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A re-examination of the reactions of cyanide with cytochrome *c* oxidase*

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Experiments were performed to examine the cyanide-binding properties of resting and pulsed cytochrome *c* oxidase in both their stable and transient turnover states. Inhibition of the oxidation of ferrocytochrome *c* was monitored as a function of cyanide concentration. Cyanide binding to partially reduced forms produced by mixing cytochrome *c* oxidase with sodium dithionite was also examined. A model is presented that accounts fully for cyanide inhibition of the enzyme, the essential feature of which is the rapid, tight, binding of cyanide to transient, partially reduced, forms of the enzyme populated during turnover. Computer fitting of the experimentally obtained data to the kinetic predictions given by this model indicate that the cyanide-sensitive form of the enzyme binds the ligand with combination constants in excess of $10^6\,M^{-1}\cdot s^{-1}$ and with K_D values of 50 nM or less. Kinetic difference spectra indicate that cyanide binds to oxidized cytochrome a_3^{3+} and that this occurs rapidly only when cytochrome *a* and Cu_A are reduced.

The reactions of cytochrome *c* oxidase with classic inhibitors of respiration, such as CO and CN^-, have been widely studied and are of general concern (see Wikström *et al.*, 1981). Whereas the inhibition of cytochrome *c* oxidase by CO seems relatively simple, in that binding of this ligand to ferrocytochrome a_3 in the enzyme can fully explain the inhibitory effects, a simple description of the inhibition by cyanide is not available. Binding of cyanide to stable forms of the enzyme (e.g. fully oxidized and/or fully reduced) cannot account for the potency of this inhibitor.

The work of van Buuren *et al.* (1972*a*,*b*) suggested that transient, partially reduced, species, populated only in turnover, are the forms that are responsible for inhibition by cyanide. It has become evident, since that earlier report, that at least two enzymic forms exist with different catalytic-centre activities (turnover numbers). The two forms, termed resting and pulsed, have been identified and their properties described (Antonini *et al.*, 1977; Brunori *et al.*, 1979). These forms are possibly conformational variants, and transitions from the less-active resting (R) to the more-active pulsed (P) form accounts for activation of the enzyme during turnover. It is essential, therefore, to examine the cyanide-binding characteristics of each of these forms in their various oxidation states, in order to arrive at a complete understanding of cyanide inhibition. In the present paper we go part way towards this goal by reporting experiments on the cyanide-binding properties of resting and of pulsed cytochrome *c* oxidase in both their stable and transient states.

We present results below that allow us to discount binding of cyanide to the resting or the pulsed enzyme either in their oxidized or fully reduced states as the source of cyanide inhibition during turnover. On the contrary, and in agreement with the work of van Buuren *et al.* (1972*a*,*b*), we propose that it is a transient, partly reduced, form that binds cyanide. We have developed a model to account fully for cyanide inhibition of the enzyme, and in order to facilitate understanding of the Results and Discussion sections, we present the scheme here (see Scheme 1).

The essential features of the model are as follows.

*Dedicated to the memory of Professor Eraldo Antonini, a dear friend and colleague.

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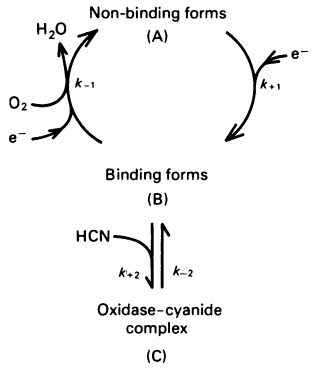


Scheme 1. *Model of HCN binding to cytochrome c oxidase during turnover*

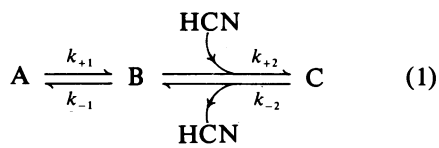
(1) The catalytic cycle of the enzyme involves intermediates that can be classified into cyanide-binding and non-cyanide-binding populations.

(2) The binding form(s) (B) is depicted as a partially reduced species by having electron-entry steps previous to its formation and subsequent to its decay. The number of electrons in the binding form will be discussed later below. The non-binding form(s) (A) includes all the other states of the enzyme. Other electron-transfer reactions and the reactions with O_2 involve the non-binding form.

(3) k_{+1} and k_{-1} are first-order rate constants for steps in the turnover cycle that are rate-limiting for the production and decay of the cyanide-binding species.

(4) k_{+2} represents the second-order combination rate constant for cyanide with the transient binding forms of the enzyme, and k_{-2} the cyanide dissociation rate constant from the bound form (C).

(5) For simplicity, and so that we may use this model for calculations, we have supposed that during turnover the forms A and B are sequentially connected such that only one step in either direction (those labelled k_{+1} and k_{-1}) link those forms. In essence, then, the model collapses to a quasi-pre-equilibrium form where intermediates other than those preceding rate-limiting steps are at negligible concentration, i.e.:



(1)

A mathematical description of this model is presented in the Methods section, and the computed time courses for cytochrome *c* oxidation are compared with experimental findings in the Results section.

Comparisons between the results of computation based on the model and experiments monitoring the oxidation of cytochrome *c* lead us to conclude that it is indeed a partially reduced form of the enzyme that is responsible for rapid cyanide binding and thus inhibition during turnover.

We have also carried out experiments to examine cyanide binding to partially reduced forms of the enzyme by mixing cytochrome *c* oxidase with $Na_2S_2O_4$ in the presence of cyanide. These experiments show that cyanide binds rapidly and tightly to cytochrome a_3^{3+}, in general agreement with the conclusion derived from turnover experiments.

