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Rates of cyanide binding to the catalytic intermediates of mammalian cytochrome *c* oxidase, and the effects of cytochrome *c* and poly(L-lysine)

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Rate constants of cyanide binding to 'fast' oxidase have been measured in the fully-oxidised (O), peroxy (P) and ferryl (F) states at pH 8.0. Values of 2.2, 8 and 10 $M^{-1} s^{-1}$, respectively, were obtained. Thus, none of these states appears to exhibit a rate that would identify it as the species responsible for the extremely rapid cyanide binding observed during turnover. On the other hand, with 'oxidised' enzyme as prepared, containing a very small fraction of one-electron-reduced (E state) oxidase, a corresponding fraction of enzyme exhibited spectral changes consistent with cyanide binding with a rate constant in excess of 10^3 $M^{-1} s^{-1}$. Evidence is presented suggesting that mediation of electron transfer from one-electron-reduced, cyanide-liganded enzyme to free, ferric oxidase, rather than a global protein conformational change of the enzyme, is responsible for the greatly enhanced cyanide binding rates seen in the presence of cytochrome *c* or poly(L-lysine). Inter-oxidase electron exchange in 'oxidised' enzyme can result in a complicated dependence of the binding rate on cyanide concentration. We have demonstrated that this may give rise to a saturation of the rate of cyanide binding.

Introduction

Binding to cyanide cytochrome oxidase, and its consequent inhibition, has been the subject of a considerable research effort spanning several decades. However, important aspects remain unclear. Nicholls et al. [1] recognised that, although the rate of binding to fully-reduced enzyme was much more rapid than to the fully-oxidised species, the rate of onset of inhibition under turnover conditions was at least an order of magnitude greater still. Jones et al. [2] proposed that during turnover a transient, 2- or 3-electron-reduced form of the enzyme is responsible for the observed rapid binding. In terms of the total enzyme population, the observed rate constant was $4.5 \cdot 10^3$ $M^{-1} s^{-1}$ (Thörnström et al. [3] obtained similar values). If the occupancy of the cyanide-binding form is as low as these workers suggest, its rate constant would be in the region of 10^6 $M^{-1} s^{-1}$, compared with only about 2 $M^{-1} s^{-1}$ for the fully-oxidised form. This dramatic increase in rate has been interpreted in terms of a conformational change to an 'open' configuration [3,4], although the number of electrons required to bring about the change remains controversial [5,6].

Many earlier studies were complicated by the use of mixed enzyme preparations containing a proportion of slowly reacting, resting oxidase. In an attempt to measure the cyanide-binding rate to a pulsed preparation, Jones et al. [2] used an aerated sample that had been dithionite-reduced, a procedure that is likely, however, to yield a considerable proportion of enzyme in the peroxy (i.e., 2-electron-reduced, oxygenated) state [7]. The slightly enhanced rate thus obtained was similar to that reported by Brittain and Greenwood for 'oxygenated' oxidase [8] produced by photolysis of the mixed-valence CO compound, presumably also mainly in the peroxy (Compound C) state [9]. Naqui et al. [10], however, found the rate of the initial phase of binding to be unchanged after dithionite reduction and reoxidation, but now showing simple kinetics rather than the two or three distinct phases of binding formerly occurring. With the present availability of stable, fast preparations, and increased awareness of the occurrence and spectral characteristics of the partly reduced intermediates of the oxidase reaction, further progress in identifying the rapid binding form(s) appeared to be possible. This led us to examine the rates of binding to the fully oxidised (O), peroxy (P) and the three-electron-re-

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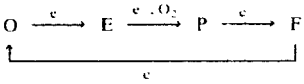
duced oxy-ferryl (F) forms. (See simplified scheme below, where uptake and release of protons and water are omitted.)



A further incentive was the realisation that, because of the enormously enhanced binding rate on partial reduction [2], it is crucially important to control the access of electrons when attempting to measure the binding of cyanide to the O state. It appeared possible that availability of reducing equivalents might explain the greatly enhanced O-state binding rate reported in intact mitochondria [11], or in the presence of added cytochrome *c* and other polycations [12].

Materials and Methods

'Fast' beef heart cytochrome *c* oxidase was isolated by a modification of the Kuboyama method as previously described (prep. D of Ref. 13). The resulting preparation reacts nearly monophasically with cyanide, with typically about 10% of the progress curve of cyanide binding exhibiting a rate constant two orders of magnitude less than that of the bulk population.

Reagents were used as commercially supplied, except for cytochrome *c* (Sigma Type VI), which was pretreated for 30 min with a 2-fold excess of potassium ferricyanide before passing through a Sephadex G-25 column in order to obtain the purely ferric form. Poly(L-lysine) hydrobromide (4–15 kDa) was obtained from Sigma. In some experiments, the presence of low levels (tens of parts per million) of ferrocyanide in some batches of analytical grade ferricyanide was found to have a significant effect. That which appeared to be least contaminated, as judged from the formation of Prussian blue on addition of ferric chloride, was purchased from BDH, Poole. Stock solutions of 0.1 or 1 M cyanide were prepared daily, without neutralisation, and kept on ice in a stoppered glass container. When lower concentrations were required to provide convenient volumes for addition, these were diluted immediately before use.

