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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME aa_3

V. BINDING OF CYANIDE TO CYTOCHROME aa_3

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SUMMARY

1. The effect of cyanide on the enzymic activity of cytochrome aa_3 shows that 1 mole cyanide is tightly bound to 1 mole cytochrome aa_3. This is confirmed by the isolation of this complex (cyano-cytochrome aa_3).

2. The rate constant for cyanide binding is 2 $M^{-1} \cdot sec^{-1}$ (pH 8.0, 0). From the K_D of $7 \cdot 10^{-7}$ M (obtained from equilibrium dialyses), a dissociation rate constant of $1.4 \cdot 10^{-6}$ sec^{-1} is calculated.

3. The time needed for equilibration of cyanide and cytochrome aa_3 depends on the redox state of the enzyme.

4. Under conditions of reducing preincubation with ascorbate and cytochrome *c* the inhibition is noncompetitive towards cytochrome *c* with a K_i of $8 \cdot 10^{-8}$–$9 \cdot 10^{-8}$ M.

5. In the presence of reducing equivalents cyanide dissociates readily from cyano-cytochrome aa_3 to form enzymically active cytochrome aa_3. The dissociation rate constant is $2 \cdot 10^{-3}$ sec^{-1} (pH 6.0, 25°).

6. It is suggested, that the cavity in which the haem of cytochrome aa_3 is buried is more closed in the oxidized than in the reduced form and that the conformation is determined by the redox state of cytochrome *a*.

INTRODUCTION

The effect of cyanide on the spectrum of the cytochrome system led KEILIN AND HARTREE[1–3] to the discovery of the cyanide sensitive cytochrome a_3. In two decades following this discovery several groups[3–16] studied the effect of cyanide on cytochrome oxidase and confirmed the observations of KEILIN AND HARTREE, but some did not agree on the separate identity of components *a* and a_3. In the last decade the effects of cyanide were studied in more detail[17–28] and quantitative data on kinetics and inhibition constants[19,25–27] were reported. The rate of reaction with oxidized cytochrome aa_3 is slow[11,25], equilibration taking some hours. In contrast YONETANI AND RAY[25] found an almost instantaneous equilibration on adding a few grains of

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dithionite to mixtures of cyanide and cytochrome aa_3, agreeing with the observation of NICHOLLS[27] that during steady-state oxidation, equilibration between cyanide and cytochrome aa_3 is obtained within a few minutes.

Determinations of the apparent K_i for cyanide inhibition have led to values between $2 \cdot 10^{-8}$ and 10^{-5} M (refs. 6, 7, 25–27). In addition almost irreversible binding of cyanide to cytochrome aa_3 was reported by CAMERINO AND KING[28] who found, also, that the activity of this cyanide–cytochrome aa_3 complex depends on the assay system used.

WAINIO AND GREENLEES[26] incubated heart-muscle preparation with cyanide under reducing conditions and found an apparent K_i of $5 \cdot 10^{-8}$ M, a value similar to that reported by ALBAUM *et al.*[6] ($2 \cdot 10^{-8}$ M). However, under such conditions a second K_i of $5 \cdot 10^{-6}$ M was also found resembling the K_i obtained by incubation of cyanide with the oxidized enzyme. Most other K_i values reported lie between these extremes and may depend on the assay method used.

Such disagreements concerning the effects of cyanide on the activity of cytochrome aa_3 have led to a reinvestigation of the effects of stoichiometric amounts of cyanide on the activity of cytochrome aa_3.

Some of this work has been reported in a symposium[29].

MATERIALS AND METHODS

Enzyme preparations

Cytochrome *c* and cytochrome aa_3 were isolated from beef heart by modification of the methods of MARGOLIASH AND WALASEK[30] and FOWLER *et al.*[31], respectively, as described in a previous communication[32]. Ferrocytochrome *c* (96–99 %) was prepared by Sephadex G-25 gel filtration as described by YONETANI AND RAY[25] (see also ref. 33)

Cytochrome *c* and cytochrome aa_3 concentrations were calculated from the reduced *minus* oxidized spectrum using a $\Delta A_{550\,nm}$ of 21 $mM^{-1} \cdot cm^{-1}$ and a $\Delta A_{605\,nm}$ of 24 $mM^{-1} \cdot cm^{-1}$, respectively[34,35].

Protein concentration was determined according to the method of GORNALL *et al.*[36] as modified by YONETANI[17]. In the presence of cyanide or at low enzyme concentration the cytochrome aa_3 concentration was determined from $A_{280\,nm}$, which was standardized against $\Delta A_{605\,nm}$. The $A_{280\,nm}$ of a particular preparation does not change upon addition of cyanide.

Determination of cyanide concentration

Cyanide solutions were standardized according to the method of MÖLLER AND STEFANSSON[37] using as reference wavelength 470 nm. It is possible to determine accurately 50 nmoles of cyanide by this method.

Cyanide concentrations in the presence of protein or at concentrations below 50 μM were measured with the liquid scintillation method, using $K^{14}CN$. 0.1–1 ml samples were mixed with 6.7 ml toluene, 2.4 ml ethanol, 3.4 ml Triton X-100, 26.6 mg 2,5-diphenyloxazole, 0.66 mg 1,4-di-2-(5-phenyloxazolyl)-benzene (*cf.* ref. 38). Radioactivity was measured in a Nuclear Chicago, Mark I or Unilux, Type II, scintillation counter.

Enzyme activity

The enzymic activity of cytochrome aa_3 preparations was measured spectrophotometrically at 25° according to a modification of the method of SMITH AND CONRAD[39]. The reaction medium contained, in a total volume of 2.5 ml, 160 μmoles potassium phosphate, 100 μg Asolectin, 12.5 μl Tween 80, 2.5 μmoles EDTA, 12.5 μmoles sucrose, 15–100 nmoles ferrocytochrome *c* and 0–25 nmoles cyanide (cyanide concentration equal to that in the incubation mixture). The pH was 6.0. The reaction was started with 1–15 pmoles cytochrome aa_3. The enzyme was diluted in an ice-cold mixture of 0.25 M sucrose, 2 mg/ml Asolectin, 0.5 % Tween 80 and 10 mM phosphate (pH 7). Rate is expressed as μM ferrocytochrome *c* oxidized per sec and activity as molecular activity (MA), which is rate per μM cytochrome aa_3.