Materials

Bovine heart cytochrome *c* oxidase was prepared by the method of Yonetani (1961), and its concentration is expressed in terms of functional units containing two haem *a* groups ($\varepsilon^{red.}_{605} = 42000\,M^{-1}\cdot cm^{-1}$). The preparation of the forms of the enzyme used in the present study are described in the Figure legends.

Cytochrome *c* (type III) ($\varepsilon^{red.}_{550} = 27000\,M^{-1}\cdot cm^{1-}$) was obtained from Sigma Chemical Co. Ferrocytochrome *c* solutions were prepared by reduction with $Na_2S_2O_4$ followed by removal of the reductant by passage down a 1cm × 10cm column of Sephadex G-25. All protein solutions were prepared in 100mM-sodium phosphate buffer, pH7.4, containing 0.1% Tween 80.

KCN solutions were prepared in 100mM-sodium phosphate buffer and adjusted to neutrality with conc. HCl. The solutions were kept tightly sealed with a slight positive N_2 pressure, and samples were removed with a syringe. The cyanide concentrations were checked by titration against standard $AgNO_3$ solution and were found to conform to expectation to within 1%.

Solutions of $Na_2S_2O_4$ at known concentrations were prepared assuming M_r 174 and 92% purity, as determined by redox titration against lumiflavin acetate (Lambeth & Palmer, 1973). Dithionite solutions were prepared under N_2 with degassed buffer and were used within a few hours of preparation.

Static spectra and static titrations were recorded on a Perkin–Elmer type 575 spectrophotometer. Kinetic measurements were made with a Durrum–Gibson stopped-flow apparatus equipped with a 2cm observation chamber (dead time approx. 3ms). A DataLab DL 901 transient recorder interfaced to a Commodore PET microcomputer was used to store and display the data. Numerical integration of differential rate equations was performed with a PDP-11 Minc minicomputer (see below).

Methods

We have used the model given in the introduction to determine the time course of cyanide binding and thus the concentration of the oxidase–cyanide complex at any time. We have then used this information, together with the well-documented exponential time course for the oxidation of cytochrome *c* catalysed by cytochrome *c* oxidase without inhibitors, to calculate the concentration of ferrocytochrome *c* as a function of time, i.e.:

$$C^{2+} = C_0^{2+} \cdot \exp(-k_{app.} \cdot t)$$

where C represents cytochrome *c*, $k_{app.}$ is the apparent rate constant for the exponential cytochrome *c* oxidation, which we have assumed to be directly proportional to the free oxidase concentration. [In fact this is found experimentally not to be strictly true, but it is closely approximated over the oxidase concentration range that we are considering (Errede & Kamen, 1978).] In the presence of cyanide the value of $k_{app.}$ is thus time-dependent, falling as the active oxidase is depleted on binding cyanide. The value of $k_{app.}$ is given as follows:

$$k_{app.} = k_{cat.} \cdot \frac{[\text{oxidase}]_{active}}{[\text{oxidase}]_{total}} \qquad (2)$$

where $[\text{oxidase}]_{total}$ is the total concentration of oxidase, $[\text{oxidase}]_{active}$ the concentration of the uninhibited (cyanide-free) enzyme and $k_{cat.}$ is the value of the rate constant for cytochrome *c* oxidation determined in the absence of cyanide.

Knowing the concentration of oxidase–cyanide complex (C of Scheme 1) at any time allows the concentration of active, non-cyanide-bound, forms (A plus B) to be calculated by subtracting the concentration of bound enzyme from the total oxidase concentration.

The concentration of the oxidase–cyanide complex was calculated from a set of first-order differential equations (see below). These equations were solved numerically by taking very short time intervals (approx. 100 μs) and assuming that the differential of a concentration with respect to time, i.e. d(concentration)/d*t*, could be approximated to an algebraic form Δ(concentration)/Δt. As this procedure is not ideal for 'stiff' equations, the method was tested by comparing the analytical solutions with the results of this method. The analytical solution can, however, only be found by assuming that the HCN concentration does not change during binding, i.e. under pseudo-first-order conditions. Under such conditions we obtained identical fits (within our quoted errors) by both the analytical and the numerical procedures. We report in our Figures the results of our numerical analyses, as these are also applicable to low-cyanide regimes.

The equations describing the scheme for cyanide binding are as follows:

$$\frac{d[A]}{dt} = -k_{+1}[A] + k_{-1}[B] \approx \frac{\Delta[A]}{\Delta t} \qquad (3)$$

$$\frac{d[B]}{dt} = k_{+1}[A] - k_{-1}[B] + k_{-2}[C] - k_{+2}[B][HCN] \approx \frac{\Delta[B]}{\Delta t} \qquad (4)$$

$$\frac{d[C]}{dt} = k_{+2}[B][HCN] - k_{-2}[C] \approx \frac{\Delta[C]}{\Delta t} = -\frac{\Delta[HCN]}{\Delta t} \qquad (5)$$

Results

Inhibition of cytochrome c oxidation by cyanide

We have conducted a number of stopped-flow experiments in which reduced cytochrome *c* was mixed with either oxidized resting (R_o) or oxidized pulsed (P_o) cytochrome *c* oxidase in the presence of O_2 (air-equilibrated buffer) and in the absence or in the presence of known concentrations of cyanide.

