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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME aa_3

VIII. EFFECT OF CYANIDE ON THE CATALYTIC ACTIVITY

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SUMMARY

1. Cyanide inhibits the catalytic activity of cytochrome aa_3 in both polarographic and spectrophotometric assay systems with an apparent velocity constant of $4 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ and a K_i that varies from 0.1 to 1.0 μM at 22 °C, pH 7.3.

2. When cyanide is added to the ascorbate–cytochrome *c*–cytochrome aa_3–O_2 system a biphasic reduction of cytochrome *c* occurs corresponding to an initial K_i of 0.8 μM and a final K_i of about 0.1 μM for the cytochrome aa_3–cyanide reaction.

3. The inhibited species ($a^{2+}a_3{}^{3+}$HCN) is formed when $a^{2+}a_3{}^{3+}$ reacts with HCN, when $a^{2+}a_3{}^{2+}$HCN reacts with oxygen, or when $a^{3+}a_3{}^{3+}$HCN (cyano-cytochrome aa_3) is reduced. Cyanide dissociates from $a^{2+}a_3{}^{3+}$HCN at a rate of $2 \cdot 10^{-3}$ s^{-1} at 22 °C, pH 7.3.

4. The results are interpreted in terms of a scheme in which one mole of cyanide binds more tightly and more rapidly to $a^{2+}a_3{}^{3+}$ than to $a^{3+}a_3{}^{3+}$.

INTRODUCTION

We have already shown that cyanide binds tightly to cytochrome aa_3 when the two are incubated with ascorbate, cytochrome *c* and O_2 (ref. 1) while a weaker and very slow reaction occurs with the completely oxidized enzyme[2]. In addition a relatively rapid reaction occurs between cyanide and the fully reduced enzyme[2-4]. The reaction of cyanide with fully oxidized cytochrome aa_3 is too slow to account for the known rates of inhibition, although Keilin and Hartree[5] clearly identified the ferric form of cytochrome a_3 as the form 'stabilized' by cyanide. For reasons of this kind Chance[6] claimed that the reduced state must be the vulnerable one, and Yonetani and Ray[7] postulated a rapid reaction of cyanide with ferrous cytochrome aa_3, followed by auto-oxidation to the cyanferric species. However, the rate of inhibition measured in the polarographic or spectrophotometric assay system is at least one order of magnitude greater than the rate of reaction of cyanide with the fully reduced enzyme.

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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The present paper attempts to clarify the conflicting spectrophotometric and catalytic observations, and to develop a provisional model for cyanide inhibition.

RESULTS

Fig. 1 illustrates the slow development of inhibition when the enzyme is added to a cuvette containing reduced cytochrome *c* and a relatively high concentration of cyanide (6.7 μM). The slope of the line at any time is a measure of the enzymic activity. By plotting the change in activity against time in a semi-logarithmic plot (inset in Fig. 1) a straight line is obtained and from its slope an apparent second-order rate constant for the binding of cyanide to cytochrome aa_3 of about $3 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ was calculated. In the final inhibited state (Fig. 1) the activity is less than 1.3 % of that of the uninhibited enzyme, corresponding to an effective K_i of less than 0.1 μM.

In a corresponding experiment (not shown) where the induction of inhibition was measured polarographically in the presence of 30 mM ascorbate, 0.75 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and 30 μM cytochrome *c*, a K_i of 0.8 μM was obtained, cyanide reacting with a k_{on} of about $2.5 \cdot 10^3$ $M^{-1} \cdot s^{-1}$.

Fig. 2 summarizes the results of rates of cyanide binding obtained with isolated cytochrome aa_3 and with Keilin–Hartree particles. Both preparations show an 'on constant' of about $3.5 \cdot 10^3$ $M^{-1} \cdot s^{-1}$. The 'off constant' obtained from the point of intersection on the ordinate is estimated to be $4 \cdot 10^{-3}$ s^{-1}, corresponding to a K_i of about 1 μM. This value is similar to the value of 0.8 μM obtained in the polarographic assay but differs from the K_i of 0.1 μM obtained after prolonged incubation (Fig. 1 and refs 1, 8). In an attempt to resolve this discrepancy we therefore studied the changes with time of the equilibration between cyanide and cytochrome aa_3 during catalysis.