Gel filtration

Varying amounts of cyanide and cytochrome aa_3 were incubated in a mixture of 50 mM Tris sulphate, 0.5 % Tween 80 and 0.5 % potassium cholate at pH 8.0. After incubation the excess of cyanide was removed by gel filtration through a Sephadex G-25 column (100 cm high, 0.9 cm diameter), eluting with the incubation mixture. The eluate was collected in 1–2-ml samples and the volume was determined by weight. Elution times, varying from 0.5 to 6 h, are indicated in the legends. All experiments were carried out at 2–5°.

Chemicals

$K^{14}CN$ was obtained from New England Nuclear Corp. The specific activity, calculated from the cyanide concentration measured spectrophotometrically and the radioactivity by liquid scintillation, was 7.2 ± 0.3 C/mole, in agreement with the value given by the manufacturer (7.22).

Asolectin (Associated Concentrated, Inc., New York) sols were made according to the method of WHARTON AND GRIFFITHS[40]. Stock solutions of 50 mg/ml were stored at 0–5° and discarded after 5 days. Tween 80 was obtained from Koch Light. All other chemicals were of Analar grade, obtained mainly from British Drug Houses.

RESULTS

Effect of cyanide on the activity of cytochrome aa_3

The effect of cyanide on the oxidation of ferrocytochrome *c* by cytochrome aa_3 is shown in Fig. 1. In the absence of cyanide straight lines are obtained in a first-order plot, but in the presence of cyanide the lines are convex to the time axis, indicating increasing inhibition. A straight line is obtained after a few min. Incubation of cytochrome aa_3 with cyanide for 2 h has no effect on the shape of this curve, whereas pretreatment of cytochrome aa_3 and cyanide in the presence of ascorbate and cytochrome *c* for this period abolishes the lag time. These observations indicate that the equilibrium between cyanide and cytochrome aa_3 can be reached more rapidly during active turnover of the enzyme. Activities measured after the lag period and after 2-h incubation in the presence of air and electron donor are equal, and suggest that the same equilibrium state is reached. More quantitative data of the rate of inhibition by cyanide will be given in a separate paper. As the presence of electron donor

during the incubation of enzyme with cyanide and air shortens the equilibration time needed, this pretreatment is the one used in the following experiments.

From a Lineweaver–Burk plot (Fig. 2) it is concluded that cyanide inhibition is noncompetitive towards cytochrome *c*. It is also seen from Fig. 2 that inhibition occurs at very low cyanide concentrations, indicating a high affinity for cyanide.

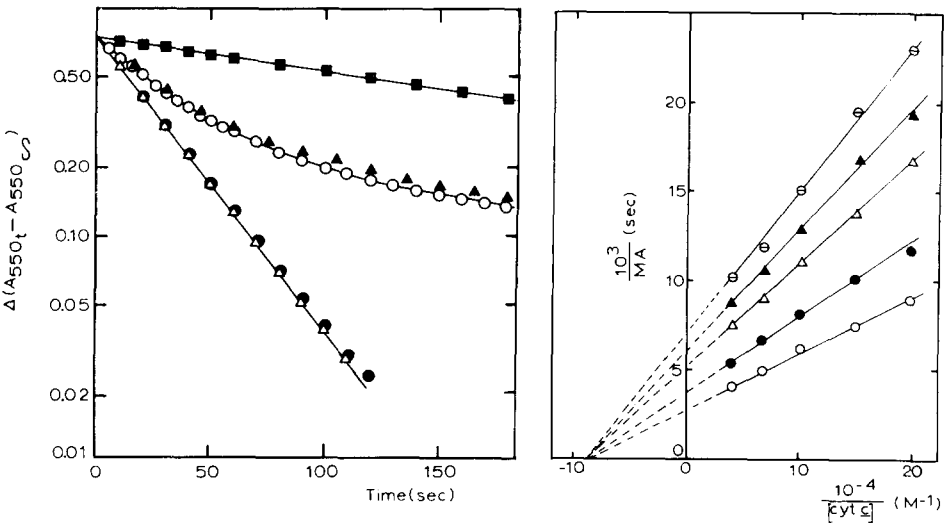


Fig. 1. Effect of cyanide on the activity of cytochrome aa_3. Rates are measured after 60-fold dilution of samples containing 90 nM cytochrome aa_3 in the assay mixture containing 35 μM ferrocytochrome *c*. △—△, no additions; ●—●, enzyme incubated 2 h at 0° in the presence of 2 mM ascorbate and 0.1 μM cytochrome *c*, and shaken under O_2; ○—○, with 4 μM cyanide; ▲—▲, with 4 μM cyanide, enzyme previously incubated 2 h at 0° in the presence of 4 μM cyanide; ■—■, with 4 μM cyanide, enzyme incubated 2 h at 0° in the dilution mixture in the presence of 2 mM ascorbate, 0.1 μM cytochrome *c* and 4 μM cyanide, with shaking under O_2.

Fig. 2. Lineweaver–Burk plot for different cyanide concentrations. The initial activities were measured after 100-fold dilution of 80 nM cytochrome aa_3 incubated for 2–3 h at 0° with 2 mM ascorbate and the following cyanide concentrations: ○—○, without cyanide; ●—●, 40 nM cyanide; △—△, 80 nM cyanide; ▲—▲, 120 nM cyanide; ⊖—⊖, 240 nM cyanide.

Previous studies[18,23], showing independent binding of 1 mole cyanide per cyanide-sensitive site ($n = 1$ in Hill plots), do not settle the question of the number of haem *a* equivalents per site[23,24,27]. The high affinity for cyanide offers a way to determine this number, but since the smallest enzyme unit contains 2 haem *a* equivalents, we have determined *m*, the number of active sites per such unit.

