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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME aa_3

VI. REACTION OF CYANIDE WITH OXIDIZED AND REDUCED ENZYME

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SUMMARY

1. The reaction of cyanide with cytochrome aa_3 in the fully oxidized and reduced states has been studied. In both cases a single molecule of cyanide is bound reversibly per molecule of aa_3 (that is, 1 mole cyanide per 2 equivalents of haem *a*).

2. The difference spectrum of fully formed ferric aa_3–cyanide complex *minus* oxidizid enzyme with maxima at 432, 540 and 585 nm resembles that of other cyanide ferrichaemoproteins; it appears to be a low-spin complex of cytochrome a_3.

3. The equilibrium constant for complex formation with ferric aa_3 (K_D) was approximately 1 μM; equilibration at 4° took several days as the rate constant for cyanide binding at low cyanide concentrations is only 1.8 $M^{-1} \cdot sec^{-1}$ (pH 7.4, 21°). At high cyanide concentrations, the rate of spectroscopic complex formation becomes independent of cyanide with a first order constant of 0.018 sec^{-1}.

4. The difference spectrum obtained on addition of cyanide to ferrous aa_3 shows a peak at 587 nm but only a small diminution of absorbance at 445 nm. The equilibrium constant for complex formation with the ferrous enzyme was 100 μM; the rate constant for cyanide binding was 150 $M^{-1} \cdot sec^{-1}$, apparently representing a simple bimolecular step.

5. The cytochrome aa_3–azide complex reacts more rapidly with cyanide than does the free ferric enzyme; the rate constant for cyanide displacement of azide is 25 $M^{-1} \cdot sec^{-1}$. The rate limiting step at high cyanide concentrations has the same velocity in the precence as in the absence of azide.

6. The reaction of ferric enzyme with cyanide is interpreted in terms of a two-step reaction involving an initial weak binding to the protein (K'_α of 10 mM) followed by binding to the a_3 haem. A conformational change accompanying azide binding is proposed that facilitates the subsequent initial weak binding of cyanide (K'_α (in presence of N_3^-) = 0.7 mM).

INTRODUCTION

The previous paper[1] has described some of the properties of a cyanide complex of cytochrome aa_3 ('cyano-cytochrome aa_3') in which 1 mole of cyanide is tightly

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bound to 1 mole of cytochrome aa_3. The formation of this complex, which is probably identical with that reported by CAMERINO AND KING[2] and by ORII AND OKUNUKI[3], requires the presence of electron donor and O_2, as had earlier been observed by WAINIO AND GREENLEES[4] for effective enzyme inhibition. However, a cyanide complex is also formed when the oxidized enzyme is treated with cyanide[5], and another complex, involving ferrous cytochrome a_3, when the fully reduced enzyme is treated with cyanide[6,7]. The reported dissociation constants are very different. Cyano-cytochrome aa_3, a ferric complex formed under conditions of turnover preincubation[1], shows an apparent K_D of 90 nM. A K_D of 90 μM has been claimed for the cyanide complex formed with fully oxidized enzyme[5,8] and of 700 μM for the ferrous a_3–cyanide complex[7]. None of these values corresponds to the K_i of about 1 μM found for inhibition of the catalytic system[9,10].

With an improved preparation of cytochrome *c* oxidase it has been possible to follow more closely the spectroscopic changes that take place on addition of cyanide. The present paper describes the kinetics and equilibrium for cyanide binding by both the oxidized ($a^{3+}a_3^{3+}$) and reduced ($a^{2+}a_3^{2+}$) forms of the oxidase. The following paper[11] will outline the essential features of the inhibition of the catalytic activity by cyanide and present a tentative model to explain some of the observed peculiarities.

Independent studies, similar to part of those reported here, have been carried out by ANTONINI, BRUNORI, GREENWOOD, MALMSTRÖM AND ROTILIO (personal communication) and ORII AND YOSHIKAWA (reported at the *2nd Int. Conf. on Oxidases and Related Redox Systems, Memphis, Tenn., June* 1971).

MATERIALS AND METHODS

Enzyme

Cytochrome aa_3 was isolated according to the method of FOWLER *et al.*[12], followed by a cholate–ammonium sulphate fractionation as described by MACLENNAN AND TZAGOLOFF[13] (*cf. ref.* 14). The final preparations had ratios of haem *a* to protein of 9.5 to 10.0 μmoles·g^{-1} and a low content of nonreducible haem as judged from the ratios: (A_{444nm}/A_{424nm}) red = 2.20–2.35 and A_{444nm} (red)/A_{424nm} (ox) = 1.30–1.38. The concentrated enzyme was stored in liquid nitrogen. Before use, it was first diluted in 0.4 M potassium phosphate (pH 7.4), 2% Tween 80. After mixing, this solution was diluted with three parts of distilled water to give a final concentration of 100 mM phosphate (pH 7.4) and 0.5% Tween 80. Enzyme solutions prepared in this way are stable for more than 48 h at room temperature and for about 1 min at 60°. Spectroscopic determinations were made at 21° unless otherwise stated.