The concentrations of enzyme and cytochrome *c* are chosen so that oxidation of all the cytochrome *c* is achieved in a few turnovers (generally about five). Such conditions allow the effect of cyanide on the resting enzyme to be studied before a significant proportion of cytochrome *c* oxidase is converted into more-active pulsed species (Brunori *et al.*, 1979).

Figs. 1 and 2 present sets of progress curves obtained at different cyanide concentrations, and compare the results of experiment with those derived from the model described in the introduction. The kinetic parameters for these simulations are given in the legends to Figs. 1 and 2. The range of cyanide concentrations explored in experiments of this type extends from 0 to 5 mM (after mixing). In the absence of cyanide the time course of cytochrome *c* oxidation was found to be exponential for both states of the enzyme. In the presence of cyanide the rate of cytochrome *c* oxidation declines with time, and above 50 μM-cyanide the velocity of the process approaches zero (i.e. the enzyme becomes completely inhibited). At low concentrations of cyanide (say less than 10 μM) the rate constant for cytochrome *c* oxidation does not approach zero because not all of the oxidase is inhibited at equilibrium. In this respect our results at lower cyanide concentrations are in agreement with results reported by van Buuren *et al.* (1972*a*).

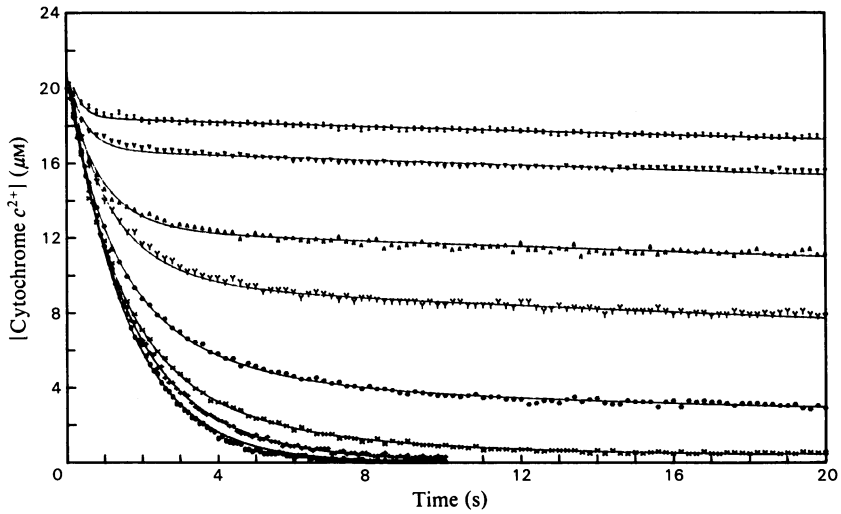


Fig. 1. *Progress curves for the cyanide inhibition of resting cytochrome c oxidase during turnover*
The reactions were performed by mixing 50 μM-ferrocytochrome *c* with 5 μM (functional units)-cytochrome *c* oxidase in the stopped-flow apparatus. Cyanide was added to the cytochrome *c* syringe to obtain the following concentrations after mixing (listed from bottom to top): 0 μM, 50 μM, 100 μM, 250 μM, 500 μM, 1 mM, 2.5 mM, 5 mM. The buffer was 100 mM-sodium phosphate, pH 7.4, containing 0.1% Tween 80. The monitoring wavelength was 550 nm and the temperature 25°C. The points show the experimental results, and the continuous lines were calculated in accordance with Scheme 1. The parameters used in the simulation were: $k_{+1} = 3.10 \pm 0.20\,s^{-1}$; $k_{-1} = 2050 \pm 200\,s^{-1}$; $k_{+2} = 10^6\,M^{-1}\cdot s^{-1}$; $k_{-2} = 0.015 \pm 0.010\,s^{-1}$. The measured value of the uninhibited rate of cytochrome *c* oxidation, $k_{cat.}$, was $0.57 \pm 0.20\,s^{-1}$. The error estimates are obtained from the final iteration step size and represent 1 S.D.