Optical measurements were made using a single-beam spectrophotometer assembled in house. Cyanide binding rates were generally measured at 432–412 nm, or, with high ferricyanide concentrations, at 587–620 nm in a 1 cm light-path cuvette with manual stirring. A cycle of three measurements was used, e.g., 412, 432, 412 nm, taking about 3 s per cycle. Further details applicable to measurements involving the peroxy and ferryl species are given in figure legends. Where cyanide binding was rapid enough for the time course to be followed nearly to completion, apparent rate constants were obtained by fitting two independent exponentials using the Simplex method [14]. The values given are for the majority, fast population of the enzyme unless otherwise stated. At low binding rates, where it was impracticable to follow the reaction to completion, the signal expected for complete cyanide binding was generally assumed to be unchanged, and the rate constant was calculated from the initial rate. The slight change of concentration of free cyanide during binding has not been corrected for, since in the low-cyanide experiments only the early part of the binding curve was normally measured.

Generation of the peroxy state

This was achieved using carbon monoxide to reduce the enzyme. In the presence of oxygen, which successfully competes with CO for the half-reduced oxidase thus formed, the peroxy compound is generated [15]. The aerobic, ferric enzyme was very briefly (2 or 3 s) bubbled with CO in the spectrophotometer cuvette. After about 4 min, when P formation appeared to be complete, the difference spectrum showed what appeared to be a virtually quantitative conversion, based on an extinction coefficient at 607 nm of about 10 mM^{-1} cm^{-1} [16], with no evidence of a shoulder at 590 nm that would indicate the presence of CO compound or an oxyferryl (F) state. The peroxy form decayed very slowly to the O state (see upper trace of Fig. 6A).

Generation of the ferryl state

The F compound was formed by addition of hydrogen peroxide. 60% (w/v) H_2O_2 was added to the ferric oxidase in the cuvette to a final concentration of 72 mM. After formation of F, taking less than 20 s, catalase (Sigma C-100) at a final dilution of 27 units/ml was added to remove excess peroxide. If this was omitted, slow spectral changes occurred, taken to indicate decomposition of the oxidase. The difference spectrum following addition of peroxide and catalase showed a broad peak at 584 nm with a smaller one at 535 nm. On the basis of an extinction coefficient of about 5 mM^{-1} cm^{-1} at 584 nm [16], about 80% conversion to the F state was indicated. During the subsequent decay to the O state, with a half-time of several minutes, a slight shoulder at about 605 nm was usually seen, the amount being so small that it was impossible to determine whether this was due to slight haem *a* reduction, or to P-state formation.

Results

Cyanide binding to 'oxidised' cytochrome oxidase

We use the term 'oxidised' to denote the enzyme state as prepared, where although a high proportion (> 95%) of enzyme molecules are in the fully oxidised

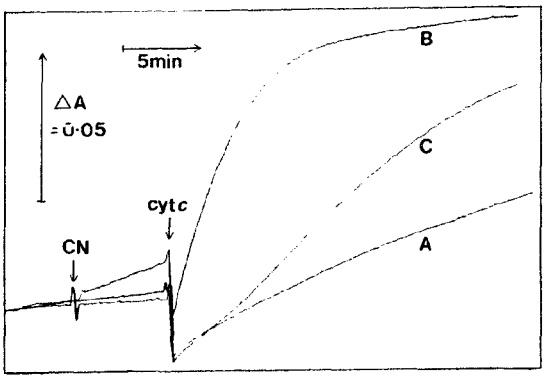


Fig. 1. Ionic strength dependence of cytochrome-*c*-stimulated cyanide binding. To a medium containing 10 mM glycylglycine and 0.1 mM EDTA, brought to pH 8.0 with KOH, were added 3 μM cytochrome oxidase and, after 8 min, 10 μM KCN followed by 0.3 μM ferricytochrome *c*, as indicated. For traces B and C, the medium was supplemented with 30 mM and 163 mM K_2SO_4, respectively, raising the ionic strength to approximately 100 and 500 mM. Absorbance was measured at 432–412 nm, where ferricytochrome *c* also absorbs, accounting for the downward step in the traces.

state, small subpopulations of partly reduced species may exist.

(i) Effect of ionic strength on the stimulatory effects of cytochrome c and poly(L-lysine)

The stimulatory effects of cytochrome *c* and poly(L-lysine) on cyanide binding to fast, 'oxidised' oxidase are illustrated in Figs. 1 and 2. It is clear that the ionic strength dependences for the two compounds are different. In the case of poly(L-lysine), significant stimulation occurs only at low ionic strength, whereas with cytochrome *c* (and also in the absence of polycations) a maximal rate is achieved at intermediate ionic strength.