Enzyme concentration (*i.e.* the concentration of aa_3) was determined using ΔA_{605nm} (red–ox) = 24 mM^{-1}·cm^{-1} (ref. 15). Protein concentration was obtained according to the method of GORNALL *et al.*[16] using serum albumin as a standard.

Instruments

For the spectroscopic determinations a Cary 17 R recording spectrophotometer was used, and for the stopped-flow experiments a Durrum stopped-flow instrument.

Chemicals

Cholic acid (Sigma) was recrystallized in ethanol and stock solutions of 20% (w/v) of the potassium salt stored at 0–4° in dark bottles. Tween 80 was obtained from

Koch Light and used from 10% (v/v) stock solutions. KCN solutions were freshly prepared usually in 1 mM KOH to prevent loss by evaporation. When high cyanide concentrations had to be added cyanide was mixed with 3/4 of its weight of citric acid and dissolved in ice-cold distilled water. No pH changes on addition of 50 mM (final concentration) cyanide were observed. When cyanide had to be used at low

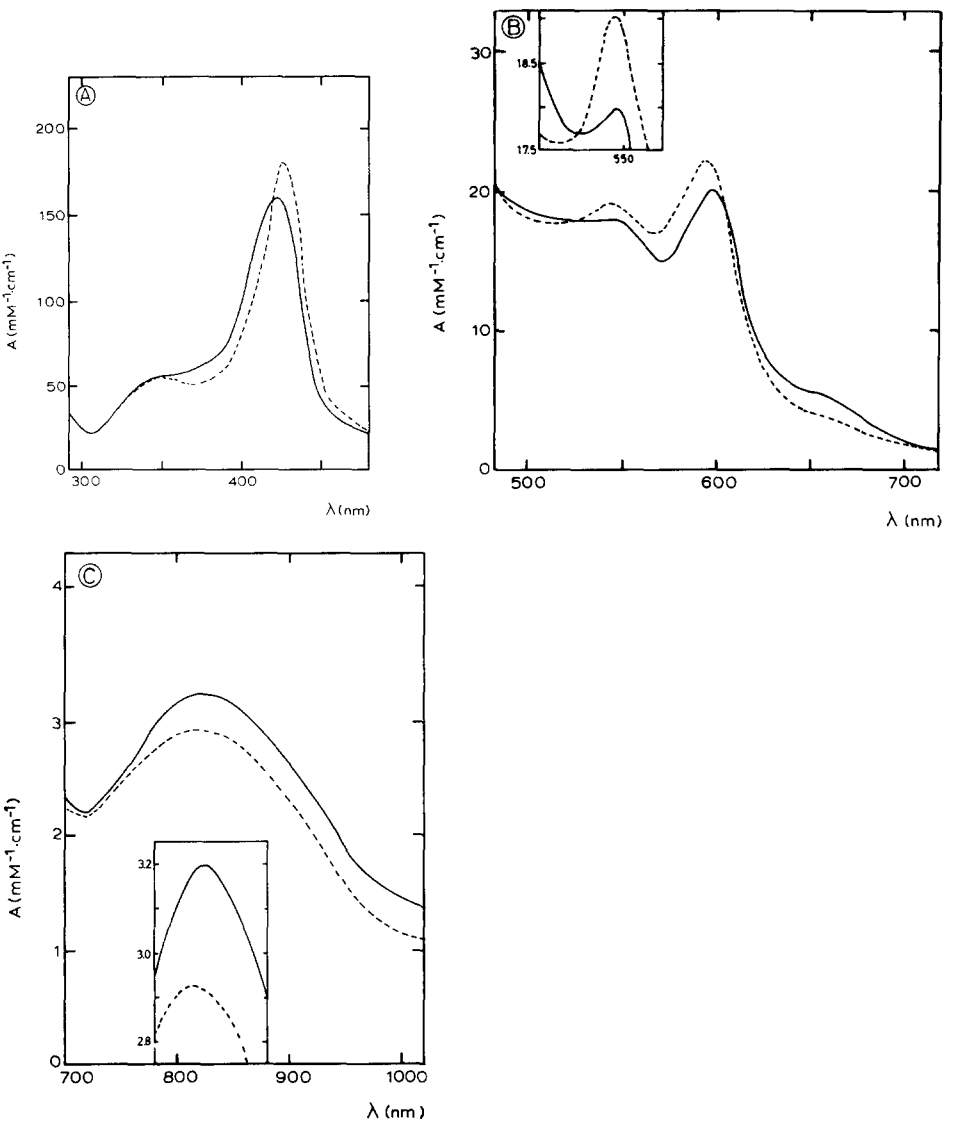

Fig. 1. Absorption spectra of oxidized cytochrome aa_3 and oxidized cytochrome aa_3 *plus* cyanide. The spectra were obtained after 18 h incubation of the enzyme at 0° in the presence (– – – –) or absence (————) of cyanide in 100 mM phosphate (pH 7.4) and 0.5% Tween 80. A. Soret region measured with 10 μM cytochrome aa_3. B. Visible region measured with 40 μM cytochrome aa_3. C. Near-infrared region, measured with 140 μM cytochrome aa_3. (The insets show the peak positions on an expanded scale.)

pressures cyanide solutions in 1 M KOH were added in μl amounts. All other chemicals were of Analar quality and were usually purchased from British Drug Houses.