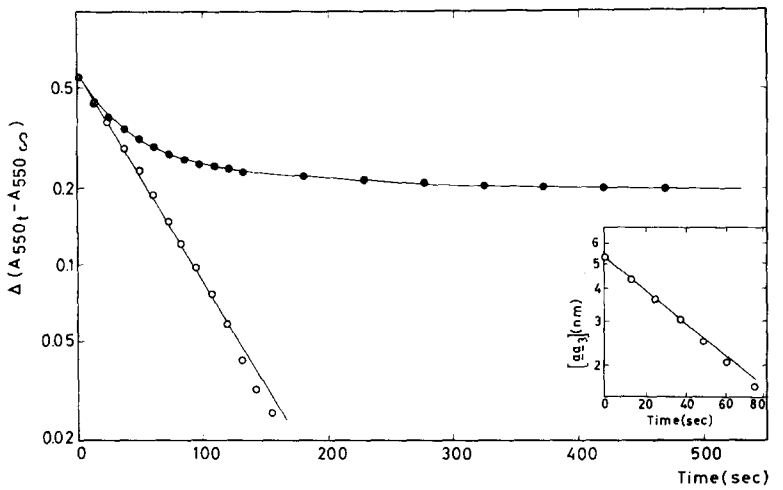

Fig. 1. Inhibition of cytochrome aa_3 by cyanide measured spectrophotometrically. Cytochrome aa_3 activity was assayed according to Experimental. At time $t = 0$, 5.3 nM enzyme was added to a cuvette containing 30 μM ferrocytochrome *c*. The reaction was followed at 550 nm in a Cary-14 recording spectrophotometer at 23 °C. ○—○, without cyanide; ●—●, in the presence of 6.7 μM cyanide. The inset shows in a semi-logarithmic plot the decrease in enzyme concentration with time. As a measure of the free enzyme concentration the slope of the line at any time is used.

Fig. 3A shows the effect of cyanide addition on the redox state of cytochrome *c* in an aerobic system containing ascorbate and cytochrome aa_3. In the absence of cyanide, addition of such low concentrations of ascorbate (1.6 mM) induces only a slight increase in 550 nm absorbance in the presence of cytochrome aa_3 and oxygen.

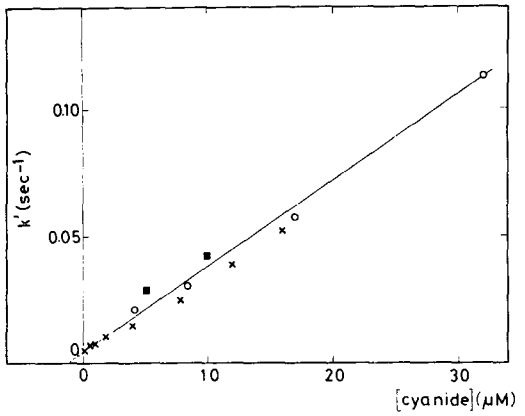

Fig. 2. Rates of cyanide inhibition. The apparent first-order rate constants are obtained from plots similar to the insets of Figs 1 and 3B. Conditions as described in Experimental, at 23 °C. ○—○, isolated cytochrome aa_3, Smith–Conrad assay; ×—×, Keilin–Hartree submitochondrial particles, Smith–Conrad assay; ■—■, isolated cytochrome aa_3, spectrophotometric assay of the ascorbate–cytochrome *c*–cytochrome aa_3–O_2 system in the initial phase.

On addition of cyanide, the cytochrome *c* reduction level changes at a rate which depends on the rate of reduction of ferricytochrome *c* by ascorbate and on the rate of cyanide binding by cytochrome aa_3. The initial steady-state change on addition of cyanide corresponds to the inhibition with a K_i of about 0.8 μM measured in the polarographic assay. This is, however, followed by a somewhat slower increase in cytochrome *c* reduction (see also ref. 9). Assay of aliquots of enzyme at several times showed that further inhibition of cytochrome aa_3 occurs during the slow second phase of the reaction. The cyanide dissociates slowly from the cyano-cytochrome aa_3, formed as described by van Buuren *et al.*[1] (see also ref. 8). This reaction appears to be responsible for the final stage of inhibition observed in the spectrophotometric assay (Fig. 1).