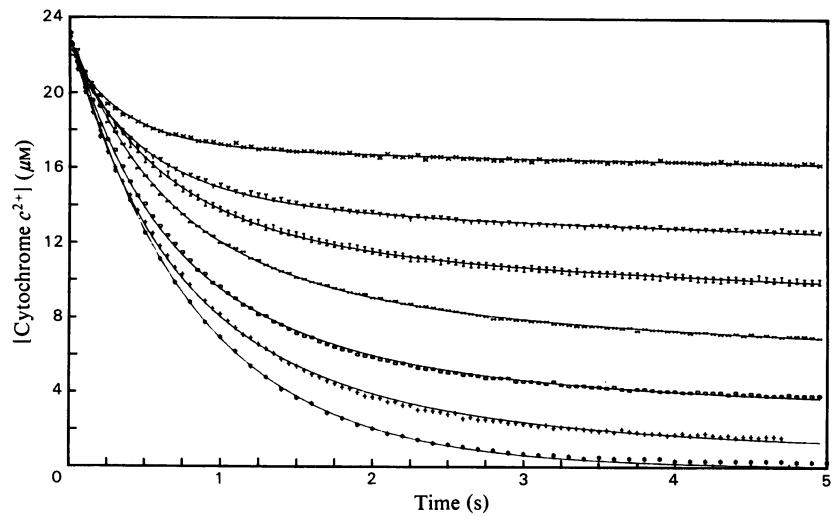


Fig. 2. *Progress curves for the cyanide inhibition of pulsed cytochrome c oxidase during turnover*
A 5 μM (functional units) solution of cytochrome *c* oxidase was fully reduced with $Na_2S_2O_4$, then re-oxidized by vigorous agitation in air to form the pulsed (P_o) species. This solution was then mixed with 50 μM-ferrocytochrome *c* in the stopped-flow apparatus, and the time course of cytochrome *c* oxidation was monitored at 550 nm. Cyanide was added to the cytochrome *c*-containing syringe to obtain the following concentrations after mixing (listed from bottom to top): 0 μM, 50 μM, 100 μM, 250 μM, 500 μM, 1 mM, 2.5 mM. The points represent the experimentally obtained data, and the continuous lines were calculated in accordance with Scheme 1. The parameters used in the simulation were: $k_{-1} = 3400\,s^{-1}$; $k_{+2} = 2 \times 10^6\,M^{-1}\cdot s^{-1}$; $k_{-2} = 0.047 \pm 0.013$. The uninhibited rate of cytochrome *c* oxidase, $k_{cat.}$, fell throughout the experiment (as the pulsed form was converted into the resting form) from 1.26 to $0.69\,s^{-1}$. In parallel with this the fitted value of k_{+1} fell from $13.5\,s^{-1}$ to $4.15\,s^{-1}$.

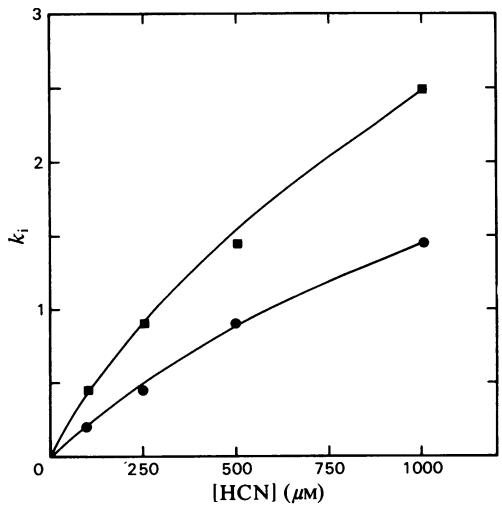


Fig. 3. *Rate constants for cyanide inhibition of pulsed and of resting cytochrome c oxidase as a function of cyanide concentration*

Values for k_i are obtained from progress curves such as those shown in Figs. 1 and 2. See the text for details. ●, Resting enzyme; ■, pulsed enzyme.

We also analysed the data in Figs. 1 and 2 by the method used by van Buuren (1972), to determine the rates of cyanide inhibition. The slope of the experimental progress curve divided by the corresponding concentration of reduced cytochrome *c* gives the apparent first-order rate constant for cytochrome *c* oxidation ($k_{app.}$) at known times after the initiation of the reaction. Thus in the absence of cyanide $k_{app.}$ is constant, whereas in the presence of the inhibitor the value of $k_{app.}$ declines throughout the reaction. This decrease in $k_{app.}$ followed an approximately exponential time course and reflects the rate of cyanide binding. The rate constant for the onset of cyanide inhibition (k_i) was calculated from the slope of plots of $\log k_{app.}$ versus time.

Estimates of the rate constants for cyanide inhibition (k_i) of resting and of pulsed oxidase as a function of cyanide concentration are reported in Fig. 3.

The initial slopes of the curves in Fig. 3 give values of the apparent second-order binding constant of cyanide to the inhibitory site. These values are $2.0 \times 10^3\ M^{-1} \cdot s^{-1}$ and $4.5 \times 10^3\ M^{-1} \cdot s^{-1}$ for the resting and the pulsed enzyme respectively. These values are in good agreement with those reported by van Buuren *et al.* (1972*b*), for the onset of cyanide inhibition. The reason that the apparent rate of cyanide binding given by this analytical procedure is much lower than the true rate of cyanide binding (k_{+2} of model) reflects the low occupancy of the cyanide-binding form during turnover (see the relative values of k_{+1} and k_{-1}.)

The non-linear nature of Fig. 3 also indicates that k_i reaches a limiting value at high cyanide concentration. This behaviour is expected from the model in Scheme 1, in which cyanide binding cannot proceed faster than entry of the enzyme into the binding form, i.e. k_i cannot exceed k_{+1}.

Nature of the inhibitory site

To obtain information on the mechanism of inhibition we have carried out a number of experiments in which spectroscopic observations of the enzyme are compared with parallel activity measurements.

Cyanide binding to fully oxidized resting cytochrome c oxidase (R_o). In experiments in which oxidized cytochrome *c* oxidase (R_o) was incubated with cyanide, both the spectral changes in the Soret region of the spectrum and the catalytic properties after addition of cyanide were followed (results not shown). The spectral changes in the Soret region occur very slowly and are multiphasic, even at high cyanide concentrations (100mM), in agreement with previous results (Antonini *et al.*, 1971; van Buuren *et al.*, 1972*b*; Wilson & Erecinska, 1977). Likewise, the activity of the enzyme is lost very slowly during incubation.

Cyanide binding to fully oxidized pulsed cytochrome c oxidase (P_o). Similar experiments were carried out with pulsed cytochrome *c* oxidase (P_o), and its loss of activity with incubation time is shown in Fig. 4. Although the loss of activity on incubation of P_o with cyanide seems to be more rapid than when cyanide is incubated with R_o, it may nevertheless be seen that even after 5min incubation the initial slope of cytochrome *c* oxidation is still unchanged, indicating slow binding to the inhibitory site. A more complete analysis is made difficult because during incubation P_o decays into R_o ($P_o \rightarrow R_o$ $t_{\frac{1}{2}}$ approx. 20–30min) (Brunori *et al.*, 1981). Parallel stopped-flow experiments, involving mixing P_o with high concentrations of cyanide (200mM) and monitoring in the Soret band, showed a complex time course, with a very slow phase comprising most of the absorbance change but also a small, faster, component (results not shown).