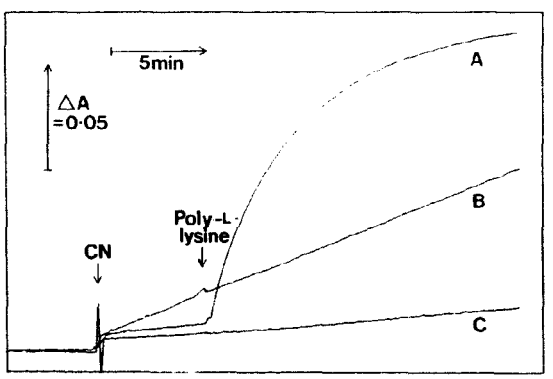


Fig. 2. Effect of ionic strength on poly(L-lysine)-stimulated cyanide binding. Conditions were as for Fig. 1, except that poly(L-lysine) was added as an 8 mg/ml solution to a final concentration of 24 μg/ml instead of cytochrome *c*. In B and C, potassium sulphate was added to 30 mM and 163 mM, respectively, as before.

With I = 200 mM (not shown), binding was marginally slower than at I = 100 mM. A similar dependence is found for the steady-state rate of oxidation of cytochrome *c* by oxidase [17], presumably because both V_{max} and K_m increase with ionic strength [18], with the latter effect becoming the more significant at high value. The increase of K_m for cytochrome *c* with increasing ionic strength is not unexpected, since the affinity of the positively charged cytochrome *c* for the negatively charged docking site on the oxidase would be affected by charge neutralisation. The observation that the rate of cytochrome-*c*-stimulated cyanide binding falls off steeply at low ionic strength would not be predicted on the basis of a conformational mechanism, and rather indicates that the stimulatory effect of cytochrome *c* on cyanide binding may arise from its ability to shuttle electrons between the enzyme molecules. Furthermore, a substoichiometric amount of cytochrome *c* is capable of causing rapid cyanide binding to all of the enzyme (Fig. 1).

In the absence of cytochrome *c* or poly(L-lysine), there is also a considerable stimulation of the rate of cyanide binding to 'oxidised' enzyme at intermediate ionic strength. (See traces immediately following cyanide addition in Figs. 1 and 2.) On the other hand, with 5 mM instead of 10 μM cyanide under otherwise similar conditions (not shown), the rate was nearly independent of the ionic strength of the medium in this range, varying by less than 2%. It thus appears that, although the rate of cyanide binding to fully oxidised oxidase is ionic-strength-independent, there may be a significant contribution resulting from inter-enzyme electron transfer at low cyanide concentrations. When the concentration of enzyme was varied from 1.2 to 4.8 μM, with the conditions otherwise as in Fig. 1B (but no cytochrome *c* added), the apparent rate constant increased by a factor of 1.5 with each 2-fold increase in concentration. This again is consistent with a major involvement of inter-enzyme electron transfer.

(ii) Effects of ferro- and ferricyanide on the stimulatory effects of cytochrome c and poly(L-lysine)

Since the above experiments indicated that movement of a small population of electrons between oxidases might be the cause of stimulation by cytochrome *c* and poly(L-lysine), it was of interest to check whether the availability of electrons was a significant factor in determining the cyanide binding rate. In experiments (not shown) similar to that of Fig. 1, trace A (i.e., at high ionic strength), addition of 0.2 mM ferricyanide completely reversed the cytochrome-*c*-induced acceleration. With ferrocyanide, on the other hand, a further 18-fold stimulation occurred. The early portion (approx. 50%) of the ferrocyanide-stimulated binding exhibits an apparent second-order rate constant of $2.3 \cdot 10^3$ M^{-1} s^{-1}, i.e., more than an order of magnitude

faster than for the fully reduced enzyme [2]. The experiment illustrates the crucial influence of a small fraction of partly reduced enzyme on the observed cyanide binding rate. It is clear that in the presence of sufficient ferricyanide, cytochrome *c* does not significantly stimulate cyanide binding. When the experiment was repeated in the absence of cytochrome *c*, only a minor change was produced by addition of ferro- or ferricyanide, reflecting the low rate of exchange of electrons between these negatively charged complexes and cytochrome oxidase in the absence of cytochrome *c*.

The effect of ferrocyanide on the poly(L-lysine)-stimulated rate is less pronounced than in the case where cytochrome *c* is able to mediate the transfer of electrons, but it is nevertheless quite significant, and the greater part of the stimulation is reversed with ferricyanide. In experiments similar to that shown in Fig. 2, trace A, but with 60 μg poly(L-lysine)/ml, nearly monophasic binding was observed with a pseudo-first-order rate constant of $3.6 \cdot 10^{-3}$ s^{-1}. When 1 mM potassium ferri- or ferrocyanide was present, the respective rate constants were $1.1 \cdot 10^{-3}$ s^{-1} and $1.4 \cdot 10^{-2}$ s^{-1}. This suggests that the poly(L-lysine) also is promoting cyanide binding by facilitating electron transfer between enzyme molecules, presumably while these are bound to the poly(L-lysine) chain. Controls indicated that the effects of potassium ferri/ferrocyanide were due to neither ionic strength nor K^+ ion concentration changes.