Fig. 3A shows a titration of cytochrome aa_3 with cyanide at three different enzyme concentrations. Initially an almost proportional decrease in the activity is observed, but at higher cyanide concentrations the lines become convex to the cyanide axis, showing reversible cyanide binding. At high enzyme and low cyanide concentration almost all cyanide will be bound and the tangent to the curve will intersect the abscissa at the ratio cyanide to cytochrome aa_3 close to, but larger than, that in the inhibitory complex. In Fig. 3A it is shown that for the highest enzyme concentration used, the tangent intersects at 1.4 cyanide per cytochrome aa_3.

The data of Fig. 4A where different cyanide concentrations were titrated with

cytochrome aa_3 are consistent with the results of Fig. 3A. Tangents with the slope of the line for the untreated enzyme, drawn to the final parts of the curves, intersect the abscissa at 0.6–0.7 mole cytochrome aa_3 per mole cyanide. The results presented in Figs. 3A and 4A indicate semi-quantitatively that $m = 1$, *i.e.* 1 mole cytochrome aa_3 contains 1 catalytic centre. Since the inhibitor is noncompetitive towards cytochrome *c* equations can be derived in which the observed activity in the absence and presence of inhibitor is related to K_i and m, and on replotting the data of Figs. 3A and 4A quantitative values for m and K_i can be obtained.

$$K_i = \frac{mE_f \cdot I_f}{mE_b} = \frac{E_f \cdot (I_t - mE_b)}{E_b} \tag{1}$$

where E_f, E_b, I_f and I_t are concentrations of free enzyme, bound enzyme, free inhibitor and total inhibitor, respectively.

$$\frac{1}{E_f} = \frac{1}{K_i} \cdot \frac{I_t}{E_t - E_f} - \frac{m}{K_i} \tag{2}$$

or

$$\frac{1}{E_t - E_f} = \frac{K_i}{I_t} \cdot \frac{1}{E_f} + \frac{m}{I_t} \tag{3}$$

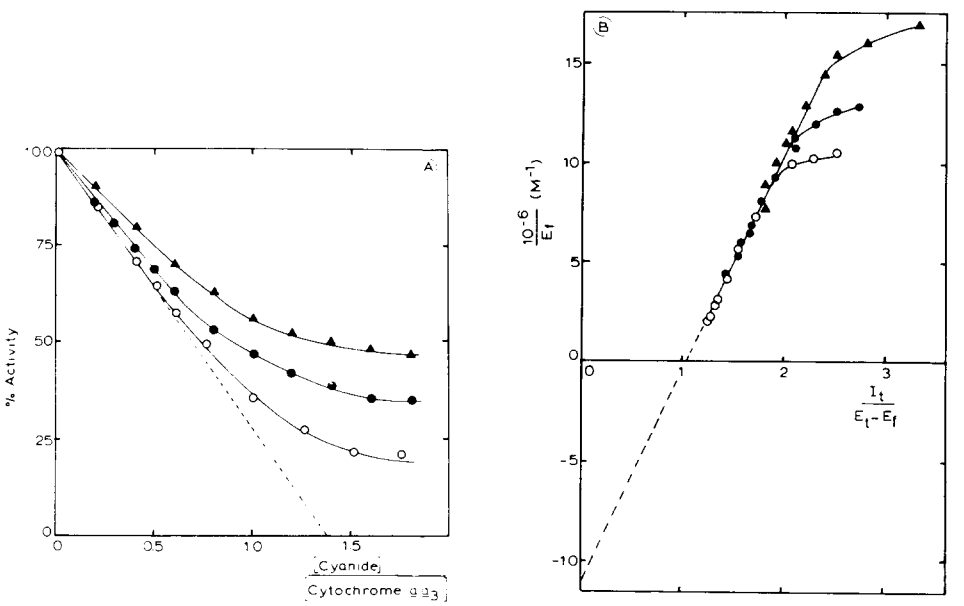


Fig. 3. Effect of stoichiometric amounts of cyanide on the enzymic activity. Cytochrome aa_3 was pretreated for 3–4 h at 0° with 2 mM ascorbate, 1 μM cytochrome *c* and varying cyanide concentrations. Activity is measured after 100- to 500-fold dilution in 30 μM ferrocytochrome *c*. ○—○, 500 nM cytochrome aa_3; ●—●, 225 nM cytochrome aa_3; ▲---▲, 125 nM cytochrome aa_3. A. Effect of the cyanide to cytochrome aa_3 ratio on the fractional activity. The broken line is the tangent to the initial part of the lines and intersects the abscissa at the apparent ratio of cyanide to cytochrome aa_3 of the inhibitory complex. B. Determination of K_i and m, from data in A and Eqn. 2 (see text).

On replotting the data of Fig. 3A as $1/E_f$ *versus* $I_t/(E_t - E_f)$ a straight line is obtained intersecting the abscissa at $m = 1.04$. From the point of intersection on the ordinate the K_i is calculated to be $9 \cdot 10^{-8}$ M.