As we have discussed previously[10] the oxidation of cytochrome *c* by cytochrome aa_3 can be best described with Mechanism IV of Minnaert[11]:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E + P$$

The corresponding rate equation is:

$$v = \frac{Ae[S]}{B + [S + P]} \qquad (1)$$

with $A = k_{-1}k_2/(k_{-1} + k_2)$; $B = k_{-1}/k_1$; e, total concentration of active cytochrome aa_3; S, ferrocytochrome *c*; P, ferricytochrome *c*; and v, the rate, in electron equiv-

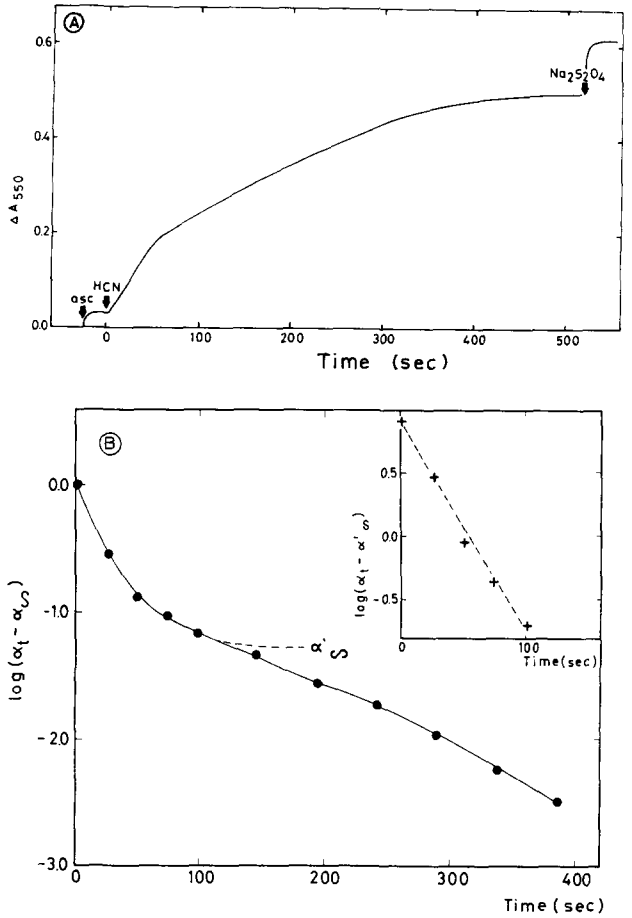

Fig. 3. Inhibition of cytochrome aa_3 by cyanide in the ascorbate–cytochrome *c*–cytochrome aa_3–O_2 system. A. The degree of reduction was measured at 550 nm in a Cary-14 recording spectrophotometer at 23 °C. At the arrow marked 'asc', 1.6 mM ascorbate was added to 30 μM cytochrome *c* and 0.3 μM cytochrome aa_3 in 100 mM phosphate buffer (pH 7.3) and 0.5 % Tween 80. At equilibrium about 6 % of the cytochrome *c* was reduced. At arrow HCN, cyanide to a final concentration of 10 μM was added. B. Decrease of cytochrome aa_3 concentration at different times during incubation with cyanide, ascorbate and cytochrome *c*. The points are obtained from the plot shown in A. On the ordinate is plotted $\alpha_t - \alpha_\infty$ which is a measure of the change in free enzyme concentration (see text). α'_∞ is a theoretical end point for the first phase of the reaction and this enzyme concentration corresponds to an apparent K_i of 0.8 μM. The inset plots $\alpha_t - \alpha'_\infty$ (*i.e.* initial decrease in enzyme concentration corrected for the secondary part of the curve) against the time semi-logarithmically. The slope of this line corresponds to an apparent 'on' constant for the initial phase of 4300 $M^{-1} \cdot s^{-1}$.

alents $\cdot l^{-1} \cdot s^{-1}$. In the steady state the rate of oxidation of cytochrome *c* by cytochrome aa_3 equals its rate of reduction by ascorbate and thus:

$$k_3[AH_2][P] = \frac{Ae[S]}{B + [S + P]} \qquad (2)$$

where k_3 is the apparent second-order rate constant for the reaction of ferricytochrome *c* with ascorbate (AH_2).