RESULTS

Spectra of complexes formed with cyanide

The reaction of the oxidized enzyme with cyanide is unusually slow[1]. Fig. 1 shows the spectra obtained with cyanide after 18 h incubation. The Soret band (Fig. 1A) shows a red shift to 427 nm, together with a slight increase in intensity. The 650-nm band is suppressed and the α-band shows a blue shift from 598 to 593 nm (Fig. 1B). In the near-infrared region (Fig. 1C) a 10% decrease in absorbance is seen. Although previous studies[17–20] have shown that most of the absorption change at 830 nm upon reduction is due to the copper of cytochrome aa_3, it is not clear to us whether the small decrease in absorption reflects an effect of cyanide on the copper or haem, since CAUGHEY[21] has stated that a contribution of haem absorption is to be expected in the near-infrared region. Moreover, as will be discussed later, a striking similarity is observed in the kinetic behaviour of the spectral shift. Thus we think that the changes in absorption are consistent with the expected stabilization of a low-spin state if the iron in cytochrome aa_3.

In contrast, the reduced enzyme reacts rather more rapidly with cyanide to give the spectrum shown in Fig. 2. Unlike the complexes formed by reduced hemoglobin, myoglobin and peroxidase, the cyanferro a_3 complex is apparently quite stable, provided that the system is maintained strictly anaerobic. The spectra in Fig. 2 were obtained in argon-flushed Thunberg cuvettes; not only is the cyanide ferrous complex oxidized easily but the cyanide–ferric cytochrome a_3 often remains even in presence of $Na_2S_2O_4$[22]. A small change in the Soret region (Fig. 2A) is accom-

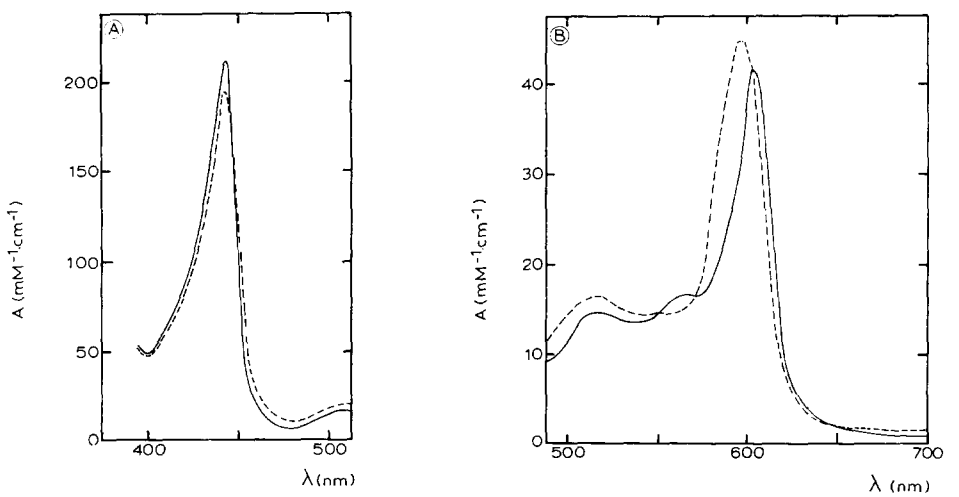

Fig. 2. Absorption spectra of reduced cytochrome aa_3 and reduced cytochrome aa_3 *plus* cyanide. The enzyme was diluted in 100 mM Tris–H_2SO_4 buffer (pH 8.0) and 0.5% potassium cholate. After fluxing with O_2-free argon in a Thunberg cuvette, dithionite was added from a side bulb to a final concentration of 10 mM. After about 10 min the spectrum of the reduced form was recorded (———). Finally cyanide from a second side bulb to a final concentration of 1 mM was added after which the spectrum was again recorded (– – – –).