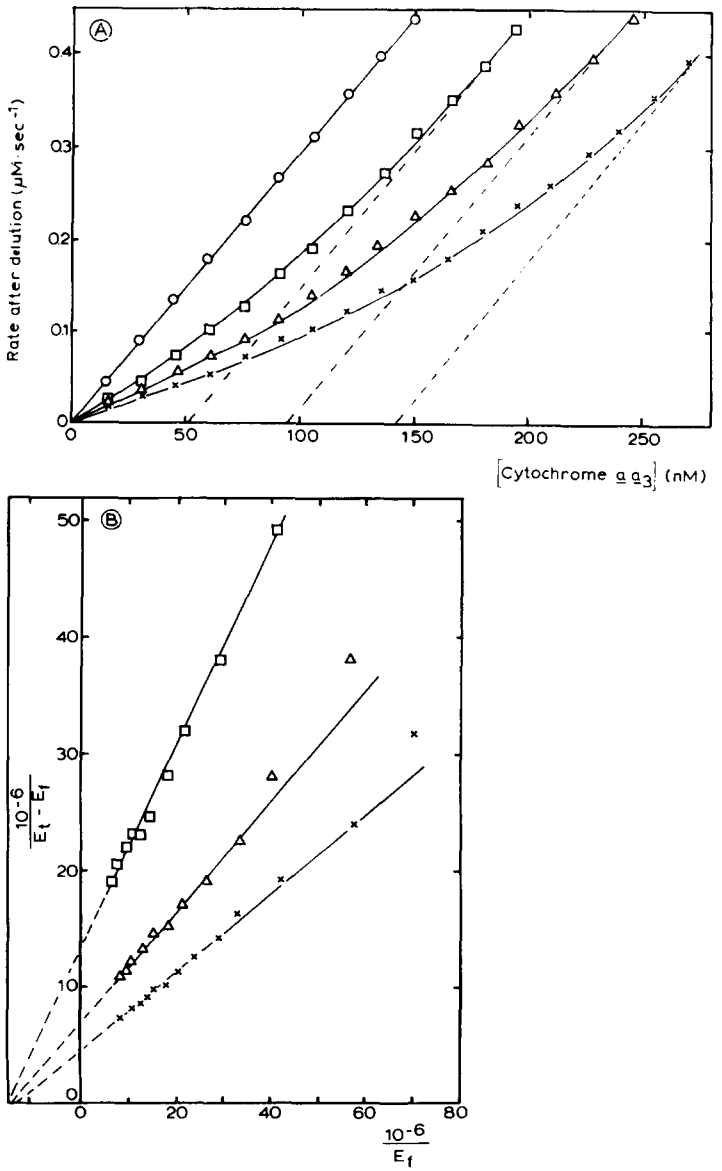


Fig. 4. Enzymic activity after preincubation of cyanide with stoichiometric amounts of cytochrome aa_3. Enzyme was pretreated as described in Fig. 3. Activity was measured after 100-fold dilution in 35 μM ferrocytochrome *c*. ○—○, without cyanide; □—□, 75 nM cyanide; △—△, 150 nM cyanide; ×—×, 225 nM cyanide. A. Effect of enzyme concentration on the rate of ferrocytochrome *c* oxidation. The dashed lines are tangents drawn to the final parts of the curves with a slope identical to the slope of the uninhibited system. They intersect at the apparent ratio of cytochrome aa_3 to cyanide in the inhibitory complex. B. Determination of K_i and m, from data in A and Eqn. 3 (see text).

At cyanide to cytochrome aa_3 ratios higher than 1, a deviation from the straight-line relationship is observed (Fig. 3B). This might be due to the fact that free cyanide is titrated away from the medium by secondary binding sites present in the cytochrome aa_3 preparations. The affinity of these sites is less than that of the inhibitory site since they become evident only at higher cyanide concentrations. The fact that the free enzyme concentration at high cyanide concentration is greater than expected from Eqn. 2 indicates that cyanide bound to these secondary sites does not influence the activity.

On replotting the data of Fig. 4A as $1/(E_t - E_f)$ *versus* $1/E_f$ (Fig. 4B) straight lines are obtained for each cyanide concentration used. From the points of intersection at the ordinate, m can be calculated to be 1.01–1.05 and from the points of intersection on the abscissa K_i appears to be $7 \cdot 10^{-8}$–$8 \cdot 10^{-8}$ M in accord with the data obtained from Fig. 3B. The presence of secondary binding sites is less evident in this type of experiments since the cyanide concentrations used are much lower, but a deviation of the straight line can be seen at low free enzyme concentration.

Formation and isolation of cyano-cytochrome aa_3

After incubation of cytochrome aa_3 with $K^{14}CN$ followed by filtration through Sephadex G-25 gel, radioactivity is observed in two well separated peaks, one of which coincides with the protein peak (Fig. 5). The cyanide eluted in the first peak could not be removed by rechromatography, extended dialysis, ultrafiltration, $HgCl_2$ treatment[43], ammonium sulphate fractionation or addition of excess azide.

The ratio of tightly bound cyanide to cytochrome aa_3 in the protein peak is about 1. Careful examination of the ratio of cyanide to cytochrome aa_3 in the different fractions shows, however, that the first fractions have a ratio slightly lower than one, whereas the last fractions have ratios higher than one. This can be explained by a slight dissociation of the complex.

The formation of cyano-cytochrome aa_3 is very slow. Fig. 6A shows that equilibration takes about 18 h and that in the equilibrium state 1 mole of cyanide is

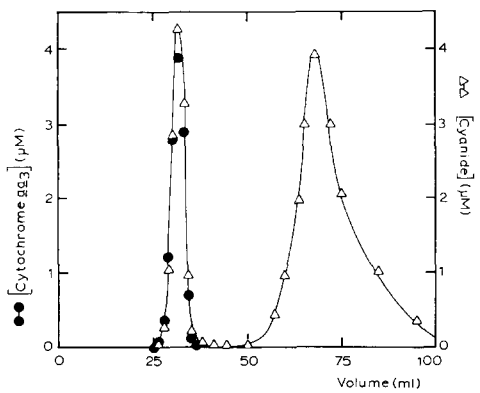


Fig. 5. Elution patterns on Sephadex G-25 of cytochrome aa_3 *plus* cyanide. 8.2 μM cytochrome aa_3 and 45 μM $H^{14}CN$ incubated for 18 h at 0°. Chromatography and determination of concentrations as described in METHODS. Flow rate, 5 ml/h; elution time for cytochrome aa_3, 6 h; protein recovery, 98%; cyanide recovery, 90%; ratio of cyanide to cytochrome aa_3 in the pooled enzyme fractions, 1.06.

bound per mole cytochrome aa_3 (2 haems). The data of Fig. 6A are used for calculating the second-order rate constant (Fig. 6B) which is 2 $M^{-1} \cdot sec^{-1}$.

