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Conformational change of cytochrome a_3 induced by oxidized cytochrome c

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Cyanide binding with the oxidized resting Yonetani-type cytochrome *c*-oxidase followed spectrophotometrically reveals a relatively rapid initial phase the rate of which shows saturation behaviour with respect to [HCN] and secondary slower absorption changes to a first approximation independent of the ligand concentration. Oxidized cytochrome *c* greatly accelerates the initial phase of cyanide binding but does not affect significantly contribution or rate constant of the slow phase. The same effect is exerted by poly-L-lysine. Within a framework of a reaction mechanism assuming Cu_B^{2+} to be the initial HCN-binding site, cytochrome c^{3+} and other polycations are likely to bring about a conformational change of cytochrome oxidase resulting in an increased affinity of Cu_B^{2+} for HCN. This could occur by virtue of loosening a bond between Cu_B^{2+} and one of its endogenous ligands facilitating displacement of the latter by HCN.

Cytochrome *c*-oxidase; Cyanide binding; Conformational change; Cu_B-site; Cytochrome *c*; Poly-L-lysine

1. INTRODUCTION

The kinetics of cyanide binding to ferric cytochrome *c*-oxidase proves to be a useful indicator of the enzyme conformational state. As shown recently by several groups, the ligand reaction with the 'resting' detergent-solubilized enzyme comprises an initial phase dependent on HCN concentration and one or two slower phases reported to be independent of ligand concentration [1–5]. The contribution of the slowest phase varies strongly in different cytochrome oxidase preparations [3,5] and may correspond to a fraction of the enzyme displaying a $g = 12$ EPR signal [5].

It was shown earlier that oxidized cytochrome *c* can greatly accelerate (more than 100-fold) the reaction of the solubilized or proteoliposome-reconstituted ferric cytochrome oxidase with KCN by lowering the K_m value of the process (i.e., HCN concentration required for half-saturation of the reaction rate [1]) from $\sim 10^{-2}$ to $\sim 10^{-5}$ M [6,7]. A conclusion was inferred that cytochrome c^{3+} binding brings about a conformational change of cytochrome a_3 allowing for easier access of the oxygen-reducing centre to ligands [6,7].

In these papers a Fowler-type preparation of cytochrome oxidase was used in which the contribution of the slow phase of cyanide binding was rather low (<30%, cf. [3.5]) and the multiphasic character of cyanide-binding kinetics was not taken into account.

Here we show that in a Yonetani-type preparation of cytochrome oxidase in which different phases of the cyanide-induced absorption changes are particularly well separated [5] cytochrome c^{3+} specifically accelerates the initial [HCN]-dependent phase of the reaction without changing significantly its contribution to the overall process or the rate of the slow phase. The same effect is exerted by poly-L-lysine.

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2. MATERIALS AND METHODS

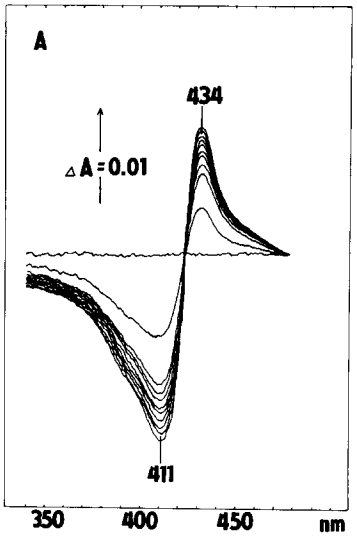
Cytochrome *c* (type III) was from Sigma; KCN and $K_3Fe(CN)_6$ (analytical grade) from Lachema; Hepes and poly-L-lysine·HBr (6–9 kDa) from Serva.

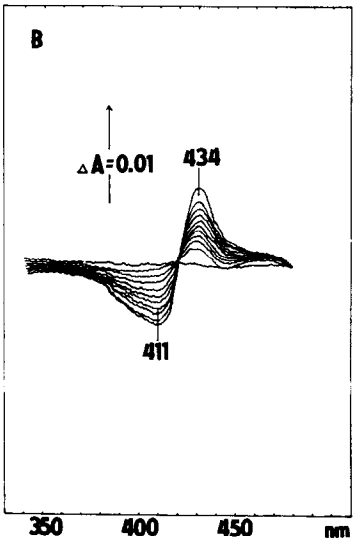
Cytochrome *c*-oxidase was isolated from bovine heart Keilin-Hartree particles by the method of Yonetani [8]. The enzyme contained 7–9 nmol heme *a*/mg protein.

Experiments were carried out in a basic reaction medium containing 50 mM Hepes-KOH, pH 7.4, 0.5% (v/v) Tween-80 and 55 μM ferricyanide. Cyanide binding was measured spectrophotometrically in a Shimadzu 3000 UV/VIS dual-wavelength split-beam spectrophotometer in standard 10 mm optical pathway quartz cells thermostatted at 27°C.