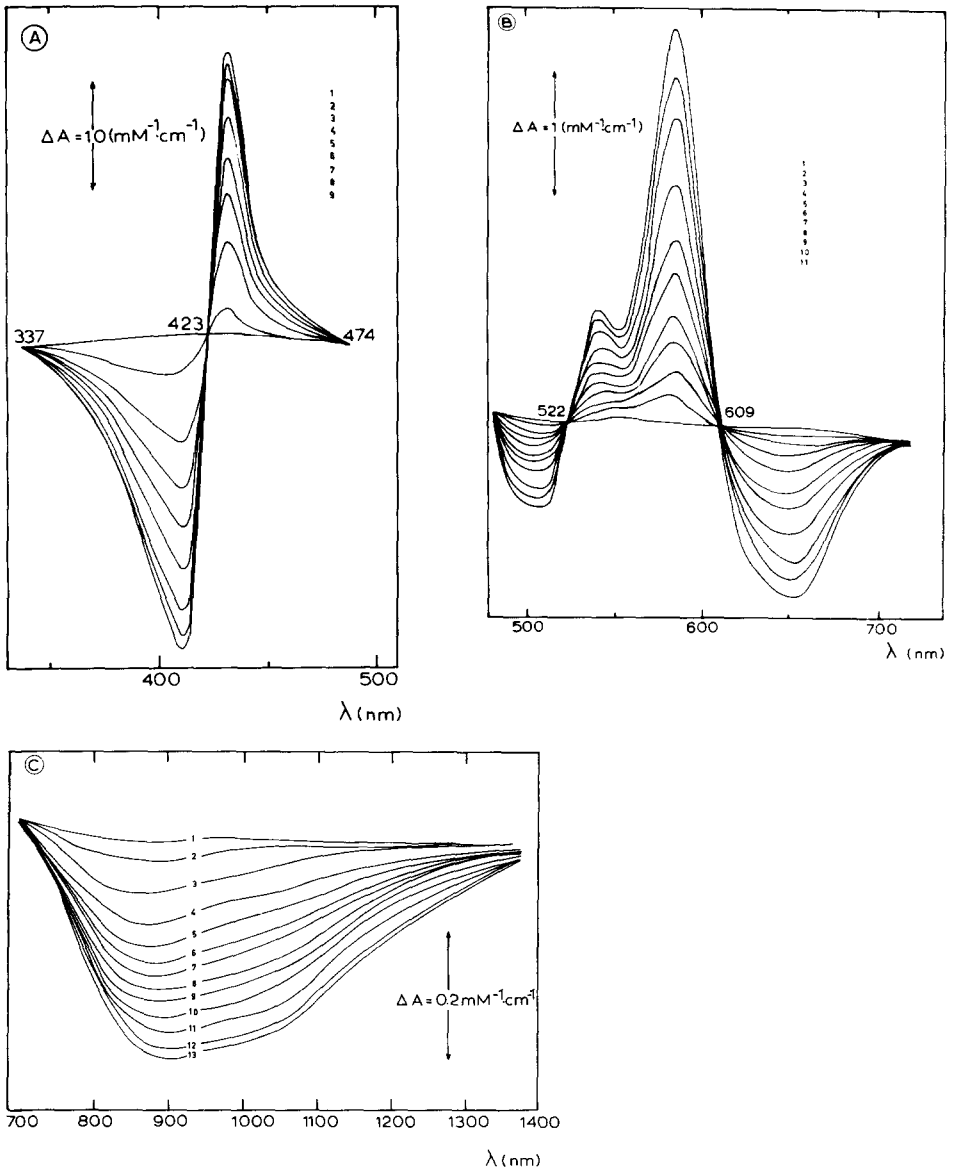

Fig. 3. Effect of time on the difference spectrum of oxidized cytochrome aa_3 *plus* cyanide *minus* oxidized cytochrome aa_3. Enzyme was diluted as described in METHODS. Scan speed 2 nm/sec and maximal spectral band width less than 0.5 nm in Soret and visible region, 5 nm/sec and maximal spectral band width of less than 1 nm in the near-infrared region. A. Soret region with 7.5 μM cytochrome aa_3 and 2 mM cyanide. Scan initiated: 9, without cyanide; 8, immediately after mixing; 7, after 1.5 min; 6, after 3.0 min; 5, after 4.5 min; 4, after 7.5 min; 3, after 10.5 min; 2, after 30 min; 1, after 4 h. B. Visible region, with 40 μM cytochrome aa_3 and 0.5 mM cyanide. Scan: 11, without cyanide; 10, immediately after mixing; 9, after 2.5 min; 8, after 5.0 min; 7, after 7.5 min; 6, after 12.5 min; 5, after 20 min; 4, after 30 min; 3, after 50 min; 2, after 90 min; 1, after 4 h. C. Near-infrared region, with 140 μM cytochrome aa_3 and 1.0 mM cyanide. Scan: 1, without cyanide; 2, immediately after mixing; 3, after 2.5 min; 4, after 5.0 min; 5, after 7.5 min; 6, after 10.0 min; 7, after 12.5 min; 8, after 15 min; 9, after 20 min; 10, after 30 min; 11, after 45 min; 12, after 60 min; 13, after 4 h.

panied by the previously reported[6,7] shift of the α-band from 603 to 590 nm with marked increase in absorbance (Fig. 2B). No changes could been seen in the near-infrared region.

