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The possible role of the closed-open transition in proton pumping by cytochrome *c* oxidase: the pH dependence of cyanide inhibition

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The rate of oxidation of reduced cytochrome *c* catalyzed by cytochrome oxidase in the presence and absence of cyanide has been measured spectrophotometrically at pH 5.5, 6.4, 7.4 and 8.3. At the cytochrome *c* concentration used (272 μM), the uninhibited rate is maximal at pH 6.4 and drops to a value about one sixth of this maximum at pH 8.3. In the presence of cyanide, the rate initially drops rapidly, but with the cyanide concentration used (5.5 μM) there is still a measurable rate of oxidation when maximal inhibition has been reached. This inhibited rate decreases as the pH increases, whereas the apparent rate constant for cyanide binding is almost independent of pH. The results have been analyzed on the basis of a model in which two-electron reduction of the oxidized enzyme triggers a transition from a closed to an open conformation. It is assumed that cyanide can only bind to the open conformation and, furthermore, that rapid internal electron transfer to the dioxygen-reducing site occurs in this state alone. The analysis shows that the true rate constant for cyanide binding decreases with decreasing pH to a constant value at low pH. It also indicates that the increase in the catalytic constant with decreasing pH is associated with an increase in the rate of the closed-open conformational transition on protonation of the enzyme, and it is proposed that this transition is operative in electron gating in the proton-pump function of the enzyme.

Introduction

It has long been known that when cyanide inhibits cytochrome *c* oxidase, it combines with oxidized cytochrome a_3 [1]. It was consequently a dilemma when it was discovered [2,3] that the combination of cyanide with the oxidized enzyme is too slow to account for the rate of the onset of inhibition during turnover. A solution to this problem was provided by Nicholls et al. [4], whose analysis suggested that in the partially reduced enzyme oxidized cytochrome a_3 reacts rapidly with cyanide. This idea was further explored by Jones et al. [5] on the basis of a kinetic model in which the oxidase cycles between nonbinding and binding forms (also called closed and open conformations [6]) during turnover. The computer simulations of their kinetic results indicated that in the open conformation cyanide combines very rapidly with oxidized cytochrome a_3, the second-order rate constant being estimated to be $2 \cdot 10^6$ $M^{-1} \cdot s^{-1}$. It should be noted that this is five orders of magnitude faster than the rapidly reacting form

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-morpholineethanesulfonic acid.

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found in mitochondria [7] or in the pulsed form of the isolated enzyme [8,9].

Several investigators have suggested [10–13] that the closed and open conformations correspond to the separate input and output states for electrons, as required for electron gating in the mechanism of cytochrome oxidase as a proton pump [14,15]. Recently it has been demonstrated [12,13] that the closed-open transition can be followed directly by fluorescence methods. It could then be shown [13] that it is rapid enough to be part of turnover, which is, of course, necessary if it should be involved in the proton pump.

As there is evidence [11,16] that the rate of the conformational switch provides the main limit on the catalytic constant (k_0), and as k_0 increases substantially with decreasing pH [17,18], we deemed it of interest to investigate the effect of pH on cyanide inhibition. Here we report the pH dependence of the onset of inhibition and of the rate of cytochrome *c* oxidation in the maximally inhibited state. The results have been analyzed in terms of an extended version of the model of Jones et al. [5]. The analysis shows that, in fact, a change in k_0 has no effect on the onset of inhibition. Instead, the changes in this and in the inhibited rate that we observe must be ascribed to changes in the rate constant for the binding of cyanide to the open conformation. It is suggested that different mechanisms of binding dominate at low and high pH, because the concentration of CN^- is negligible below pH 7 but becomes appreciable (> 1%) above that pH.

Materials and Methods

Cytochrome oxidase was isolated from bo[illegible] hearts essentially as described by Van Buuren [19]. Cytochrome *c* was prepared by the method of Brautigan et al. [20] and was further purified by ion-exchange chromatography. Dithionite was used to reduce cytochrome *c*; excess dithionite was removed on a Sephadex G-25 column. Reduced cytochrome *c* was frozen as droplets in liquid nitrogen; these were thawed immediately before use, and the protein was then typically 98% reduced.