This faster kinetic phase, which was not observed with the resting enzyme but only with the pulsed form, has a half-time of approx. 500ms at 200mM-cyanide, and its amplitude was dependent on the time elapsed since the formation of pulsed oxidase. In fact, the time-dependence of the amplitude of this faster component indicated a half-time of approx. 15min, corresponding closely to the decay time for the transition $P_o \rightarrow R_o$.

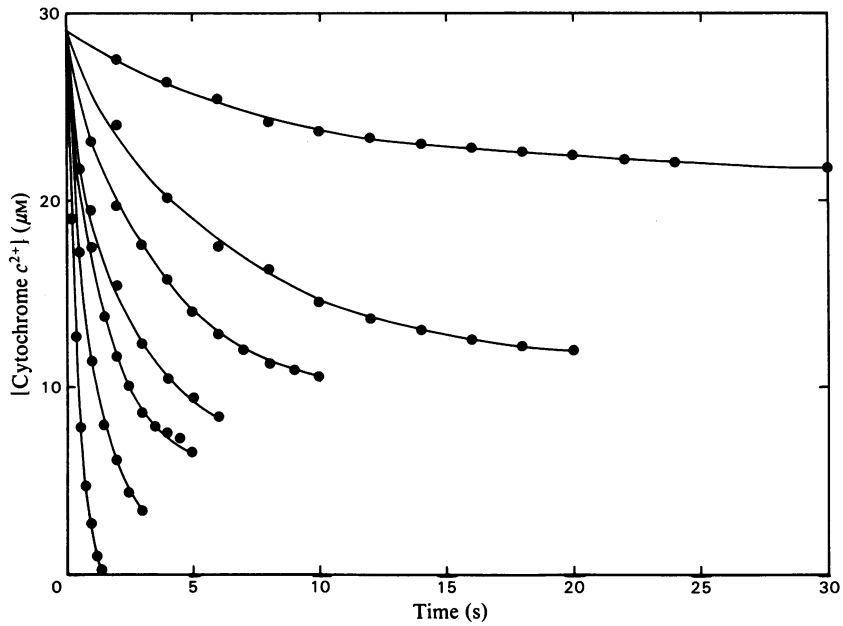


Fig. 4. *Inhibition of pulsed cytochrome c oxidase (P_o) on incubation with cyanide*
A 5 μM (functional units) solution of pulsed oxidase (P_o) was prepared as described in the legend to Fig. 2. The time course of its oxidation of a 50 μM-ferrocytochrome *c* solution was followed at 550nm in a stopped-flow apparatus. The oxidase activity after the addition of 240 μM-cyanide was measured (from bottom to top) at 0, 5, 10, 15, 20, 40 and 60min. Other conditions as given in the legend to Fig. 1.

On the basis of these results, we conclude that neither binding to R_o nor binding to P_o can account for the onset of inhibition seen in Fig. 1. However, pulsed cytochrome *c* oxidase seems to be more receptive to cyanide. It may be recalled that this finding is in agreement with the flow–flash results obtained by Brittain & Greenwood (1976), who showed that the product of the reaction of mixed-valence cytochrome *c* oxidase with O_2 yielded a species that reacted with cyanide at the haem of cytochrome a_3 more rapidly ($k = 22 M^{-1} \cdot s^{-1}$) than did the resting enzyme.

Cyanide binding to fully reduced cytochrome c oxidase. In agreement with early reports (van Buuren *et al.*, 1972*b*; Wilson & Erecinska, 1977), we have found that dithionite-reduced cytochrome *c* oxidase binds cyanide slowly and with low affinity. The rate constant for cyanide binding (as determined from stopped-flow experiments) was $35 M^{-1} \cdot s^{-1}$, slower than that previously reported (Antonini *et al.*, 1971; van Buuren *et al.*, 1972*b*; Wilson & Erecinska, 1977) and certainly much slower than the rate constant for the cyanide inhibition indicated by Figs. 1 and 2. In addition, the binding of cyanide to the reduced enzyme (binding constant $4 \times 10^3 M^{-1}$ as determined from the stopped-flow and $1 \times 10^3 M^{-1}$ from static titrations) is far too weak to account for the inhibition that we observed at low cyanide concentrations. Thus we discount binding of cyanide to the fully reduced enzyme as the basis for cyanide inhibition.

Cyanide binding to partially reduced cytochrome c oxidase (pulsed and resting). It has previously been suggested (van Buuren *et al.*, 1972*b*) that it is partially reduced forms of the enzyme that bind cyanide during turnover, and this hypothesis has been incorporated into Scheme 1. In order to examine this idea experimentally, we generated half-reduced species of cytochrome *c* oxidase in which cytochrome *a* (and presumably Cu_A) was reduced while cytochrome a_3 (and presumably Cu_B) was oxidized (Jones *et al.*, 1983). Stopped-flow experiments were performed in which cytochrome *c* oxidase (R_o) was mixed with a solution of $Na_2S_2O_4$ in the absence and in the presence of cyanide at inhibitory concentrations. In the absence of cyanide cytochrome *a* is rapidly reduced, followed by the very slow reduction of cytochrome a_3 ($t_{\frac{1}{2}}$ approx. $0.1 s^{-1}$) (Jones *et al.*, 1983). Thus an essentially half-reduced species exists for a period of time after the mixing.