From Eqn 2 the enzyme concentration may be calculated as

$$e = \frac{B + [S + P]}{A} \cdot k_3[AH_2] \cdot \frac{[P]}{[S]} \tag{3}$$

In the presence of cyanide the concentration of active cytochrome aa_3 decreases with time and the proportion of active enzyme at any stage of the reaction can be calculated from the following Eqn 4:

$$\frac{e_t}{e_0} = \frac{[S]_0[P]_t}{[P]_0[S]_t} = \alpha \tag{4}$$

where 0 and t refer to time = zero and time = t, respectively. Eqn 4 will be valid provided the rate of electron flow is rapid compared with the rate of reaction of cytochrome aa_3 with cyanide. Thus the system can be regarded as proceeding through a series of "micro steady states" governed by Eqn 2. Under the conditions used this holds for cyanide concentrations below 100 μM.

Fig. 3B illustrates the result of transposing the steady-state reduction levels of an ascorbate–cytochrome c–cytochrome aa_3–cyanide system in this way. From the initial and final steady-state reduction levels (6 and 81 %, respectively) the relative amount of active cytochrome aa_3 in the presence of 10 μM cyanide is calculated to be less than 1.3 %, corresponding to an effective K_i of 0.13 μM. The results indicate that the reaction is biphasic showing an initial phase with an apparent K_i of about 0.8 μM and a rate constant of $4.3 \cdot 10^3$ $M^{-1} \cdot s^{-1}$. The final phase with a K_i of 0.13 μM has a rate constant of 0.014 s^{-1} at 10 μM cyanide.

The dissociation of cyanide from the inhibited cytochrome aa_3 may be assayed spectrophotometrically by dilution of the complex into a medium containing reduced cytochrome c but no cyanide (*cf.* Fig. 12 of ref. 1). The apparent k_{off}, obtained from a semi-logarithmic plot of the change in bound enzyme against time, was $2.1 \cdot 10^{-3}$ s^{-1}. The initial rate shown by the inhibited enzyme corresponds to less than 0.5 % of the control activity and the final rate to at least 66 %.

Various authors (Wainio and Greenlees[15], Camerino and King[8], Antonini *et al.*[4]) have suggested secondary binding sites to account for the cyanide inhibition. It has, however, already been shown[1] that a completely inactive cyanide–cytochrome aa_3 complex contains only one mole of cyanide per mole of cytochrome aa_3, *i.e.* one mole of cyanide per two haems. Fig. 4 illustrates an experiment where the steady-state reduction level was measured at a high cytochrome aa_3 and ascorbate concentration. Equilibration was brought about rapidly by allowing the system to become anaerobic and then pulsing with an appropriate addition of cyanide and a small amount of air or pure oxygen. Little change in reduction level was observed until the amount of added cyanide exceeded the amount of cytochrome aa_3 present. The percentage reduction then began to increase. The amounts of free cytochrome aa_3 present can be plotted against the cyanide concentration. Initially all the cyanide is bound by the enzyme since on extrapolation of the first part of the curve the cyanide/cytochrome aa_3 ratio in the inhibited complex was found to be 1.0. When a plot was made of $1/E_f$ against $I_t/E_t - E_f$ (*cf.* ref. 1) the K_i was found to be 0.54 μM, decreasing to 0.14 μM after longer equilibration with oxygen bubbling through the solution (inset Fig. 4).