(iii) The E-state subpopulation of 'oxidised' enzyme and its rate of cyanide binding

In the absence of cytochrome *c* or poly(L-lysine), (see, for example, the early portions of Figs. 1 and 2) the apparent second-order rate constant of cyanide binding to 'oxidised' enzyme may be as high as 40 M^{-1} s^{-1}, considerably higher than the value of about 2 M^{-1} s^{-1} generally accepted for isolated fully oxidised oxidase [19]. Addition of cyanide initially caused a small, rapid absorbance step. The spectral changes responsible for this feature, while difficult to measure precisely, include a peak at about 430 nm and a broad peak around 580 nm, consistent with cyanide binding, and also a small, sharp peak at 604 nm with no accompanying change at 444 nm, consistent with electron transfer from haem a_3 to haem *a*. The extent of the absorbance step was found to show considerable variation between different enzyme samples and tended to decline slowly over several hours subsequent to thawing. It could be virtually eliminated by 20 min. incubation in the presence of ferricyanide at 25°C. The rate of this fast step was approximately proportional to the cyanide concentration for the range 0.5 to 10 μM, with the extent remaining unchanged. Time-courses of absorbance at 432–412 nm following addition of 1 or 10 μM cyanide are shown in Fig. 3. With a second-order rate constant

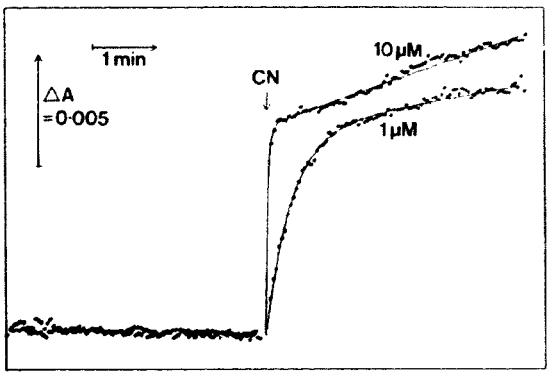


Fig. 3. The small, rapid cyanide binding step. Conditions as for Fig 1A, measured at 432–412 nm. 1 or 10 μM cyanide additions. The data points are fitted with the sum of two exponentials. 1 μM cyanide: $k_1 = 0.042$ s^{-1}, $k_2 = 7.2 \cdot 10^{-5}$ s^{-1} 10 μM cyanide: $k_1 = 0.42$ s^{-1}, $k_2 = 1.4 \cdot 10^{-4}$ s^{-1} with the final extent of the second phase taken to be $\Delta A = 0.13$ in both cases.

of approx. $4 \cdot 10^4$ M^{-1} s^{-1}, it appeared likely that a fraction of the enzyme was either in the partly reduced state(s) exhibiting extremely fast cyanide binding [2], or in a form in rapid equilibrium with it.

We have previously observed [20,21] that the aerobic enzyme as prepared commonly contains a small fraction of the one-electron-reduced species, referred to as the E state, not oxidisable by oxygen, but slowly oxidised by ferricyanide. Fig. 4 shows the inverse of the difference spectrum obtained following ferricyanide addition and several minutes incubation. The extent of the absorbance change at 605 nm would be consistent

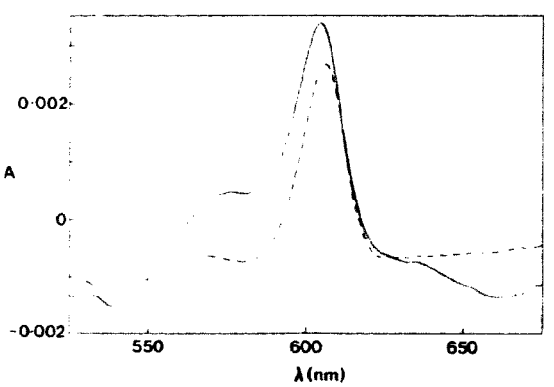


Fig. 4. Difference spectrum following incubation with ferricyanide. Cytochrome oxidase (4.8 μM) was incubated for 8 min in the same medium as for Fig. 1A. After adding 5 mM potassium ferricyanide, incubation was continued for a further 80 min. Spectra were taken at intervals throughout the experiment. The final spectrum is subtracted from that taken immediately before the ferricyanide addition, and the difference compared with the reduced-oxidised spectrum of haem *a* at about 0.15 μM (broken line), obtained by reduction of cyanide-liganded enzyme.