The formation of the cyanide complex of the oxidized enzyme (Fig. 1) can be followed by difference spectroscopy. Fig. 3 shows such difference spectra recorded at various times after the addition of cyanide. It can be seen that all peaks (432, 535 and 587 nm) and troughs (411, 512, 645 and 880 nm) develop at the same rate (in the Soret (3A), visible (3B) and near-infrared (3C) regions) and a single set of isosbestic points at 337, 423, 474, 522 and 609 nm exists. Despite the kinetic complexities (discussed below) we conclude that only two distinct spectroscopic entities are involved, the original enzyme and the cyanide complex. Experiments were therefore carried out to determine the equilibrium constant in this system.

Equilibrium in the formation of the cyanide complex with fully oxidized cytochrome aa_3

Fig. 4 shows the approach to equilibrium obtained at various wavelengths (*cf.* Fig. 3) on the addition of 2 mM cyanide to the enzyme. The reaction time course deviates from the expected first order line, indicating that despite the spectroscopic

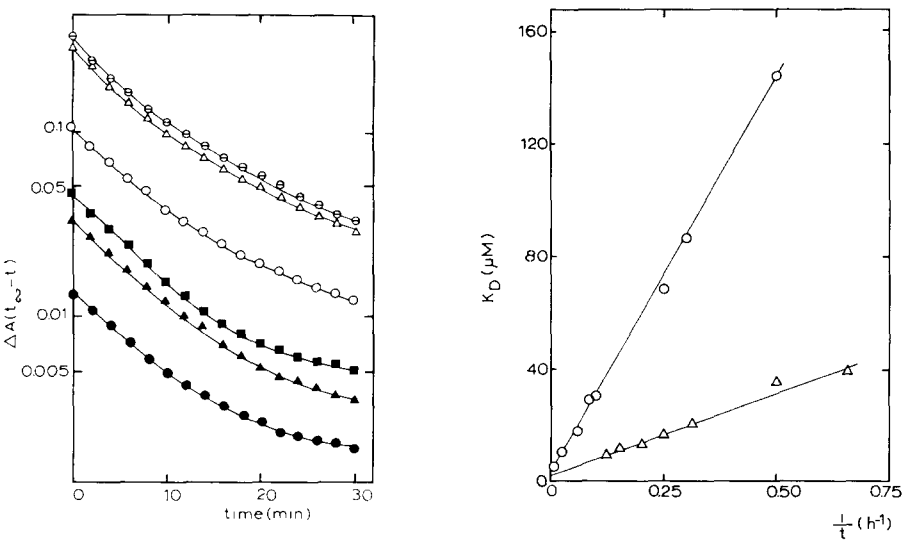

Fig. 4. Time course for reaction of oxidized cytochrome aa_3 with cyanide at different wavelengths. The reaction was started by the addition of 2 mM cyanide to 35 μM cytochrome aa_3 except for △---△ and ○---○ where the enzyme concentration was 10 μM. Enzyme was diluted as described in METHODS. ●—●, $-\Delta A_{830\ nm}$; ▲---▲, $\Delta A_{530\ nm}$; ■—■, $-\Delta A_{645\ nm}$; ○—○, $\Delta A_{586\ nm}$; △---△, $\Delta A_{432\ nm}$; ⊖—⊖, $-\Delta A_{411\ nm}$.

Fig. 5. Determination of the dissociation constant for the oxidized cytochrome aa_3–cyanide complex. Effect of incubation time on the apparent spectroscopic dissociation constant for the oxidized cytochrome aa_3–cyanide complex at 21° and 0°. For each determination at 0°, seven completely filled test tubes covered with parafilm were warmed to room temperature 5–10 min before the determination. For the room temperature experiment (21–23°) one single set of completely filled cuvettes covered with parafilm were used. For the calculations of the apparent K_D, ΔA (432–411 nm) was used. Cytochrome aa_3 diluted to 5.8 μM as described in METHODS. ○---○, incubation at 0°; △---△, incubation at room temperature.

simplicity (Fig. 3) the reaction does involve more than two components (for example, heterogenicity in binding sites, or an equilibrium between two forms of the enzyme only one of which reacts rapidly with cyanide, could give rise to the curves of Fig. 4). The slowness of the initial reaction, together with the slowing down seen as the reaction proceeds, creates the difficulties previously observed in determining the 'end point' and hence calculating an equilibrium constant. This is because apparent Hill plots can be constructed with straight line relationships between log $[EI]/([E]-[EI])$ and log $[I]$ even when equilibrium has not been reached. The apparent dissociation constants obtained in this way have time-dependent values, as shown in Fig. 5. Here the apparent K_D at various incubation times has been plotted against the reciprocal of the incubation time ($1/t$). Incubation at 0° for about 3 h gives the value of 90 μM reported by ORII AND OKUNUKI[5] and by NICHOLLS[8], and the figure shows that this is far from the values of about 4 μM (0°) or 2 μM (22°) pertaining on extrapolation to infinite incubation time. These may be compared with the value of 0.8 μM or less obtained by equilibrium dialysis[1].