In the presence of cyanide, we again observed (at 420nm) the reduction of cytochrome *a* with unaltered rate, but the slower phase, attributed to cytochrome a_3 reduction, was absent. This result suggests that, during the time of the reduction of cytochrome *a*, cyanide may combine with cytochrome a_3^{3+}, and prevent its reduction.

This conclusion was tested by experiments in which the reduction of cytochrome oxidase by dithionite was monitored at a wavelength isosbestic (427nm) for the reduction of cytochrome a_3. A comparison of the reduction profiles of cytochrome *a* in the absence and in the presence of cyanide at this wavelength showed that, although the rate of the process was unchanged by cyanide addition, the amplitude of the absorbance change was unaltered. We attribute this alteration in the absorbance change of cytochrome *a* reduction to the presence of a second spectral component arising from the binding of cyanide to cytochrome a_3, i.e. the wavelength, though isosbestic for cytochrome a_3 reduction, is not isosbestic for cyanide binding to the oxidized cytochrome a_3. The magnitude of this spectral contribution (difference in amplitude of the progress curve for cytochrome *a* reduction in the absence and in the presence of cyanide) as a function of cyanide concentration is shown in Fig. 5. This Figure also reports the comparable experiment starting with the P_o form of the enzyme. The hyperbolic nature of the plots indicates a single binding process, and the accompanying Hill plots derived from Figs. 5(*a*) and 5(*c*), showing Hill coefficients close to unity, support this conclusion. The Hill plots yield binding constants for cyanide to the R_o and P_o forms of $2.1 \times 10^5 M^{-1}$ and $1.1 \times 10^5 M^{-1}$ respectively.

Fig. 6 shows the kinetic difference spectrum

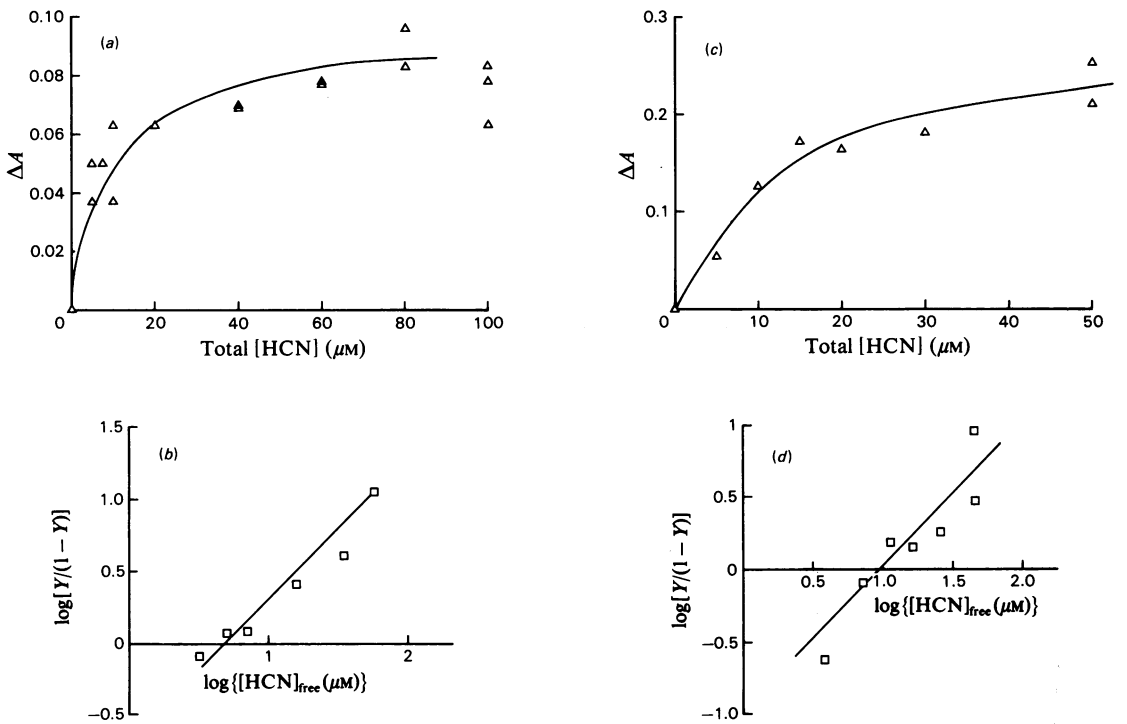


Fig. 5. *Cyanide binding to transient, partially reduced, forms of cytochrome c oxidase*

(*a*) Cyanide titration of the partially reduced species of cytochrome *c* oxidase formed on reduction of the resting oxidized enzyme (R_o) by $Na_2S_2O_4$. The titration was performed by mixing an anaerobic 5 μM-cytochrome *c* oxidase solution with 25mM-$Na_2S_2O_4$, containing an increasing concentration of cyanide (0–100 μM after mixing), in a stopped-flow apparatus. The reaction was monitored at 427nm, isosbestic for the reduction of cytochrome a_3. The vertical scale shows the maximum absorbance change relative to that obtained in the absence of cyanide for the fast phase of the reaction only. (*b*) Hill plot of the data (up to 90% saturation) obtained in the experiment with R_o oxidase described above. The continuous straight line has a slope of 1. *Y* represents the proportion of sites occupied by cyanide. (*c*) Cyanide titration of the partially reduced species of cytochrome *c* oxidase formed on reduction of the pulsed oxidized enzyme (P_o) by $Na_2S_2O_4$. The titration was performed by mixing a solution containing 5 μM-cytochrome *c* oxidase and 25mM-$Na_2S_2O_4$ with air-equilibrated buffer in the stopped-flow apparatus. The P_o form is thus generated by oxidation of the enzyme by O_2, and then its re-reduction by the dithionite could be followed at 427nm. Increasing concentrations of cyanide (0–50 μM) were added to the buffer. The vertical scale shows the maximum absorbance change relative to that obtained in the absence of cyanide. (*d*) Hill plot of the data (up to 90% saturation) obtained in the experiment with P_o oxidase described above. The continuous straight line has a slope of 1. *Y* represents the proportion of sites occupied by cyanide.