An attempt was made to obtain a value for the equilibrium constant of oxidized enzyme with cyanide by equilibrium dialysis. From a Scatchard plot (Fig. 7) it was found that 0.9 mole of cyanide is bound per mole cytochrome aa_3 with a K_D of 0.7 μM. From the K_D and the association rate constant (2 $M^{-1} \cdot sec^{-1}$) a dissociation rate con-

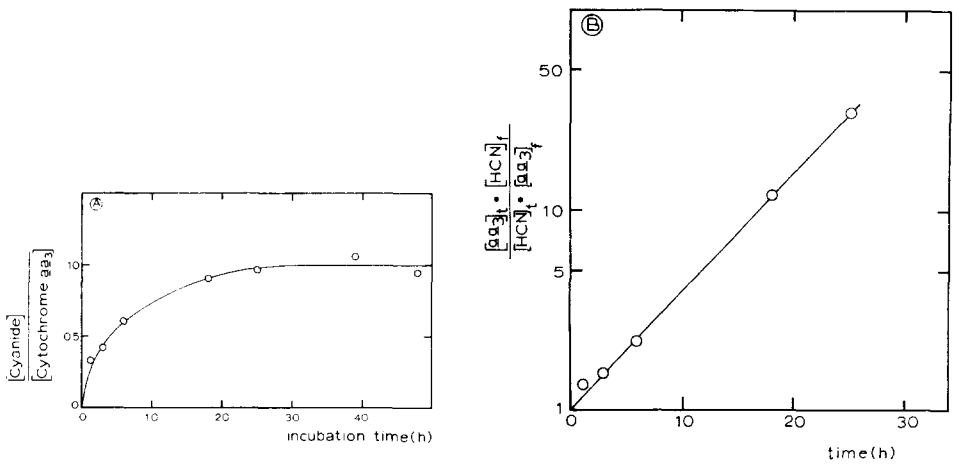


Fig. 6. Rate of formation of cyano-cytochrome aa_3. 8.2 μM cytochrome aa_3 and 31 μM $H^{14}CN$ were incubated at 0°. At the times indicated samples were withdrawn and chromatographed, and the ratio cyanide to cytochrome aa_3 was determined, as described in METHODS. A. Time course of the reaction. B. Second-order plot.

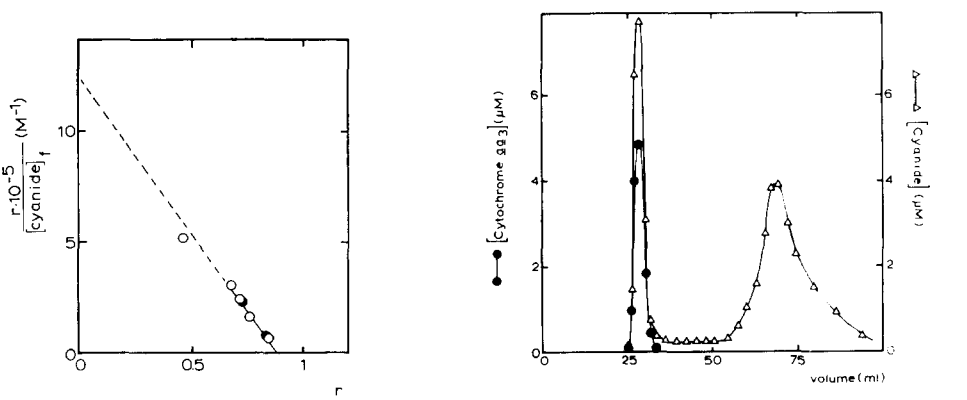


Fig. 7. Equilibrium dialysis of cytochrome aa_3 against $H^{14}CN$. 0.25 inch tubes were shaken for 24 h in 0.5% potassium cholate, 0.5% Tween 80 and 10 mM Tris sulphate (pH 8.0). Bags with 1–2 ml cytochrome aa_3 were put in stoppered centrifuge tubes, that had been filled with 12 ml Tween–Tris–cholate and mounted on a tilted, rotating wheel. Mixing was accomplished by air bubbles inside the bags and tubes. $K^{14}CN$ was added either to the enzyme or to the dialysate and after 100 h equilibration radioactivity was measured inside as well as outside the bags (recoveries were close to 100%). All experiments are carried out at 0–4°. Radioactivity was measured as described in METHODS. r equals $H^{14}CN$ bound per cytochrome aa_3.

Fig. 8. Elution pattern on Sephadex G-25 of cytochrome aa_3 *plus* cyanide with rapid elution. 8 μM cytochrome aa_3 and 50 μM $H^{14}CN$. Flow rate, 30 ml/h; elution time, 1 h; protein recovery, 97%; ratio cyanide to cytochrome aa_3 in the pooled enzyme fractions, 1.64 (*cf.* Table I).

stant of $1.4 \cdot 10^{-6}$ sec^{-1} can be calculated. This small rate constant explains why it was possible to isolate a cyano-cytochrome aa_3 complex by gel filtration.

Table I summarizes the observations on the binding of cyanide to cytochrome aa_3 under different conditions. When oxidized enzyme was incubated with a 6-fold excess of cyanide and the protein eluted slowly from the column, only cyano-cytochrome aa_3 was eluted. Shortening the elution time, however, produces some tailing of the cyanide peak (Fig. 8), and the ratio of cyanide to enzyme increases to 1.64. From this it can be concluded, that more than 1 mole of cyanide can be bound to cytochrome aa_3. As can also be seen from Table I, increasing amounts of cyanide in the incubation mixture give more secondarily bound cyanide.

TABLE I

EFFECT OF INCUBATION ON THE BINDING OF CYANIDE TO CYTOCHROME aa_3

Oxidized incubation, chromatography on Sephadex G-25 and determination of enzyme and cyanide concentrations as indicated in METHODS. Turnover incubation: 2 mM ascorbate and 0.1 μM cytochrome *c* were added, and the mixture was shaken in an oxygen atmosphere. Reduced incubation: $Na_2S_2O_4$ was added; after 10 min the mixture was vigorously shaken with air for about 15 sec. Ferricyanide (15 mM) was added before gel filtration when turnover or reduced incubations were applied. Cytochrome aa_3, 1–8 μM. Cyanide to cytochrome aa_3 was determined from the total amount of cyanide present in the protein peak. Elution time: time for eluting the protein from the column.