Equilibrium in the formation of the cyanide complex with fully reduced cytochrome aa_3

In contrast to the slow reaction with the ferric enzyme, the ferrous enzyme equilibrates readily with cyanide under anaerobic conditions. Addition of cyanide to the reduced enzyme in a Thunberg cuvette flushed with argon gives rise immediately to the difference spectrum of Fig. 6. A sharp minimum is seen at 441 nm (Fig. 6A) and the characteristic shoulder on the α-peak (Fig. 2) appears as a peak at 587 nm in the difference spectrum (Fig. 6B). Fig. 7 shows a Hill plot for the equilibrium at pH 8.0 and 22°, with K_D equal to 104 μM and $n = 0.98$, obtained from ΔA (587–611 nm) of the difference spectrum. The kinetics of appearance of the 587-nm band were studied in the stopped-flow spectrophotometer; typical results for two enzyme and two cyanide concentrations are plotted in Fig. 8. The reaction shows first-order kinetics in both enzyme and cyanide concentrations, with a calculated association

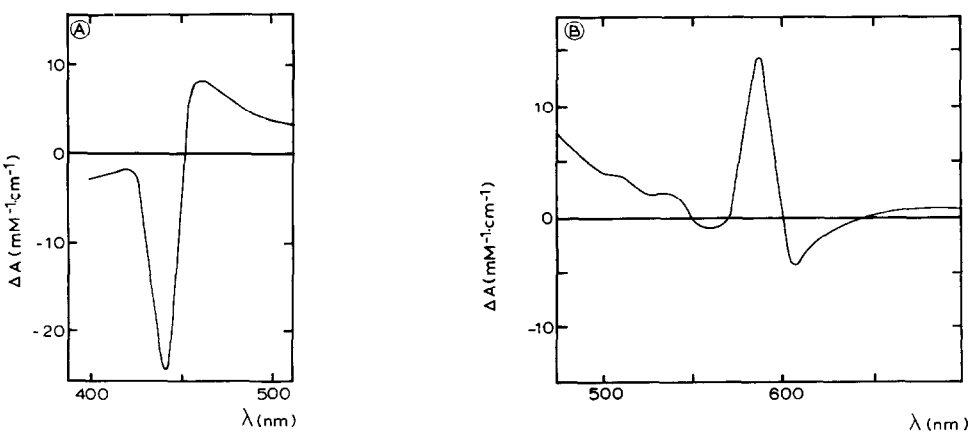

Fig. 6. Difference spectrum of reduced cytochrome aa_3 in presence and absence of cyanide. Conditions as described in Fig. 2. A. 5 μM cytochrome aa_3 and 1 mM cyanide; B. 14.2 μM cytochrome aa_3 and 1 mM cyanide.

rate constant of 150 $M^{-1} \cdot sec^{-1}$ at 25°. From this value and the equilibrium constant of 0.1 mM a dissociation rate constant equal to 0.015 sec^{-1} is calculated.

Unlike the reaction of ferric enzyme, this reaction is readily reversible. Six ml of enzyme solution (17.4 μM cytochrome aa_3 in 100 mM Tris–H_2SO_4 buffer (pH 8) with 0.5% potassium cholate, 10 mM $Na_2S_2O_4$, 25% sucrose) was flushed with argon and 1 mM cyanide then added (6 μl 1 M KCN from a side arm). Two ml of this solution was then passed through a Sephadex G-25 column (1.5 cm × 60 cm) equilibrated with Tris–cholate–$Na_2S_2O_4$ buffer (with a 25% sucrose solution it is possible to make the necessary transfer to the column without admixing air using a 5-ml pipette with a fine point, layering the contents on the top of the gel *underneath* a layer of the dithionite-saturated buffer). Spectra were recorded before and after addition of cyanide (*cf.* Fig. 2) and after gel filtration. The eluate had a spectrum (not shown) identical with that of free ferrocytochrome aa_3, and was fully reactive with fresh cyanide with no sign of autoxidation to form a^{2+} $a_3{}^{3+}$HCN, provided that the precautions stated were observed.

Although there is some difference between our K_D value of 100 μM and that reported by GIBSON AND GREENWOOD[7] (0.7 mM), we are not inclined to attribute much significance to this. The previous result[7] was obtained by quite indirect methods, and alternative calculations from the same data give much lower apparent K_D values.

Kinetics of formation of the cyanide complex of oxidized cytochrome aa_3

In view of the anomalous behaviour of the cyanide reaction with the ferric enzyme (Fig. 4), the initial rates of cyanide binding (as shown by the spectroscopic changes, Fig. 3) were studied at varying cyanide and enzyme concentrations. At low concentrations of cyanide, the initial rates are proportional to both cyanide