TABLE I

Apparent pseudo-first-order (k_1) and second order (k_2) rate constants of cyanide binding in the absence (−) and presence (+) of 20 mM ferricyanide

The basic medium contained 0.1 M glycylglycine and 1 mM EDTA, all as potassium salts, pH 8.0, 25°C. Each value is the mean of at least three experiments, with standard error.

CN (mM)	$K_3Fe(CN)_6$		$+ K_3Fe(CN)_6$		$10^4 (k_1 - k_1')$ (s^{-1})
	$10^4 k_1$ (s^{-1})	k_2 ($M^{-1} s^{-1}$)	$10^4 k_1'$ (s^{-1})	k_2' ($M^{-1} s^{-1}$)	
0.01	2.9 ± 0.4	29	0.21 ± 0.02	2.1	2.7 ± 0.4
0.2	6.4 ± 0.4	3.2	3.7 ± 0.5	1.9	2.7 ± 0.6
10	225 ± 4	2.3	223 ± 4	2.2	2.0 ± 5

with oxidation of just over 3% of the haem *a* present. However, in comparison with a pure haem *a* spectrum, a shoulder is evident on the low-wavelength side of the 605 nm peak, consistent with reduced haem a_3, and a trough at about 655 nm indicates that either haem a_3 or Cu_B [22,23] was reduced before incubation with ferricyanide. The spectrum resembles that obtained following a one-electron flash-reduction (see Fig. 2 of Ref. 20) to produce the E state. The one electron in the E state thus appears to be free to equilibrate between the redox centres. In the present case, however, the above-mentioned shoulder is more prominent, probably indicating that some ferryl enzyme was also present. The fraction of enzyme that appears to be in the E state is sufficient readily to account for the small proportion showing extremely rapid cyanide binding.

(iv) The rate of cyanide binding to the O-state enzyme

The question now arises as to what proportion of the observed binding rate to the 'oxidised' enzyme is in fact due rather to slow, continuing E state formation, with subsequent rapid cyanide binding, as the electron is transferred from a cyanide-bound enzyme molecule to a free, fully oxidised oxidase. This was addressed by measuring the inhibitory effect of ferricyanide at a range of cyanide concentrations (Table I). In the absence of ferricyanide, we find that the rate becomes less dependent on cyanide concentration at higher levels. However, within experimental error, a constant amount of the cyanide binding rate is abolished by ferricyanide at all cyanide concentrations tested (last column of Table I). The remaining, ferricyanide-insensitive rate is proportional to cyanide concentration. In other words, 'saturation' behaviour is no longer observed in this range. The second-order rate constant of this ferricyanide-insensitive reaction, which may be taken as the actual O state binding reaction, is found to be 2.2 $M^{-1} s^{-1}$ at pH 8.0 and 25°C, similar to published values for the early part of the binding curve for ferric enzyme [10,19].

Binding of cyanide to the ferryl state

Following the generation of the ferryl enzyme with hydrogen peroxide (see Materials and Methods), a slow reversion to the O state occurred. The effect of cyanide on the disappearance of the ferryl compound is shown in Fig. 5A as measured at 562–546 nm. At this wavelength pair, the absorbance of the O and cyanide-bound

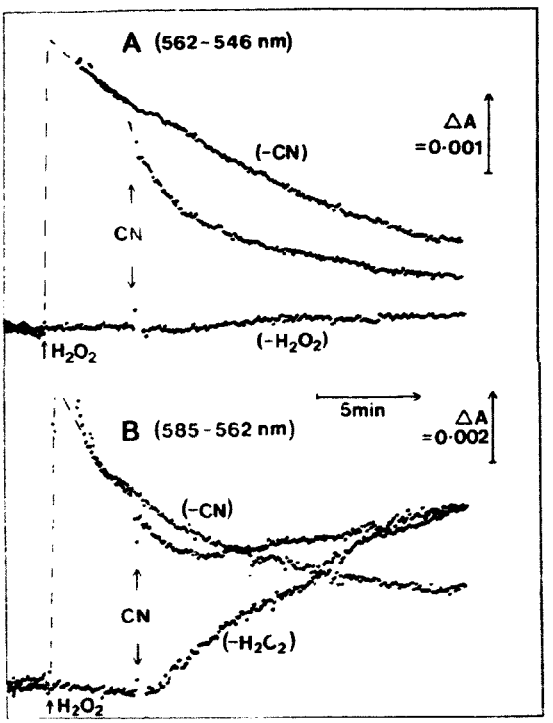


Fig. 5. Addition of cyanide to the ferryl compound. The medium contained 0.1 M glycylglycine (pH 8.0), 1 mM EDTA, 0.1 mM $K_3Fe(CN)_6$, to which was added 2.5 μM cytochrome oxidase. After 2 min, hydrogen peroxide was added, followed by catalase (see Methods) and, after a further 4 min, 0.5 mM cyanide. Control experiments are also shown, where either H_2O_2 /catalase or cyanide was omitted.