An experiment was also carried out in which cytochrome aa_3 was titrated to

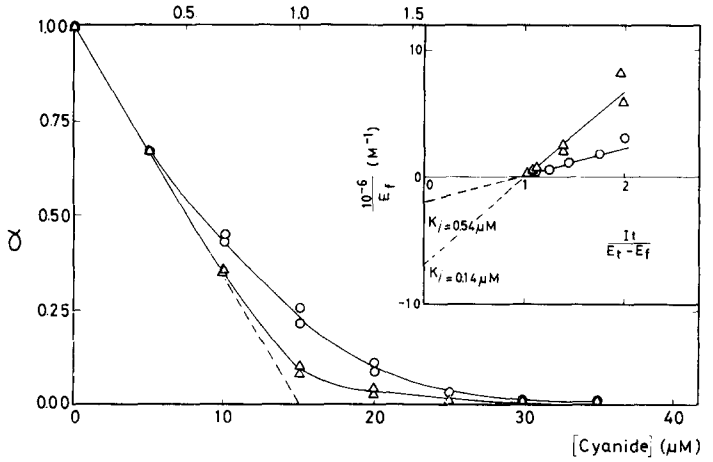

Fig. 4. Titration of cytochrome aa_3 with cyanide under catalytic conditions. The reaction was followed by measuring 550 nm-absorbance on addition of 17 mM ascorbate to 30 μM cytochrome *c* and 15 μM cytochrome aa_3. After anaerobiosis cyanide was added and oxygen was introduced and the steady-state reduction level was monitored. After the system had become anaerobic again cyanide and oxygen were added and the new steady-state reduction level was recorded. The figure shows the decrease in relative enzyme concentration, plotted as α (see text) against the cyanide concentration added. ○—○, results obtained when air was mixed together with the cyanide; △—△, results obtained when, after cyanide addition, pure O_2 was bubbled through the cuvettes for 30 s. The inset shows the determination of the apparent K_i in a plot of $1/E_f$ against $I_t/E_t - E_f$ (*cf.* ref. 1). E_t, E_f and I_t are concentrations of total enzyme, free enzyme and total inhibitor, respectively. Temperature, 23 °C.

complete reduction with phenazine methosulphate + 4 electron equivalents (2 moles of NADH per mole cytochrome aa_3), according to van Gelder and Slater[12], and an excess of cyanide (10 mM) was then added. Under these conditions the product was $a^{2+}a_3{}^{2+}$HCN. Subsequent addition of oxygen then gave rise to $a^{2+}a_3{}^{3+}$HCN (*cf.* ref. 5). The latter species can thus be formed by three routes, two of them rapid and one slow:
(i) reduction of $a^{3+}a_3{}^{3+}$ in the presence of cyanide (rapid)[13];
(ii) oxidation of $a^{2+}a_3{}^{2+}$HCN by molecular oxygen (rapid)[14];
(iii) binding of cyanide to $a^{3+}a_3{}^{3+}$ (slow) followed by the addition of the reductant.

DISCUSSION

The results presented show that cyanide reacts with cytochrome aa_3 at a rate of about $4 \cdot 10^3$ $M^{-1} \cdot s^{-1}$, more than 20-fold faster than the reaction of cyanide with the fully reduced enzyme. Moreover the reaction seems to occur in two stages with an initial equilibrium at a K_i of about 0.8 μM and a final equilibrium with a K_i of about 0.1 μM.

Fig. 5 extends the previously[2] proposed reaction scheme to include the possible reactions of cyanide with partially reduced enzyme to form the inhibited $a^{2+}a_3{}^{3+}$HCN species. Although the scheme is capable of accounting for all the observations, it is not clear whether it is the minimum hypothesis necessary, nor are sufficient data available to determine all individual rate constants contained in the scheme. Two possible simplifications of the overall scheme of Fig. 5 seem to be worth consideration, differing in their interpretation of the initial K_i of 0.8 μM in the catalytic system.