	Incubation conditions					
	Oxidized		*Turnover*		*Reduced*	
Moles of cyanide per mole cytochrome aa_3 during incubation	6	30	100*	6	6	6
Incubation time	18 h	18 h	18 h	10 min	1 h	10 min
Cyanide bound per mole cytochrome aa_3 after gel filtration:						
at elution time 1 h	1.64**	4.88**	6.9**	0.23	0.95	0.97
at elution time 6 h	0.95	1.58**	—	—	1.08	0.96

* In this experiment 1 mg Asolectin per ml was present instead of 0.5% potassium cholate.
** "Tailing" of the cyanide peak is observed (see text).

In Fig. 6A it was shown that 50 μM cyanide and 8 μM enzyme need about 18 h for equilibration when the enzyme is in the oxidized form. When the enzyme was pretreated with cyanide in the presence of ascorbate and cytochrome *c* the formation of cyano-cytochrome aa_3 was much faster, with 0.23 mole of cyanide bound per mole aa_3 after 10 min and 0.95 within 1 h. Fully reduced enzyme reacts still more rapidly with cyanide when oxygen is introduced into the mixture. Pretreatment with dithionite and cyanide followed by shaking the mixture with air equilibrates cyanide and enzyme within 10 min.

The data of Table I show that cyano-cytochrome aa_3 can be isolated after various pretreatments. Although pretreatment with dithionite and air gives the quickest result, the incubation with ascorbate and cytochrome *c* is preferred since it gives more reproducible ratios of cyanide to cytochrome aa_3. Cyano-cytochrome aa_3 was concentrated by ammonium sulphate precipitation and stored at −195°. Further-

more the cytochrome *c* content decreases to less than 0.5 % of that of cytochrome aa_3 by the ammonium sulphate fractionation.

Absorption spectra of cyano-cytochrome aa_3

In Fig. 9 the spectra of oxidized and reduced cyano-cytochrome aa_3 are compared with those of cytochrome aa_3. These spectra show almost identical peak positions as those of cytochrome aa_3 in the presence of excess cyanide, indicating that only 1 mole cyanide produces the spectral shifts. This was also concluded from

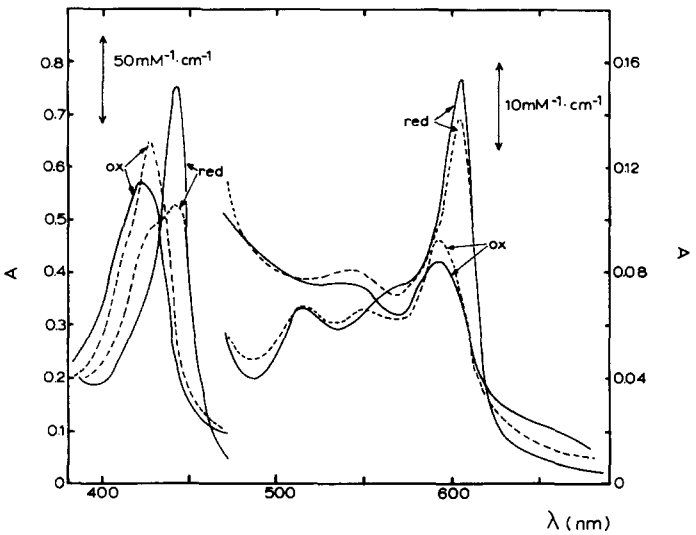


Fig. 9. Absorption spectra of cyano-cytochrome aa_3 and cytochrome aa_3. 3.5 μM enzyme was diluted in 100 mM Tris sulphate (pH 8.0), 0.5 % potassium cholate and 0.5 % Tween 80. Reduced spectrum of cyano-cytochrome aa_3 measured 15 sec, and of cytochrome aa_3 15 min after addition of $Na_2S_2O_4$. ———, cytochrome aa_3, – – – –, cyano-cytochrome aa_3.

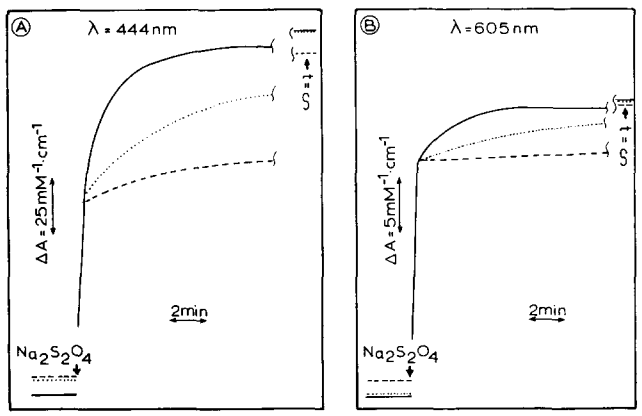


Fig. 10. Rate of reduction of cyano-cytochrome aa_3 and cytochrome aa_3 with $Na_2S_2O_4$. Conditions as described in Fig. 9. Temperature, 22°. ———, cytochrome aa_3; · · · · · ·, cyano-cytochrome aa_3; – – – –, cyano-cytochrome aa_3 *plus* 10 mM cyanide. Final absorptions measured after 45 min. A. 5 μM enzyme, 444 nm. B. 15 μM enzyme, 605 nm.

the CD spectrum (not shown). Thus the cyanide bound to the secondary binding sites does not contribute to the spectral changes.

When the difference spectrum of oxidized cyano-cytochrome aa_3 *minus* oxidized cytochrome aa_3 is compared with that of cytochrome aa_3 *plus* excess cyanide *minus* cytochrome aa_3 it appears that the peak and trough positions are identical but the extinction coefficients for cyano-cytochrome aa_3 are some 10 % lower[44]. This may be due to the presence of free cytochrome aa_3 in the cyano-cytochrome aa_3 preparations. The amount of free cytochrome aa_3 was estimated from the changes in absorbance obtained after addition of cyanide (10 mM, 4 h) or azide (0.5 mM, 10 min) to cyano-cytochrome aa_3. For example the spectroscopic effect of azide and cyanide on a particular preparation that contained 0.98 mole cyanide per mole cytochrome aa_3 was 5–6 % of that obtained for cytochrome aa_3. These values varied from preparation to preparation; the highest amount of free enzyme being about 10 % and obtained after pretreatment with dithionite and the lowest being 2 % obtained after incubation with ascorbate and cytochrome *c*. The free cytochrome aa_3 present causes the enzymic activity of the complex, as will be discussed below.