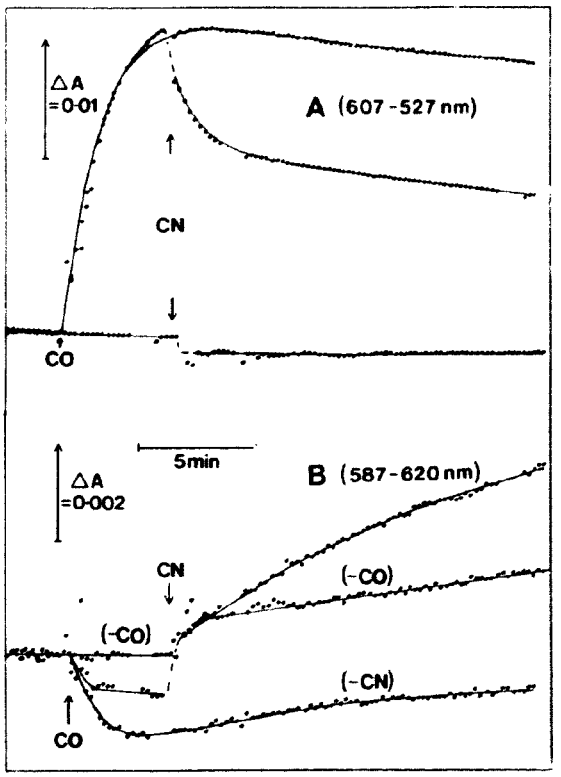


Fig. 6. Addition of cyanide to the peroxy compound. Conditions were as for Fig. 5, except 1 mM ferricyanide present. CO was added instead of H_2O_2 and catalase (see Materials and Methods), cyanide was decreased to 50 μM final concentration.

states is the same, so that, in the absence of peroxide pretreatment, cyanide has little or no observed effect. Although cyanide clearly accelerates the decline of the ferryl signal, it cannot be determined whether cyanide binding is occurring or not. At 585–562 nm, however, the ferryl and cyanide-bound states absorb nearly equally, and, as shown in Fig. 5B, using data taken from the same experiments as Fig. 5A, cyanide does not significantly accelerate the decline of the peroxide-induced signal. It thus appears that with the cyanide-stimulated disappearance of the ferryl compound there is concomitant cyanide binding. The apparent rate constant, taken from the data of Fig. 5A, subtracting the background decay, was found to be $4.9 \cdot 10^3$ s^{-1}, i.e., a second-order cyanide binding rate constant of about 10 M^{-1} s^{-1}.

Cyanide binding to the peroxy state

A procedure was adopted similar to that used in the case of the ferryl compound, except that the peroxy enzyme was most conveniently formed by bubbling briefly with carbon monoxide in the presence of oxygen. Fig. 6A shows formation of the P state at 607–527 nm and its cyanide-accelerated decay. Taking the extinction coefficient to be about 10 mM^{-1} cm^{-1}, conversion was approximately complete. At this wavelength pair, cyanide binding to the O state is spectrally silent. At 587–620 nm (Fig. 6B), on the other hand, the O → P transition has little effect, but a strong signal is obtained from formation of the cyanide compound. The rate of cyanide binding is increased in the sample gassed with CO, but there is no transient fast binding corresponding to the partial disappearance of the 607 nm signal. Spectra taken before addition of cyanide and immediately after the rapid decline at 607 nm (not shown) show an apparent reversion to the O state with very little cyanide binding, consistent with Fig. 6B. If the cyanide concentration is increased from 50 to 500 μM (not shown), the rate and extent of this reversion are also increased, but again with no corresponding feature at 587–620 nm.

Unlike the case of the F state, where the cyanide-induced acceleration of the disappearance of the ferryl compound can be taken as a measure of the binding rate, in order to estimate the approximate rate of cyanide binding to the peroxy compound from these data, it is necessary to know the proportion of enzyme in each state. The apparent rate constant for the upper trace of Fig. 6B declines with time, presumably as the concentration of P state enzyme decreases. Taking the fairly stable region about 10 min after addition of cyanide, where, assuming an initial 100% conversion to the P state, the 607 nm trace indicates some 54% remaining, cyanide binding (Fig. 6B) is consistent with a second-order rate constant of 8 M^{-1} s^{-1}, at which point about 28% of the enzyme appears to be cyanide-bound as measured at 587–620 nm. (Any traces of ferryl enzyme would be included in this estimate, but would make no contribution to the observed rate, since the cyanide-bound and ferryl forms have the same extinction coefficient at this wavelength pair.) This is reasonably close to the value of 10 M^{-1} s^{-1} obtained by Jones et al. for the dithionite-reduced and re-aerated enzyme (a procedure which also presumably generated predominantly peroxy enzyme).