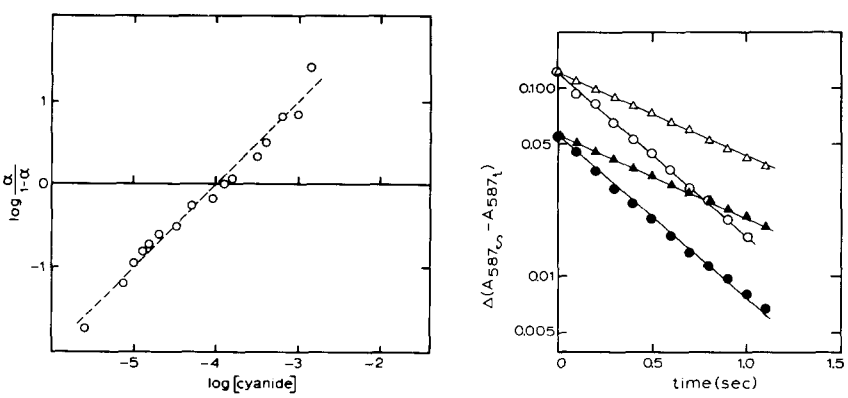

Fig. 7. Determination of the spectroscopic dissociation constant for the reduced cytochrome aa_3–cyanide complex. 14 μM cytochrome aa_3 in 100 mM Tris–H_2SO_4 buffer (pH 8.0), 0.5% potassium cholate and 10 mM $Na_2S_2O_4$ was flushed with O_2-free argon in a Thunberg cuvette. To prevent evaporation 2 M cyanide stock solutions were made in 0.5 M KOH and during argon flushing the pressure was kept above 20 cm Hg. For the calculation of α, ΔA (587–611 nm) was used.

Fig. 8. Time course for reaction of cyanide with fully reduced cytochrome aa_3. Cyanide and enzyme were diluted in 100 mM Tris–H_2SO_4 buffer (pH 8.0), 0.5% potassium cholate and 10 mM $Na_2S_2O_4$ at 25°. ●—●, 5 μM cytochrome aa_3 and 12.5 mM cyanide; ▲—▲, 5 μM cytochrome aa_3 and 6.25 mM cyanide; ○—○, 10 μM cytochrome aa_3 and 12.5 mM cyanide; △—△, 10 μM cytochrome aa_3 and 6.25 mM cyanide.

and enzyme concentration (Fig. 9A) with an apparent rate constant of 1.8 $M^{-1} \cdot sec^{-1}$ (at pH 7.4, 21°), in accord with the value obtained by binding studies[1] with $K^{14}CN$. No rapid changes preceding those discussed above were observed when the reaction was studied in the 10–10^3-msec range between 400 and 850 nm. At higher cyanide concentrations, however, the rate of spectroscopic change becomes independent of the cyanide concentration. Fig. 9B represents a set of double-reciprocal plots (1/reaction