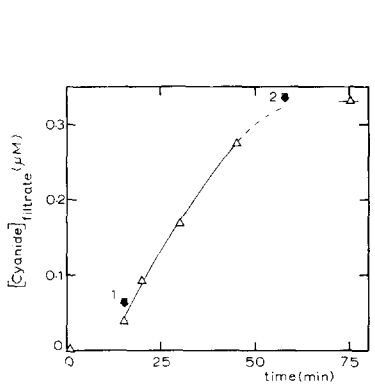
Stability of cyano-cytochrome aa_3

Oxidized cyano-cytochrome aa_3 is extremely stable but the complex dissociates readily on reduction. This is illustrated in Fig. 10 where the time course for reduction with $Na_2S_2O_4$ of cyano-cytochrome aa_3 in the presence and absence of extra added cyanide is compared with that of cytochrome aa_3. Two phases can be distinguished and from the contributions to the absorbance at 605 and 444 nm it may be concluded that the first phase represents the reduction of cytochrome *a* and the second phase that of cytochrome a_3. The reduction of cytochrome a_3 in cyano-cytochrome aa_3 is much slower than in the enzyme itself whereas in the presence of 10 mM cyanide the reduction is even yet slower. The reduction level of cyano-cytochrome aa_3 reached after 45 min equals that of cytochrome aa_3. These data show that before cytochrome a_3 can be reduced cyanide has to dissociate from the complex. The absorbance changes of the second phase can thus be used for the determination of the dissociation rate constant for cyano-cytochrome aa_3 in the presence of dithionite. The k_{off} obtained from Fig. 10 is $2 \cdot 10^{-3}$–$3 \cdot 10^{-3}$ sec^{-1}. This constant is considerably greater than that for the oxidized enzyme ($1.4 \cdot 10^{-6}$ sec^{-1}).

The enhancement of the rate of dissociation of cyanide on reduction is also observed by measuring the formation of free cyanide. Fig. 11 shows the appearance of $H^{14}CN$ in the filtrate during ultrafiltration of cyano-cytochrome aa_3 in the presence and absence of ascorbate and cytochrome *c*. Without electron donor hardly any cyanide was present in the filtrate but on addition of electron donor the concentration of cyanide in the filtrate increased gradually until all the ascorbate was consumed. By this treatment more than 65 % of the cyanide originally present was removed.

The loss of cyanide during turnover is demonstrated by the appearance of enzymic activity as shown in Fig. 12. The slow increase in the rate of oxidation of cytochrome *c* is due to dissociation of the complex during turnover. It cannot be due to a dissociation of cyanide from the oxidized enzyme, since preincubation of cyano-cytochrome aa_3 in the reaction mixture for 1 or 5 min did not change the pattern of restoration of the activity. If initial rates are calculated from tangents of the curve of Fig. 12 the enzymic activity of cyano-cytochrome aa_3 is overestimated. This was

demonstrated by measuring the rates at different times after addition of cyano-cytochrome aa_3 to various cytochrome *c* concentrations. Fig. 13 shows that in a Lineweaver–Burk plot the lines for each time intersect on the abscissa, indicating that the K_m for cytochrome *c* is not affected. The increase in activity during the turn-



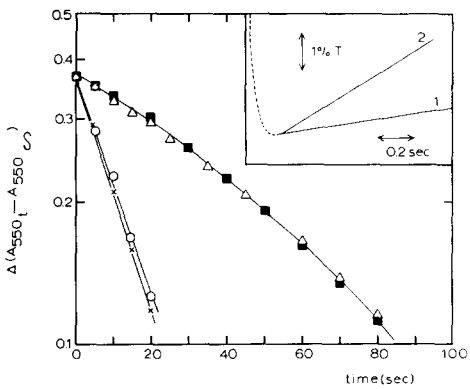


Fig. 11. Ultrafiltration of cyano-cytochrome aa_3. Cyano-cytochrome aa_3 was prepared by addition of dithionite to 10 μM cytochrome aa_3 and 60 μM cyanide and chromatographed on Sephadex G-25. Incubation mixture and chromatography as described in METHODS. Before gel filtration the mixture was shaken with air for about 15 sec after which $K_3Fe(CN)_6$ (5 mM) was added. The ratio of cyanide to cytochrome aa_3 was 1.05. 50 ml incubation mixture containing 0.9 μM cyano-cytochrome aa_3 was put in an Amicon ultrafiltration cell fitted with a PM 30 filter. Filtration was carried out at 0° with 3.5 atm O_2 with a filtration rate of 0.3–0.1 ml/min. At the first arrow 50 mM ascorbate and 300 μM cytochrome *c* were added, at the second arrow the colour of the mixture changed suddenly from bright red to dark brown indicating that all ascorbate was consumed. Filtration was continued for another 20 min.

Fig. 12. Enzymic activity of cyano-cytochrome aa_3. The enzyme was diluted to 5.0 nM and ferrocytochrome *c* to a final concentration of 23 μM was added. Cyano-cytochrome aa_3 was prepared as described in Fig. 6. The ratio of cyanide to cytochrome aa_3 was 0.96. ×—×, fresh enzyme; ○—○, enzyme incubated and gel-filtrated without cyanide; ■—■, cytochrome *c* added immediately after 60-fold dilution of cyano-cytochrome aa_3; △—△, cytochrome *c* added 5 min after 60-fold dilution of cyano-cytochrome aa_3. Inset: Expanded scale reading on a Durrum stopped-flow screen. Full scale transmission change, 36%. The tracings are obtained from a free run, the dotted part of the curves representing the flow. Curve 1, 0.8 μM cyano-cytochrome aa_3 (ratio cyanide to cytochrome aa_3, 0.97); Curve 2, 0.07 μM cytochrome aa_3 (note more than 10-fold difference in enzyme concentration in Curves 1 and 2). Reaction started with 6.8 μM ferrocytochrome *c* in 100 mM phosphate buffer (pH 7.4), 0.5% Tween 80 and 1 mM EDTA. Temperature, 25°.

over was therefore due to the generation of free enzyme with the same kinetic properties as cytochrome aa_3. When the rates at infinite cytochrome *c* concentration are plotted against time (inset) the initial rate can be obtained by extrapolation. This is 30 sec^{-1}, in comparison with 375 sec^{-1} for the untreated enzyme, indicating that 8% free enzyme was present in the cyano-cytochrome aa_3. The k_{off}, calculated from the initial rate of increase of activity (inset in Fig. 13), is $1.8 \cdot 10^{-3}$ sec^{-1}.