3. RESULTS

Fig.1A shows a typical series of difference spectra induced in the Soret region by addition of a nearly saturating concentration of KCN (4 mM ~ K_m, see below) to the oxidized cytochrome *c*-oxidase. The time course of the reaction is clearly biphasic (fig.2a) and comprises an initial burst contributing about 35% to the overall absorption changes (ΔA_{max} 434–411 $\approx$ 60 $mM^{-1} \cdot cm^{-1}$ as observed after 20 h of incubation, cf. [1]) and a slower second phase. A similar contribution of the





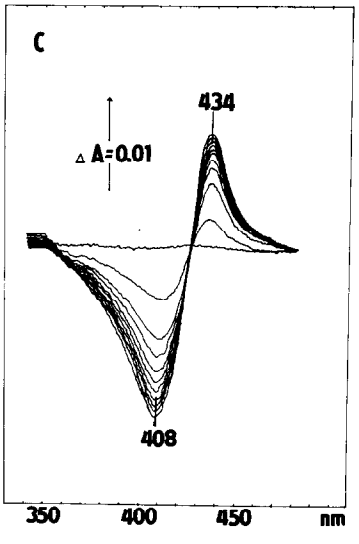


Fig.1. Cyanide-induced spectral changes of cytochrome *c*-oxidase. The sample and reference cells contain cytochrome *c*-oxidase (aa_3 = 1.1 μM) in the basic medium. In C, 2.8 μM cytochrome c^{3+} is also present. KCN has been added to the sample at zero time in concentrations of 4 mM (A) or 40 μM (B,C) and difference spectra have been recorded at a rate of 100 nm/min. Series of the first ten 3 min-spaced spectra is shown in each case, the first scan started 30 s after KCN addition.

initial phase was reported earlier for the Yonetani-type enzyme by Baker et al. [5].

The rate of the initial phase of cyanide binding depends on the ligand concentration showing a saturation behaviour (not shown); accordingly, kinetics of this phase can be characterized by the k_{max} and K_m (K_α) parameters [1,6,7,9,10]. For the enzyme solubilized in 0.5% Tween 80 at pH 7.4 and 27°C, K_m was typically 1 mM and k_{max} about 0.005 s^{-1} which may be compared to the corresponding values for the Fowler-type enzyme (5–10 mM and 0.006–0.02 s^{-1}, respectively [1,6]).

In variance with the earlier reports [3,5] we found the rate of the slow phase to depend on KCN concentration, although in a rather complex way. The apparent k_v value increased from $0.4 \times 10^{-4} \cdot s^{-1}$ at 1 mM KCN to $5 \times 10^{-4} \cdot s^{-1}$ at 100 mM of the ligand which allows the reconciliation of the data given for the Yonetani-type enzyme by Naqui et al. [3] ($k_v = 0.6 \times 10^{-4} \cdot s^{-1}$ at 1.33 mM KCN) and Baker et al. [5] ($k_v = 5 \times 10^{-4} \cdot s^{-1}$ at 100 mM KCN).

Fig.1B,C and fig.2b,c show absorption changes induced by a 100-fold lower concentration of cyanide. In the absence of other additions the reaction becomes very slow and monophasic (fig.1B, fig.2b). However, when the experiment is performed in the presence of 2.8 μM oxidized cytochrome *c*, the kinetics pattern returns to that observed with 4 mM cyanide (fig.1C, fig.2c) and once again comprises an initial burst of nearly the same amplitude as in figs 1A, 2a and a slow secondary increase of absorption.

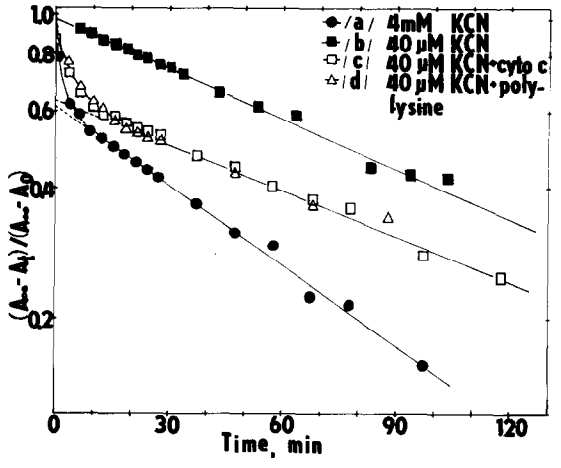


Fig.2. Kinetics of cyanide binding with heme a_3^{3+}. (a–c) Corresponds to A–C in fig.1. (d) As c but 10 μg/ml of poly-L-lysine is present instead of cytochrome c^{3+}.