Discussion

Identification of the form of oxidase that binds cyanide rapidly

As well as measuring the spectrally observable rates of cyanide binding to the partly reduced states of oxidase that are easily obtained in a reasonably stable and pure state, i.e., the peroxy and ferryl forms, we have attempted to discover whether a specific intermediate of the catalytic cycle can be identified as the rapidly binding form, and to determine whether there

is evidence pointing to global protein conformational changes. The observed rates of cyanide binding to various reduction states of the enzyme are summarised in Table II. Clearly, the rapidly binding form(s) of the enzyme must be looked for in the 'E state' category. We feel reasonably confident that this is indeed largely a one-electron-reduced state from its stability in the presence of oxygen, and its spectral similarity to the species formed following photoreduction with low levels of reductant, where the likelihood of two-electron reduction is small [20,29]. There is good evidence from these flash-induced reduction studies that, in contrast to the resting enzyme, with our fast preparations a single electron on haem *a* is able rapidly to equilibrate with the binuclear centre. Preliminary experiments (Moody, J., unpublished data) on flash-induced generation of E-state enzyme indicate that rapid cyanide binding ($> 10^4$ M^{-1} s^{-1}) could be induced. Clearly, other states are possible that may also exhibit rapid cyanide binding, e.g., the two-electron-reduced species before reaction with oxygen. Under aerobic conditions and with micromolar concentrations of cyanide, as reported here, however, an enzyme molecule in this state would be extremely unlikely to react with cyanide before combining with oxygen [28].

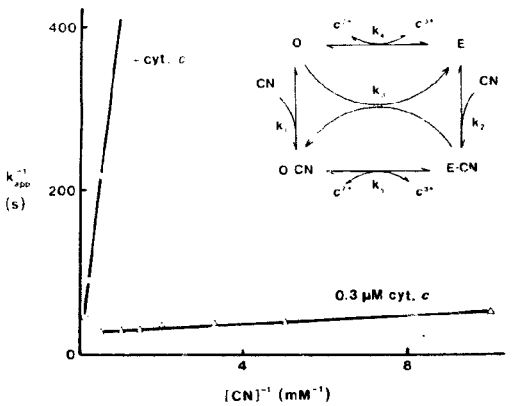


Fig. 7. Variation of apparent cyanide binding rate with cyanide concentration. Data points were generated from the model (inset), taking the enzyme concentration to be 3 μM with initially 98% O state, 2% E state. Inset: Model for cyanide binding to oxidised cytochrome oxidase with a proportion of E-state enzyme present. 'Forward' reactions are in the direction of reduction and cyanide binding. For reaction 3, only the forward direction is shown for the sake of clarity. Rate constants were taken to be: $k_1 = 2.25$ M^{-1} s^{-1}, $k_{-1} = 2 \cdot 10^{-6}$ s^{-1}, $k_2 = 4 \cdot 10^4$ M^{-1} s^{-1}, $k_{-2} = 4 \cdot 10^{-3}$ s^{-1}, $k_3 = k_{-3} = 4.15 \cdot 10^3$ M^{-1} s^{-1}, $k_4 = k_{-4} = k_5 = k_{-5} = 2 \cdot 10^6$ M^{-1} s^{-1}.

Simulation of results

A model that appears sufficient to account for our observations on the effect of electron mediation between enzyme molecules on the cyanide binding rate and on the cyanide concentration dependence of observable cyanide binding is shown in Fig. 7 (inset). Inter-enzyme electron transfers causing no net change are not shown. From the rate constants given in the figure legend, none of which appears to be inconsistent with published data, taking the cytochrome *c* concentration as zero, and the initial E-state fraction as 2% (− ferricyanide) or zero (+ ferricyanide), apparent constants within experimental error of those given in Table I were generated using an iterative simulation. The observed small, rapid binding phase is also closely simulated. The rate of disproportionation of E-state enzyme, i.e.:

$$2E + O_2 \rightarrow \text{fully-oxidised} + \text{peroxy}$$

using the value assigned to k_3, would be consistent

TABLE II

Second-order rate constants of ligand binding to mammalian cytochrome c oxidase

Form	pH	Ligand	k (M^{-1} s^{-1})	References
O 'Slow' (resting)	6.5–8	KCN	0.01–0.02	Moody et al. [13]
O 'Fast'	7–8	KCN	1.3–2.2	Moody et al. [13]; present work
E 'Resting'		KCN	unknown	
E 'Fast'	8.0	KCN	$> 10^4$	present work
'Mixed valence'		KCN	unknown	
'Mixed valence'	7.4	O_2	$(1–2) \cdot 10^8$	Varotsis et al. [24] Oliveberg et al. [25] Gru [26]
Peroxy	8.0	KCN	8	present work
Ferryl	8.0	KCN	10	present work
Reduced	7.4	KCN	35–130	Antonini et al. [27]
Reduced	7.4	O_2	$(1–2) \cdot 10^9$	Hill et al. [28]
'Open'	5.5–8.3	KCN	$> 10^4$	Jones et al. [2]
O 'Fast' + cyt c^{3+}	7	KCN	≈ 250	Musatov and Konstantinov [12]

with a half-life of 46 s at a concentration of 60 nM (i.e., 2% of 3 μM total enzyme), at which level autoreduction appears to maintain a kinetic equilibrium.