A better method to measure initial rates is to follow the ferrocytochrome *c* oxidation in a stopped-flow apparatus. The inset of Fig. 12 shows the time course of ferrocytochrome *c* oxidation on an expanded scale. The dashed line represents the flow and Curve 1 is obtained after mixing of 6.8 μM ferrocytochrome *c* with 0.8 μM cyano-cytochrome aa_3 and Curve 2 with 0.07 μM cytochrome aa_3 itself. The activity

of cyano-cytochrome aa_3 is 2.5 % of that of the enzyme without cyanide, agreeing with the free enzyme content of 3–4 % obtained after measurement of the spectroscopic effects on addition of azide and cyanide.

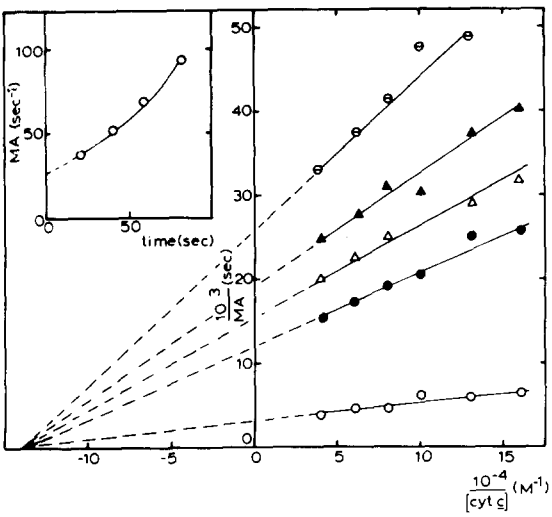


Fig. 13. Formation of cytochrome aa_3 from cyano-cytochrome aa_3. At zero time, cyano-cytochrome aa_3 (5 nM final concentration) or cytochrome aa_3 (1 nM) were added to 65 mM potassium phosphate (pH 6.0), 0.5 % Tween 80 and 0.5 mM EDTA. Temperature, 25°. Rates were measured at different time intervals from the slopes of the lines in a semi-logarithmic plot (*cf.* Fig. 12). ⊖—⊖, 20 sec after dilution; ▲—▲, 40 sec; △—△, 60 sec; ●—●, 80 sec; ○—○, cytochrome aa_3. Inset: Effect of time on the molecular activity at infinite cytochrome *c* concentrations.

DISCUSSION

It is clear from the binding studies as well as from the effect of cyanide on the activity that either the rate of binding of cyanide to cytochrome aa_3 or the equilibrium between them or both depends on the redox state of the enzyme. This is in agreement with earlier observations[11,25–27]. The observed enhancement of the rate under reducing conditions is unexpected, since the rate of binding of cyanide to ferrihaemoproteins, such as peroxidase and metmyoglobin, is greater than to ferrohaemoproteins[45–47]. Therefore it is concluded that the binding site of cyanide in oxidized cytochrome aa_3 is more masked than when the enzyme is reduced. As in other haemoproteins[48–50] the iron of cytochrome a_3 is probably buried in a cavity, as judged from its nonpolar environment[51] and the sterically hindered but diffusion-controlled reaction with molecules like O_2 (ref. 52) and azide (unpublished results). The dependence of the rate of cyanide binding on the redox state of the enzyme suggests that the cavity in oxidized cytochrome aa_3 is more closed than when the enzyme is reduced. A difference in conformation between oxidized and reduced cytochrome aa_3 has also been shown by VAN GELDER[53] and YAMAMOTO AND OKUNUKI[54]. Since the reduction of cytochrome *a* facilitates considerably the dissociation of cyanide (k_{off} differs three orders of magnitude) it seems that the conformation of the enzyme is mainly determined by the redox state of cytochrome *a*.

The noncompetitive inhibition of cyanide towards cytochrome *c* observed

after a reducing preincubation of the components is in disagreement with the uncompetitive inhibition observed after incubation of cyanide with the oxidized enzyme[25]. However, the rates obtained after the latter incubation are determined from the slope of a complicated curve consisting of three logarithmic components: the liberation of cyanide from an enzyme–cyanide complex, oxidation of ferrocytochrome *c* by the liberated enzyme and inactivation of the liberated enzyme by cyanide under turnover conditions. It is likely that the difference in type of inhibition originates from a determination of enzymic activity under nonequilibrium conditions.

The K_D value obtained by equilibrium dialysis is about one order of magnitude greater than the K_i obtained from inhibition studies, where cytochrome aa_3 was pretreated with cyanide in the presence of electron donor and air. The very long incubation time (4 days) needed for equilibration might have changed the affinity for cyanide just as the rate constant for cyanide binding is lowered on freezing and thawing[44]. Other conditions influencing the rate of cyanide binding and the affinity for cyanide will be discussed in a separate paper

The K_i value of $9 \cdot 10^{-8}$ M corresponds with the lowest values for the K_i found in particulate preparations of cytochrome aa_3, pretreated with electron donors. Since the rate of association as well as the rate of dissociation depends on the redox state of cytochrome *a*, it is likely that also the affinity for cyanide will depend on the redox state. This explains the difference in K_i values reported as well as the observation that the cyanide inhibition depends on the assay system.

It has been shown by VAN GELDER AND MUIJSERS[20,55] that with a large excess of cyanide the reduction of half of the haem and copper is blocked. The same phenomenon is also observed when cyano-cytochrome aa_3 is titrated with NADH and phenazine methosulphate. The data and an explanation of it will be offered in a separate paper.

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