In the first such simplification (Model A), the overall equilibrium for the binding

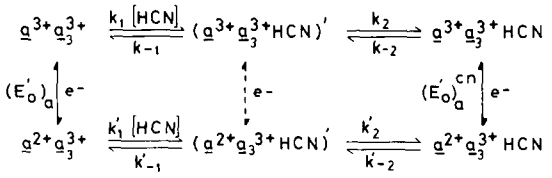

Fig. 5. A scheme for the reaction of cyanide with oxidized cytochrome a_3 in the cytochrome aa_3 system.

of cyanide by the oxidized enzyme (upper reaction in Fig. 5) is responsible for this apparent K_i. The lower (partially reduced enzyme–cyanide) reaction then catalyses this equilibrium. The final equilibrium (K_i of 0.1 μM) corresponds to that in the lower reaction. This model requires $k'_2 > k_2$ and $k'_{-2} > k_{-2}$, and rapid equilibration of the $a^{2+}a_3{}^{3+}$HCN species with cytochrome c^{3+}. So far no data are available to support the former requirements, but, since ferricytochrome c is rapidly reduced by cytochrome $a^{2+}a_3{}^{3+}$HCN (unpublished observations) the latter assumption is likely to be correct.

In the alternative (Model B) the initial apparent K of 0.8 μM (as found in Fig. 3B) represents in the lower reaction scheme of Fig. 5 the equilibrium between $a^{2+}a_3{}^{3+}$ and $(a^{2+}a_3{}^{3+}\text{HCN})'$. The relatively rapid formation of $(a^{2+}a_3{}^{3+}\text{HCN})'$ is then followed by a slower conversion into $a^{2+}a_3{}^{3+}$HCN with a K_i of 0.1 μM. This model requires both $a^{2+}a_3{}^{3+}$HCN species to be inhibitory.

In Model A the rate of formation and dissociation of the $a_3{}^{3+}$HCN species will be strongly influenced by changes in the ratio of cytochrome c^{2+} to cytochrome c^{3+} during the reaction since this determines the ratio of cytochrome $a^{2+}a_3{}^{3+}$HCN to cytochrome $a^{3+}a_3{}^{3+}$HCN. In Model B the reaction scheme for the binding of cyanide in the presence of reducing agent is analogous to that discussed in an earlier paper[2] for the fully oxidized enzyme. Table I summarizes the data obtained earlier with the completely oxidized enzyme together with those for the enzyme in the catalytic system. The determination of k_2 in the 'turnover' system needs some explanation. According to Model B, the final rate in Fig. 3B represents the reaction

$$(a^{2+}a_3{}^{3+}\text{HCN})' \xrightarrow{k_2} a^{2+}a_3{}^{3+}\text{HCN}$$

and k'_2, which can be determined from the slope of the line, equals $1.4 \cdot 10^{-2}$ s^{-1}. This value agrees well with the value $1.6 \cdot 10^{-2}$ s^{-1} calculated from $K_\alpha \cdot k_{off} \cdot K_i{}^{-1}$ and is also similar to the k_2 observed for the fully oxidized enzyme in absence and presence of azide. As can be seen from Table I according to Model B the addition of either azide or reducing equivalents increases the apparent second-order rate constant for cyanide binding (k_{on_1}) and decreases the K_α.

Model B accounts more satisfactorily than Model A for the failure of the polarographic assay system in presence of TMPD to approach more rapidly the highly inhibited state. Model A would predict that the high degree of reduction in this system should eliminate the apparent intermediate equilibrium. On the other hand Model A accounts more satisfactorily for the lag phase sometimes observed between initial and final inhibition (Fig. 3B and ref. 9). The delay is attributed to the slow build-up of the vulnerable species $a^{2+}a_3{}^{3+}$, with the low-ascorbate assay system giving rise to an autocatalytic process—the greater the inhibition the more reduced the cytochrome c and hence the greater the concentration of $a^{2+}a_3{}^{3+}$.

TABLE I

RATE CONSTANTS FOR THE REACTION OF CYANIDE WITH CYTOCHROME aa_3

Data from ref. 2 and this paper.