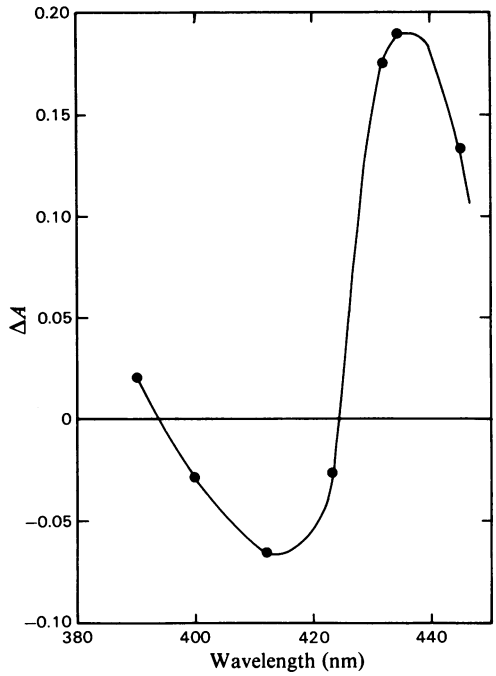


Fig. 6. *Kinetic difference spectrum for binding of cyanide to partially reduced cytochrome c oxidase*

Resting (R_o) cytochrome oxidase (5 μM) was mixed with 25 μM-$Na_2S_2O_4$ in the absence or in the presence of 200 μM-KCN. The differences in the absorbance change in the fast phase of reduction between the progress curves in the absence and in the presence of cyanide are plotted as a function of wavelength. The continuous line is hand-drawn to aid visualization.

arising from cyanide combination to the partially reduced R form of the enzyme. It was not possible to generate a kinetic difference spectrum for the pulsed (P) form of the enzyme, as in this case the reduction of cytochrome a_3, in the absence of cyanide, is rapid and cannot be unambiguously resolved from the reduction of cytochrome *a* (Peterson & Cox, 1980).

Fig. 6 displays an isosbestic point (424nm), maximum (435nm) and minimum (413nm) similar to that reported by van Buuren *et al.* (1972*b*) for the static difference spectrum of cytochrome a_3^{3+}–cyanide complex minus cytochrome a_3^{3+}. However, the relative amplitudes of the curves are somewhat different. Nevertheless, comparison with van Buuren *et al.* (1972*b*) indicates that cyanide binds to cytochrome a_3^{3+} rapidly, inhibiting the partially reduced form of the enzyme. However, we must point out that the amplitude of the kinetic difference spectrum suggests either that the molar absorption coefficient for cyanide binding to the partially reduced form is small ($\varepsilon_{420} = 20000 M^{-1} \cdot cm^{-1}$) or that we are observing cyanide binding to a portion of the enzyme only.

Although Figs. 5 and 6 give information on the site of cyanide binding and the affinity of this site for cyanide, they yield little information on the rate of this process. Cyanide combination must be faster than reduction of cytochrome *a* by dithionite. In order to obtain an estimate of this rate we added Benzyl Viologen as an electron mediator between dithionite and cytochrome *a* (Peterson & Cox, 1980). The reduction of cytochrome *a* now becomes very rapid [completed within the dead time (3ms) of the apparatus] at 25mM-dithionite and 40 μM-Benzyl Viologen. In the presence of 20 μM-cyanide, a fast process, $t_{\frac{1}{2}}$ approx. 5ms, was observed (results not shown), indicating rapid cyanide binding, in general accord with the results derived from Figs. 1 and 2.

Discussion

Table 1 shows the rate of cyanide binding to, and its affinity for, several forms of cytochrome *c* oxidase. This body of data supports the hypothesis that it is the partially reduced form that is responsible for rapid cyanide binding and for rapid cyanide-induced inhibition of the enzyme during turnover.

Although the oxidation state of the cyanide-binding site is the same in either the fully oxidized enzyme or the partially reduced form, the rate of binding is significantly different. Binding of cyanide to the partially reduced enzyme (at $>10^6 M^{-1} \cdot s^{-1}$) is at least 100000 times faster than the fastest rate of cyanide binding to either the pulsed or the resting form of the fully oxidized enzyme ($10 M^{-1} \cdot s^{-1}$ and $2 M^{-1} \cdot s^{-1}$ respectively). Even this rather remarkable comparison tends to underestimate the contrast between the rate of cyanide reaction with the partially reduced and the fully oxidized enzyme. This is because only a small fraction of the resting enzyme molecules react with the 'fast' concentration-dependent rate, whereas most react with a much slower, concentration-independent, rate (see Table 1, line 1). Since cyanide toxicity is due to its effect on cytochrome *c* oxidase, and cyanide is a notably rapid toxin, the effects of cyanide *in vivo* also appear to be due to its reaction with the partially reduced form of cytochrome *c* oxidase.