Absorption coefficients used for the difference between reduced and oxidized proteins were $21.1 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ for cytochrome *c* at 550 nm and $24 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ for cytochrome oxidase at 605 nm. Solutions of KCN were prepared a few hours before use. They were kept in totally filled, tightly sealed test tubes. Cyanide concentrations were checked with the argentimetric method [21]. Oxygenated oxidase (the 428-nm form) was prepared by anaerobic reduction of the enzyme with a small amount of dithionite and subsequent aeration. The buffer used in the oxygenation procedure was 0.05 M Hepes with 0.167 M K_2SO_4 and 0.5% Tween 80 (pH 8.0) at 20 °C. Oxygenated oxidase was never used more than 20 min after oxygenation; within this time small spectral changes only could be seen.

The cytochrome *c* oxidation rates were determined spectrophotometrically in a Zeiss PMQ II spectrophotometer coupled to a potentiometric recorder, as described earlier [18]. The measuring temperature was 25 °C. The buffers used were 0.05 M Mes or Hepes, depending on pH, with 0.167 M K_2SO_4 and 0.5% Tween 80, giving an ionic strength of 0.5 M.

Results

The rate of oxidation of reduced cytochrome *c* catalyzed by cytochrome oxidase in the absence and presence of cyanide at four pH values is shown in Fig. 1. A high concentration of cytochrome *c* was used, so that the uninhibited rate should be limited largely by the catalytic constant (k_0), and not by the bimolecular reaction between cytochrome *c* and the oxidase. The cyanide concentration, on the other hand, was relatively low, so that we could follow the onset of inhibition, and so that there was a measurable rate of oxidation even after maximal inhibition had set in.

From the curves in Fig. 1 we have estimated the initial uninhibited rate (v_0), i.e. the molecular activity in the absence of cyanide, which is identical to the initial molecular activity in the presence of cyanide. We have also determined the inhibited rate, which is the molecular activity in the second phase of the experiments in the presence of cyanide. The values of these parameters at four pH:s are listed in Table I, which also includes estimates of the number of complete four-electron turnovers that the oxidase has gone through be-

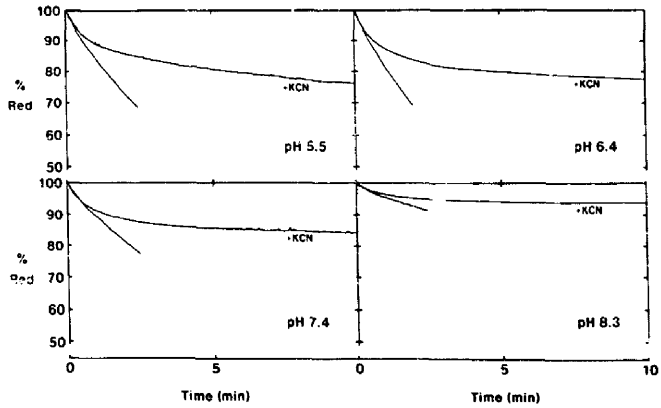

Fig. 1. Time course of the oxidation of cytochrome *c* at pH 5.5, 6.4, 7.4 and 8.3 in the absence and presence of cyanide. The concentrations used were: cytochrome oxidase, 17 nM; cytochrome *c*, 272 μM; cyanide 5.5 μM. The measuring times were generally longer than shown to give reliable estimates of the slopes of the inhibited reactions in the slow phase.

fore reaching maximal inhibition, and the apparent rate constant for cyanide binding, determined by the method of Nicholls et al. [4]. The table also includes rate constants which have been calculated as described in the Discussion. It can be seen that both the inhibited rate and the number of turnovers decrease as the pH increases.

Discussion

We have analyzed our results on the basis of the reaction scheme shown in Fig. 2, which represents a slight modification of the model of Jones et al. [5]. An intermediate (E_c') has been added between the closed (E_c) and the open (E_o) state. This is formed on the addition of two electrons, as a two-electron requirement for the conformational transition has been demonstrated [10,13]. Scholes and Malmström [10] suggested that both cytochrome *a* and Cu_A must be reduced to trigger the transition, but an alternative explanation has recently been put forth by Copeland et al. [13]. According to this, Cu_A is the trigger, but this ion is not reduced until two electrons have been added, because cytochrome *a* has a considerably higher reduction potential than Cu_A.