Fig. 7 shows a simulated double reciprocal plot of pseudo-first-order rate constants, using apparent rates generated by the model, versus cyanide concentration. Over a limited range of concentration, saturation behaviour is closely approximated. The simulated effect of cytochrome *c* is greatly to lower the apparent $K_{1/2}$ for cyanide (from about 70 mM to 50 μM with 0.3 μM cytochrome *c*), while the apparent V_{max} is nearly unchanged (cf. Ref. 12). It seems unnecessary to postulate, on the basis of the experimentally observed apparent saturation, that cytochrome oxidase forms a spectrally undetected prebinding complex with cyanide [11,19].

A spectrally silent cyanide complex has also been proposed in order to account for observations [5] of a slow spectral change subsequent to a relatively rapid onset of inhibition following cyanide addition. These experiments were carried out, however, with largely resting oxidase, suggesting a different interpretation. We would expect most of the enzyme activity to be due to a small 'fast' subpopulation, which would bind cyanide rapidly, causing nearly complete inhibition with little spectral change. This would be followed by a much larger, slow spectral change accompanying cyanide binding to the major, resting fraction, but with little further inhibition. It appears also from the present data that spectral indications of cyanide binding to a small, rapidly binding population may be observed with kinetics comparable with those reported for the onset of inhibition [2].

The chemical origin of rapid cyanide binding to the E-state enzyme

Since it appears that a one-electron reduction of the enzyme is able to bring about an acceleration of the cyanide binding rate by at least a factor of 10^4, it is of great interest to discover by what means this conversion to a rapidly binding state is achieved. It may be that this process is intimately involved in other enzyme functions, e.g., that of proton translocation across the osmotic barrier region. It is interesting to compare the rates of association of cyanide with other haemoproteins. Apparent binding rate constants to oxidised cytochrome *c* peroxidase, horseradish peroxidase and lactoperoxidase of the order of 10^5 M^{-1} s^{-1} or greater may be observed, comparable with those of the rapid binding species of cytochrome oxidase [30]. Metmyoglobin exhibits a lower rate, but one that is still more than two orders of magnitude greater than that of fully oxidised cytochrome oxidase. It appears, therefore, that it is the rate of cyanide binding to the O state rather than to the E state that is anomalous, and we should think in terms of the blocking of cyanide access.

Since electron entry at the cytochrome *c* binding site is remote from the binuclear centre where cyanide is bound, it is perhaps understandable that the effect of partial reduction has been thought of in terms of protein conformational changes [3,4,31,32], or 'action at a distance.' However, in 'fast' oxidase in the E state, it has been shown [29] that the single electron can equilibrate rapidly with the binuclear centre, and is thus in a position to influence the ligand binding site in a direct manner. (Rapid single-electron transfer between the binuclear centre and haem a/Cu_A is also reported in Ref. 33.) A comparable effect of partial reduction on azide binding kinetics has been observed by Nicholas Fisher in unpublished experiments carried out in this laboratory. Cyanide binding to *Escherichia coli* cytochrome *bo* appears to be similarly regulated (Rich, P.R., unpublished work). At present, it seems logical to investigate the simpler possibility of direct chemical action to account for the profound binding rate changes before considering long-range protein conformational mechanisms. It may be possible from examination of the kinetics of photoreduction-induced binding to determine whether Cu_B reduction is a requirement. If that proves to be the case, one might envisage a gating action of Cu_B whereby the access of cyanide to haem a_B is restricted. Such a mechanism could readily be accommodated in the type of proton-translocating configurational cycle envisaged by Mitchell [34].

Conclusions

Our interpretation of the above data may be summarised as follows. The rate of cyanide binding to fast, fully-oxidised cytochrome oxidase is approximately as previously found for the initial phase of binding to less homogeneous preparations. The somewhat higher rates found with 'pulsed' [2] or 'oxygenated' [8] enzyme are characteristic of the peroxy form. Fully oxidised, peroxy and ferryl oxidase can probably be ruled out as the rapid binding species populated during turnover. One-electron-reduced oxidase is capable of binding cyanide at a high ($> 10^4$ M^{-1} s^{-1}) rate, and therefore cytochrome *c* and other polycations that facilitate intermolecular electron transfer may greatly enhance the rate of ligation to predominantly ferric enzyme. The slow transfer of electrons, even in the absence of added cytochrome *c*, from one-electron-reduced cyanide complex to fully oxidised oxidase results in a background rate of cyanide binding that is nearly independent of cyanide concentration down to micromolar levels, giving a resemblance to saturation behaviour. There seems therefore to be no need to invoke an initial formation of an invisible cyanide complex to explain such behaviour [11,19]. In addition, from the available data, either in this report or in other pub-

lished material, there appears to be no necessity to consider global protein conformational changes, as opposed to local chemical changes of the binuclear centre itself, in order to account for the cyanide binding characteristics.

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