The model of cyanide reaction with cytochrome *c* oxidase shown in Scheme 1 has several steps; and, because these steps are interacting, the Scheme alone cannot provide a unique solution to fit the inhibition data shown in Figs. 1 and 2. We do, however, have independent experimental estimates for some of the kinetic constants in the Scheme for cyanide binding. For example, from

Table 1. *Binding parameters of HCN to different forms of cytochrome c oxidase*

Species	k_{on} ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})	Binding constant (M^{-1})	Reference
Oxidized (R_o)	≈ 2.0 (rate-limited at $2 \times 10^{-5} s^{-1}$)	$\sim 2 \times 10^{-6}$	$\sim 10^6$	Antonini *et al.* (1971); van Buuren (1972)
Oxidized (P_o)	~ 10	–	–	Present work; Brittain & Greenwood (1976)
	22 (rate-limited at $2 \times 10^{-3} s^{-1}$)	–	–	Estimated from Fig. 4 of present work
Reduced	130	6.5×10^{-2}	$\sim 2 \times 10^3$	Antonini *et al.* (1971)
	35	8.8×10^{-3}	4×10^3 (stop flow)	Present work
	–	–	1×10^3 (static)	Present work
Partially reduced formed by dithionite from R_o	$> 10^6$	–	2.1×10^5	Present work
Partially reduced formed by dithionite from P_o	$> 10^6$	–	1.1×10^5	Present work
Partially reduced formed during turnover starting with R_o	10^6	1.5×10^{-2}	6.7×10^7	Present work, Fig. 1
Partially reduced formed during turnover starting with P_o	2×10^6	4.7×10^{-2}	4×10^7	Present work, Fig. 2

the preliminary cyanide-binding experiments with Benzyl Viologen as the oxidase reductant, we know that the rate of cyanide binding to the partially reduced form is of the order of $10^6 M^{-1} \cdot s^{-1}$. With this lower estimate for k_{+2}, and with estimates for k_{+1} in the range of 2–10 s^{-1} (from our knowledge of the rate of cytochrome *c* oxidation in the absence of cyanide), we can use the model to predict the range of values that the rate constants may hold. Clearly the model shown in Scheme 1 simulates the time course of cytochrome *c* oxidation in the presence of cyanide well, and forms a useful basis for the study of all the inhibitors that preferentially bind ferric cytochrome a_3 (e.g. formate, azide, fluoride).

Fitting the experimentally obtained data to Scheme 1 also indicates that the (interconvertible) turnover cycles postulated for the resting and the pulsed enzymes (Wilson *et al.*, 1981) are characterized by somewhat different rate constants (see the legends to Figs. 1 and 2), the pulsed enzyme having the higher values for k_{+1} and k_{-1}, in keeping with the known higher activity of this form (Brunori *et al.*, 1979). In both cycles, however, transient cyanide-sensitive species are responsible for cyanide binding and inhibition. The binding properties of these species, though similar, may nevertheless be distinguished (see Table 1). However, as mentioned above, these properties cannot as yet be unambiguously assigned, owing to the possibility of other sets of rate constants giving equally good fits to the experimentally obtained data. For example, if k_{-1} and k_{+2} were increased in proportion, then the fits remain good, as in this case the proportion of species B (see Scheme 1) binding cyanide to form C and the proportion regenerating A remain constant.

An additional outcome of our study is a revised estimate of the dissociation constant for cyanide binding to cytochrome *c* oxidase. Previous estimates, based on the assumption that all the enzyme was capable of reacting with cyanide during turnover, have been of the order of 1 μM (Wilson & Erecinska, 1977). Recognition that only a small fraction of the enzyme can react (i.e. B of Scheme 1), together with the binding estimate supplied by the preliminary studies with Benzyl Viologen, now suggest that K_D is much lower, equal to or less than 50 nM. This value for the dissociation constant of cyanide from ferric haem and the value for k_{+2} are compatible with the known binding and combination rate constants to other ferric haem proteins possessing open binding sites (Saunders *et al.*, 1964; Nicholls, 1965).

The results with dithionite clearly corroborate our studies with cytochrome *c*, the natural reductant, insofar as they indicate rapid, tight, binding of cyanide to oxidized cytochrome a_3 in partially reduced cytochrome *c* oxidase. Reference to Table 1 shows that the binding constant of cyanide in the presence of dithionite is lower than when cytochrome *c* was employed in turnover experiments. The lower value for the binding constant and the smaller-than-expected amplitude for the difference spectrum of cyanide binding in the presence of dithionite (Fig. 6) may suggest that products of dithionite decomposition (including H_2O_2 and H_2S, known ligands of oxidized cytochrome a_3) may compete with cyanide for cytochrome a_3. Alternatively, effects of the natural reductant on the properties of the ligand-binding site in cytochrome *c* oxidase may provide the explanation for the differences.

Finally, we return to the nature of the partially

reduced form. From previous studies showing that electrons can enter either the oxidized or the oxidized cyano form of the enzyme (Johnson *et al.*, 1981) and from the fact that in the inhibited enzyme cytochrome a_3 is oxidized, we conclude that the entry of either two or three electrons is necessary to produce the partially reduced, cyanide-sensitive, form. In the former case, with two electrons, this would result in both Cu_B and cytochrome a_3 being oxidized in the cyanide-bound form. If three electrons are required for rapid cyanide binding, the Cu_B would also be reduced. A way to distinguish between these forms and to ascertain whether cyanide causes inhibition by binding solely to the haem of cytochrome a_3 or by bridging between the haem of cytochrome a_3 and Cu_B (as has been suggested for the fully oxidized enzyme; Greenwood *et al.*, 1983) is through a study of freeze–quench intermediates by e.p.r. methods.

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