Step 4, which represents the internal electron transfer from cytochrome *a* and Cu_A to cyto-

TABLE 1

KINETIC PARAMETERS ESTIMATED FROM THE CURVES IN FIG. 1

pH	Uninhibited rate (s^{-1})	Inhibited rate (s^{-1})	No. of turnovers before maximal inhibition [a]	Apparent rate constants for cyanide binding ($10^3\ M^{-1}\cdot s^{-1}$)	
				Exp. [b]	Calc. [c]
5.5	41	1.3	815	2.4	2.3
6.4	49	1.1	774	2.7	2.9
7.4	26	0.47	606	2.4	2.0
8.3	7.6	0.044	310	1.7	1.6

[a] This parameter was calculated in the following way: the linear parts of the curves were extrapolated to zero times, and the amount of cytochrome *c* oxidized was then used to calculate the number of turnovers made by each molecule of cytochrome oxidase.

[b] Calculated according to Nicholls et al. [4].

[c] Calculated as described in the text.

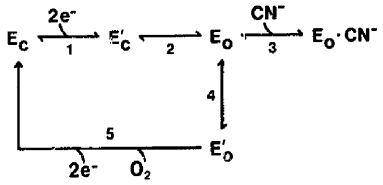

Fig. 2. Model used to describe the catalytic turnover and the onset of cyanide inhibition of cytochrome oxidase. E_c represents the fully oxidized, pulsed enzyme in the closed conformation, E_c' the two-electron reduced enzyme still in the closed state, E_o the two-electron reduced enzyme in the open conformation, and E_o' the enzyme in the open state with the primary electron acceptors oxidized and the dioxygen site reduced. For further details, see the text.

chrome a_3-Cu_B, has been added, because it is known that cyanide binds to oxidized cytochrome a_3, whereas dioxygen combines with reduced cytochrome a_3. After the addition of dioxygen and the formation of a peroxide intermediate, the enzyme returns to the closed conformation and cytochrome a as well as Cu_A become re-reduced, so that the enzyme goes through the whole cycle again. This has, however, not been shown explicitly. Instead the second cycle has been incorporated in step 5, as it is unlikely that the peroxide intermediate will bind cyanide.

There is evidence [16,22,23] that the limit on k_0, the catalytic constant, is set by the rate constant for the internal electron transfer. It has been suggested [11,16], however, that it is rather the conformational transition (step 2) which is the slow step and that the electron transfer (step 4) is rapid in E_o. The reason for this is that the slowest step in the oxidation of cytochrome a and Cu_A, starting from the fully reduced enzyme, has a rate constant of 700 s^{-1} at pH 7.4 [24], and that the internal redox equilibrium is also rapid in the mixed-valence state in the absence of dioxygen [11,25]. As the dioxygen reaction is also rapid, and the reductive steps do not enter into the limiting rate, it seems likely that the rate constant (k_2) of step 2 is the main determinant of k_0.

In the experiments illustrated in Fig. 1 we have used a high concentration of ferrocytochrome c (272 μM) in order to have the observed uninhibited rate approach k_0 and thus be determined mainly by k_2. This requires that $k_s[c^{2+}] > k_0$, where k_s is the so-called specificity constant for ferrocytochrome c (i.e. k_0 divided by the Michaelis constant). As the value of k_s under our conditions is $1 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ [16], and thus $k_s[c^{2+}] = 272$ s^{-1}, this is achieved at the two highest pH values, where $k_0 < 50$ s^{-1}. At pH 6.4, however, k_0 approaches $k_s[c^{2+}]$, so that the reductive steps also limit the observed turnover rate to some extent. This is even more pronounced at the lowest pH, where $k_0 > 272$ s^{-1}, which is also the reason why the turnover rate drops at pH 5.5 compared to pH 6.4, despite the higher k_0 value.

In the presence of cyanide the rate of cytochrome c oxidation gradually decreases and after a time reaches a nearly constant, inhibited rate (Fig. 1), which corresponds to a condition in which the forward and backward rates of step 3 are equal. It can be seen that the number of turnovers that the enzyme goes through before reaching this state becomes larger as the pH decreases (Table I). This can, however, not be explained by the fact that the turnover rate also increases with decreasing pH, because the increased concentration of E_o obtained at high turnover rates leads to increases in the forward rates of steps 3 and 4 by the same factor. Thus, the number of turnovers made before maximal inhibition is reached is entirely determined by the rate constants for these two steps. As the forward rate of step 4 does not change with pH ([11] and unpublished experiments), the changes we observe must be related to the rate constants (k_3, k_{-3}) of step 3.

The conclusion that k_0 is limited mainly by k_2 is strengthened by our finding that the number of turnovers completed before maximal inhibition shows a small variation with pH compared to the uninhibited rate (Table I). The partitioning between cyanide binding (step 3) and catalysis (step 4) thus changes rather little, and this small variation can be associated with the pH dependence in step 3. This again indicates that the pH dependence of k_0 does not originate from k_4.