Constant	*Enzyme species reacting*		
	$a^{3+}a_3{}^{3+}$	$a^{3+}a_3{}^{3+}HN_3$	$a^{2+}a_3{}^{3+*}$
$K_D = \frac{k_{-1}k_{-2}}{k_1(k_2+k_{-2})}$	10^{-6} M	—	—
$K_I = \frac{k_{-1}k_{-2}}{k_1k_2}$	—	—	10^{-7} M
$K_\alpha = \frac{k_{-1}+k_2}{k_1}$	10^{-2} M	$7\cdot10^{-4}$ M	$8\cdot10^{-7}$ M
$k_{on_1} = \frac{k_1k_2}{k_{-1}+k_2}$	1.8 $M^{-1}\cdot s^{-1}$	25 $M^{-1}\cdot s^{-1}$	$4\cdot10^3$ $M^{-1}\cdot s^{-1}$
$k_{on_2} = k_2$	$1.8\cdot10^{-2}$ s^{-1}	$1.8\cdot10^{-2}$ s^{-1}	$1.4\cdot10^{-2}$ s^{-1}
$k_{off} = \frac{k_{-1}k_{-2}}{k_{-1}+k_2}$	—	—	$2.1\cdot10^{-3}$ s^{-1}

* Based on Model B.

In either of these and all similar models, the observations on cyanide binding can be reconciled if:

(a) the oxidized form of the enzyme ($a^{3+}a_3{}^{3+}$) reacts slowly with cyanide, with a K_D in the micromolar range and a dissociation half-time measured in days; while

(b) the partially reduced form of the enzyme ($a^{2+}a_3{}^{3+}$) reacts much more rapidly with cyanide, with a K_D in the 100 nanomolar range and a dissociation half-time measured in minutes.

Some assumptions are made by any model based on Fig. 5. But the attraction of this kind of picture is that it does not require the secondary cyanide-binding sites advocated by Camerino and King[8], Wainio and Greenlees[15], and Antonini *et al.*[4]. Cyanide titrations (ref. 1 and Fig. 4) seem to rule out such sites. Nor is it necessary to assume[4,7] that cyanide inhibition involves the oxidation of $a^{2+}a_3{}^{2+}$HCN by oxygen. Although this reaction is fast[14], the binding of cyanide to $a^{2+}a_3{}^{2+}$ is too slow[2,4] to account for the rate of inhibition.

The cyanide reaction may also provide information about the overall behaviour of cytochrome aa_3. Reduction of cytochrome *a* and/or copper dramatically increases both the overall rate of binding and the affinity of ferric cytochrome a_3 for cyanide. Similarly, it seems that only in the presence of some ferrous cytochrome *a* does cytochrome a_3 become available for reduction by cytochrome *c*[16]. And only after the reduction of cytochrome *a* does the EPR signal of the low spin cytochrome a_3–azide complex appear[17]. Conformational changes affecting cytochrome a_3 evidently accompany the reduction of cytochrome *a*.

If the oxidation state of cytochrome *a* affects the binding of cyanide, the latter binding can affect the redox potential of cytochrome *a*. At thermodynamic equilibrium for a system such as that in Fig. 5, $(E'_0)_a^{CN}$ will be 60–80 mV more positive

than $(E'_0)_a$. If $(E'_0)_a$ is about 200–250 mV (Muijsers *et al.*[18]) then $(E'_0)_a^{CN}$ will be between 260 and 310 mV. Changes in redox potential of this kind are of interest in view of the postulated existence of high energy for forms of the cytochromes with altered potentials[19].

EXPERIMENTAL

The cytochrome aa_3 and cytochrome *c* preparations used were described in a previous paper[14]. Sodium ascorbate stock solutions of approximately 0.5 M were kept at −20 °C. Cyanide was added as a solution of KCN.

Other reagents were as in a previous paper[2]. Most experiments were carried out with a Cary-14 recording spectrophotometer. The usual assay medium contained 100 mM potassium phosphate buffer (pH 7.4) and 0.5 % Tween 80. For the polarographic assay 30 mM ascorbate, 1 mM EDTA and 0.75 mM TMPD were added to 30 μM cytochrome *c* and after 1–2 min the reaction was started by addition of cytochrome aa_3. All rates are corrected for auto-oxidation. Oxygen uptake was measured with a Clark electrode mounted on a Gilson oxygraph.

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