A slightly different form of the difference spectra in the presence of cytochrome *c* (λ_{min} shifts from 411 to 408 nm) is due to a small interference from the cytochrome *c* reaction with KCN [11,12]. The contribution of this effect to $\Delta A_{434-480}$ used to plot the kinetics traces is negligible.

Exactly the same effect on the cyanide-binding kinetics was exerted by poly-L-lysine (fig.2d); the shape of the KCN-induced difference spectra remained unperturbed in this case (not shown).

4. DISCUSSION

First of all, the present results obtained with the Yonetani-type cytochrome oxidase confirm the earlier finding made with the Fowler-type enzyme that oxidized cytochrome *c* and other polycations can increase the rate of ferric cytochrome oxidase reaction with cyanide [6,7].

The data given in this work lead to the conclusion that the stimulatory effect of cytochrome c^{3+} is associated specifically with the rapid, strongly [HCN]-dependent phase of the reaction. In agreement with [6,7] we found c^{3+} to lower the K_m value of this phase from ~1 mM to ~10 μM KCN (Musatov, A.P., unpublished) without affecting significantly either k_{max} or the amplitude of the phase.

The somewhat slower rate of the slow phase of cyanide binding observed with cytochrome c^{3+} or poly-L-lysine-supplemented cytochrome oxidase at 40 μM KCN (as compared to the experiment at 4 mM of the ligand) is most likely due to a certain [KCN]-dependence of the phase, not recognized earlier [3,5]. However, we defer discussion on this point until more thorough studies have been carried out. As found recently in our group (and see also [5]), detergent-solubilized oxidized cytochrome *c*-oxidase does itself undergo slow spectral changes when allowed to stay at room temperature [13]. These spectral changes are affected by many experimental conditions and can interfere with the slow phase of the cyanide-induced spectral shift, which renders quantitation of this phase problematic.

Recently much emphasis was placed on the transition of cytochrome oxidase from the 'closed' to

'open' conformation upon partial enzyme reduction by cytochrome *c* as probed by reactivity of the enzyme towards cyanide [14–16]. Our data show ([6,7], this paper) that cytochrome *c* induces a conformational transition of cytochrome oxidase to a more open state even in the absence of electron transfer, as evidenced most clearly by an analogous effect exerted by protamine [6] and poly-L-lysine (this work). Accordingly, stimulation of intramolecular electron transfer in cytochrome *c*-oxidase by polycations has been known since 1965 [17,18].

This fact ignored by Malmström and co-workers [14–16] may question the validity of their conclusion on the closed to open conformation transition being related uniquely to an input of two electrons into the enzyme. Actually, the rate of cyanide binding to heme a_3 in their experiments as measured spectrophotometrically occurs in the same time range as reaction of the ligand with the rapidly reacting part of the resting enzyme, and 6-fold stimulation of this reaction by cytochrome oxidase reduction (cf. fig.2 in [14]) is a rather modest effect as compared to the 100–1000-fold increase in the effective rate constant induced by ferric cytochrome c^{3+} at unsaturating KCN concentrations [6,7].

Finally, what is the mechanism of the cytochrome c^{3+}-induced acceleration of cyanide binding? First of all we would point to a similar effect (i.e., decrease in K_m) exerted by azide [1] and H^+ [6,19]; the reader may refer to [1,6,10,19–21] for general discussion on the meaning of the K_m parameter in the cyanide-binding kinetics.

More specifically, the following simplified reaction scheme can be considered in which Cu_B^{2+} serves as the initial (invisible) site of HCN binding [21] and where Cu and Fe denote Cu_B^{2+} and Fe_{a3}^{3+}.

Within the framework of this model, cytochrome c^{3+} and other polycations would increase Cu_B^{2+} affinity for HCN. As shown in [6,9,19], HCN binding to the intermediary site requires protonation of a cytochrome oxidase ionizable group with pK 6.6–6.8 and it is tempting to identify this group as one of the Cu_B^{2+} ligands (histidine or, possibly, hydroxyl), protonation of which facilitates its displacement by HCN [12,21]. Cytochrome c^{3+} binding can be suggested to loosen this endogenous ligand bond to Cu_B^{2+} and thus to increase the effective affinity of Cu_B^{2+} for HCN.

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slow phase — rapid phase (c^{3+}-stimulated)

$$E_{Fe}^{Cu} \xrightarrow[k_v \sim 10^{-4}\ s^{-1}]{} E_{Fe}^{Cu} \qquad E_{Fe}^{Cu} \underset{K_{eq} \approx K_m = 10^{-2}\text{–}10^{-5}\ M}{\overset{HCN}{\rightleftharpoons}} E_{Fe}^{Cu\text{-}HCN} \xrightarrow[k_{max} \sim 10^{-2}\ s^{-1}]{H^+} E_{Fe\text{-}CN}^{Cu}$$

non-reactive ($g = 12$ form [5]), ~65%	reactive, ~35%	inhibited, invisible complex	inhibited, visible complex

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