$ s^{-1}. The corresponding least-squares fit of v to a linear function, $a + bN$, yields a 240% greater sum of squared residual errors because there are significant deviations from experiment at both low and high extreme values of N. The a term refers to approx. 15% of the oxidase which reacts rapidly with cyanide without any addition of reducing equivalents, whereas the b term describes the 85% majority that requires reduction to be put into the open, cyanide-binding form.

Electron donation to the majority population of the oxidase molecules stops at $N = 2$ [8], so that for a given N value electrons will distribute themselves between nonreduced ($a^{3+}Cu_A^{2+}$), singly reduced ($a^{2+}Cu_A^{2+}$ and $a^{3+}Cu_A^{+}$), and doubly reduced ($a^{2+}Cu_A^{+}$) forms. If the quadratic variation of v with N is very similar to the variation of the concentration of one of these forms with N, then the implication is that that particular form is the predecessor of the open, cyanide-binding form B.

A simple dependence of the concentrations of the various forms on N can be derived if cytochrome a and Cu_A are independent, noninteracting sites with almost the same reduction potentials and rates of reduction by cytochrome c [18]. Let P be the probability for having cytochrome a reduced, which is also the probability for having Cu_A reduced. The probability for having the oxidase in the non-, singly, and doubly reduced forms would then be $(1 - P)^2$, $2P(1 - P)$, and P^2, respectively. Furthermore, $N = 2P$ so that the fraction of doubly reduced forms would be $(1/4)N^2$. Thus, the concentration of these forms displays the same parabolic dependence on N as observed for v in fig.2. This strongly suggests that two electrons must be added to the oxidase to convert it into the open conformation.

Recently, a moderate anticooperative interaction between cytochrome a and Cu_A has been reported [20,21] for carboxycytochrome oxidase. We have therefore also analyzed our data in terms of interacting models. Very strong interactions, cooperative or anticooperative, can be excluded, as they could not yield the parabolic dependence in fig.2. Within the accuracy of our data we cannot, on the other hand, exclude weak interactions. The important conclusion is, however, that neither in the cooperative nor in the noncooperative case can the results in fig.2 be fitted to models in which single reduction is sufficient for the conversion to the open form. Thus, the conclusion seems firm that this conversion requires the addition of two electrons to the resting oxidase.

The observation that a small fraction of the resting enzyme can react rapidly with cyanide may be related to the heterogeneity observed in other experiments with cyanide inhibition [8]. In addition, it has been found [22] that about 20% of the molecules in the resting enzyme react differently with CO compared to the majority fraction. Thus, the resting enzyme appears to be a mixture of at least two conformational states.

The fact that internal electron transfer to the cytochrome a_3-Cu_B site does not occur in the singly reduced enzyme [9,10] suggests that this transfer may also require that the enzyme is in the open conformation. If, in addition, the closed-open conformational transition furnishes the alternating access of an acid-base group, demanded in a proton pump, then this transition could also provide the obligatory coupling between electron transfer and proton translocation in cytochrome oxidase.

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