The inhibited rates also increase with decreasing pH, as seen in Fig. 1 and Table I. In this case there is, of course, a relation with the increase in k_0. This can, however, only be responsible for a small part of the observed effect, as the uninhibited rate increases at most by a factor of 6.4, whereas the inhibited rate increases by a factor of 29. Thus, this increase must also be largely de-

termined by a decrease in k_3 or an increase in k_{-3}, or a combination of these.

It can be seen (Table I) that the increases in the two inhibition parameters are largest between pH 8.3 and 6.4, but there is then very little change in going to pH 5.5. This suggests to us an explanation of the observed effects. We assume that the bound form of cyanide in the inhibited complex is CN^-, as this is usually the case with the Fe(III) derivatives of respiratory heme proteins [3,26]. As the pK_a of HCN is 9.2, the fraction of cyanide present as CN^- is, however, very small at low pH ($2 \cdot 10^{-4}$ at pH 5.5 and $1.6 \cdot 10^{-3}$ at pH 6.4), but it increases to as much as 11% at pH 8.3. It is thus possible that different mechanisms for the formation of cytochrome a_3^{3+}-CN^- dominate at high and low pH:

$$a_3^{3+} + CN^- \rightleftharpoons a_3^{3+} - CN^-$$

$$a_3^{3+} + HCN \rightleftharpoons a_3^{3+} - HCN \rightleftharpoons a_3^{3+} - CN^- + H^+$$

If the second-order rate constant in the first mechanism is higher than that in the second one, we can nicely account for the observed effects. Let α be the fraction of the total cyanide present as CN^- and $k_3 = 2 \cdot 10^6\alpha + 8 \cdot 10^4(1-\alpha)$, which gives the value of k_3 estimated by Jones et al. [5] when $\alpha = 1$. We then get a small increase in k_3 in going from pH 5.5 to 6.4 (from $8 \cdot 10^4$ to $8.4 \cdot 10^4$ $M^{-1} \cdot s^{-1}$), a somewhat larger increase at pH 7.4 ($1.1 \cdot 10^5$ $M^{-1} \cdot s^{-1}$), and a considerable increase at pH 8.3 ($2.9 \cdot 10^5$ $M^{-1} \cdot s^{-1}$). This corresponds exactly to the trends observed in the changes with pH of the number of turnovers before maximal inhibition and of the inhibited rates of cytochrome *c* oxidation.

Our analysis can be used to predict quite accurately the apparent rate constant for cyanide binding, determined by the method of Nicholls et al. [4], as seen by a comparison between the experimental and calculated values in Table I. The apparent rate constant is lower than the true one because of the low occupancy of the cyanide-binding form (E_o) in the steady state. We can estimate this occupancy on the basis of the fact that in the steady state all steps in the catalytic cycle proceed with the same velocity, namely the observed uninhibited turnover rate (v_0). To a first approximation, $([E_o]/[E]_{tot}) = v_0/2k_4$, where the factor 2 stems from the fact that about half of the enzyme is in the second cycle, in which the oxygen intermediate prevents the binding of cyanide. If $k_4 = 700\ s^{-1}$, then the occupancy is as low as 0.0054 at pH 8.3, it increases to 0.035 at pH 6.4, and then drops again to 0.029 at pH 5.5. The calculated values in Table I have been obtained by multiplying these occupancies with the values of k_3, calculated as described earlier. The good agreement with the experimental values gives additional support to the idea that the complex pH dependence of cyanide inhibition is caused by the presence of two mechanisms of cyanide binding.

Finally, we would like to argue that the closed-open conformational transition is involved in electron gating in the proton pump, as suggested earlier [10–13]. As it is oxidized cytochrome a_3 that binds cyanide, this transition must precede the internal electron transfer, as it does in our reaction cycle (Fig. 2). We have already pointed out that the internal electron transfer is rapid in step 4, but it appears slow when starting from the fully oxidized enzyme, even in the pulsed state [23,27]. This could be explained, if step 2 is the slow step, as we have already suggested. Thus, the variation in k_0 with pH is most likely ascribed to changes in the apparent rate constant of step 2. This suggests that the conformational transition becomes more rapid, when a group in the enzyme becomes protonated. In a proton pump, the conformational switch between the input and output states for electrons (and protons) should be rapid only in the protonated state [14,15,28]. Thus, the properties of the closed and open conformations conform well to the requirements for electron gating in the proton pump.

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