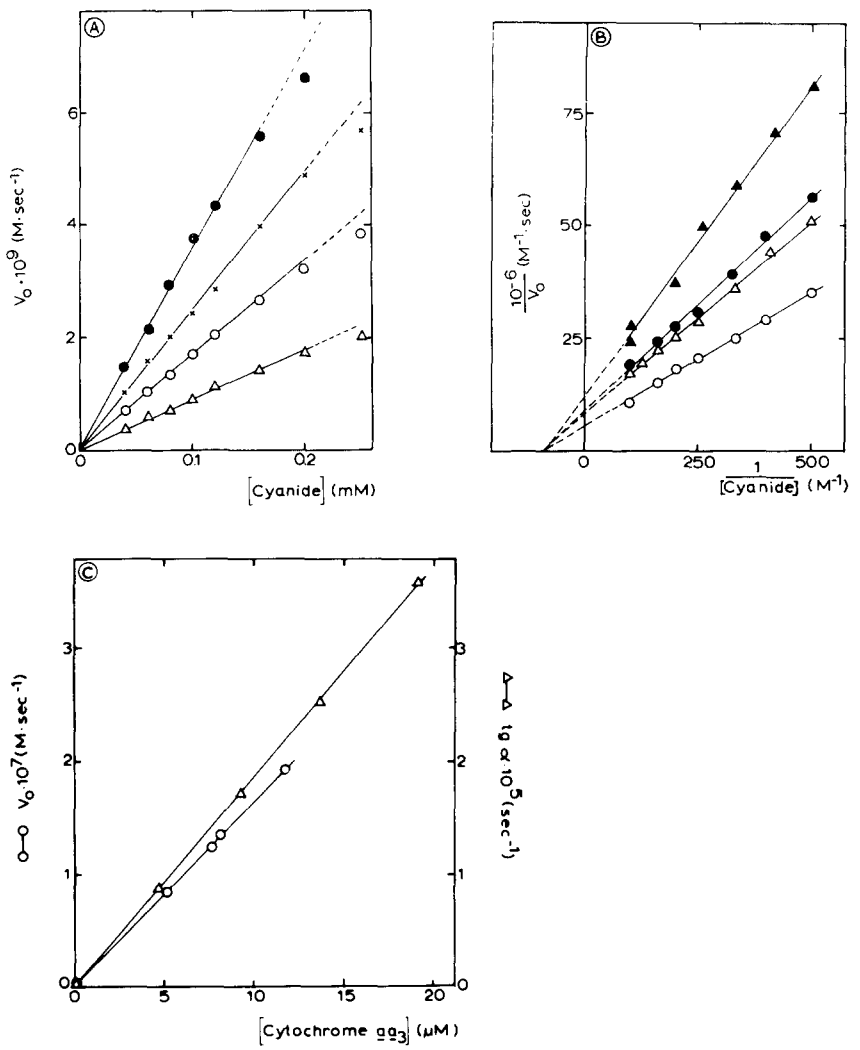

Fig. 9. Effect of cyanide concentration on the initial rate of cyanide–cytochrome aa_3 formation. A. Initial rates at low cyanide concentrations. △—△, 4.5 μM cytochrome aa_3; ○—○, 9.0 μM cytochrome aa_3; ×—×, 13.5 μM cytochrome aa_3; ●—●, 18 μM cytochrome aa_3. B. Double-reciprocal plot at different enzyme concentrations. ○—○, 11.2 μM cytochrome aa_3; △—△, 8.1 μM cytochrome aa_3; ●—●, 7.5 μM cytochrome aa_3; ▲—▲, 5.0 μM cytochrome aa_3. C. Dependence of the initial rate at infinite cyanide concentration and of the initial rate per unit cyanide on the enzyme concentration. △—△, slope of the lines in A; ○—○, rates at infinite cyanide concentration from B. Enzyme is diluted as described in METHODS.

rate *vs.* 1/[cyanide]) for different enzyme concentrations. The reaction obeys an equation of the Michaelis form (Eqn. 1)

$$v = k_{max}\, e[HCN]/(K'_\alpha + [HCN]) \quad (1)$$

where $k_{max} = 0.018$ sec^{-1} and $K'_\alpha = 10$ mM. Stopped-flow experiments carried out at high cyanide concentrations (50 mM) gave values between 0.017 and 0.020 sec^{-1}

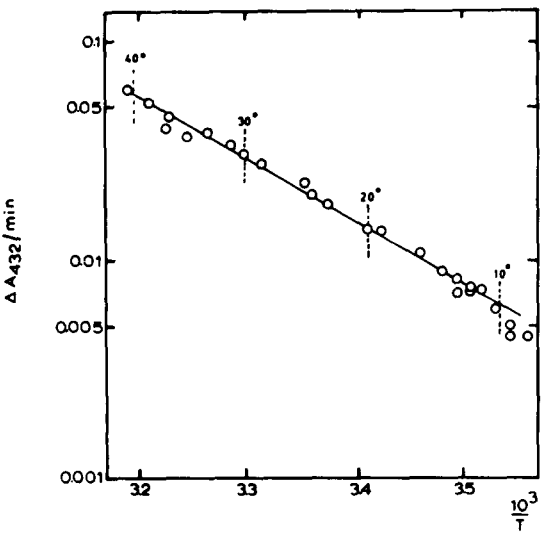

Fig. 10. Effect of temperature on the initial rate of cyanide–cytochrome aa_3 formation. 4.8 μM cytochrome aa_3 diluted as described in METHODS. Rates measured at 432 nm in the presence of 2 mM cyanide.

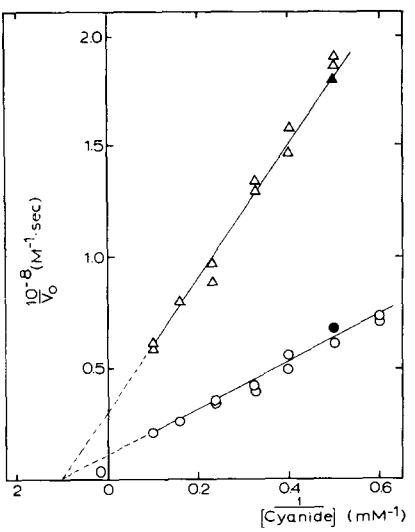

Fig. 11. Effect of temperature on the initial rate of cyanide–cytochrome aa_3 formation. Conditions as described in Fig. 10. △—△, at 16°; ○—○, at 27°. The filled points are calculated from the line in Fig. 10.

for the rate of change at 432 nm. At these cyanide concentrations the reaction is first order in enzyme; the deviations from exponential behaviour seen at lower cyanide concentrations (Fig. 4) are thus much less marked. When *initial* rates are considered, the reaction is first order with respect to enzyme concentration at both low and high cyanide concentrations (Fig. 9C).

The reaction of the oxidized enzyme with cyanide is fairly temperature sensitive. Fig. 10 shows the temperature dependence of the reaction with 2 mM cyanide as a Van 't Hoff plot. Fig. 11 illustrates the fact that the major effect of temperature is on the apparent maximal velocity of the reaction (k_{max}), and not on the amounts of cyanide (K'_a) needed for half-maximal reaction rates. The Q_{10} of 2.2 (20–30°) for this process is thus independent of cyanide concentration; the calculated Arrhenius activation energy is therefore 14 kcal/mole.

The maximal rate, and hence the apparent second-order rate constant at low cyanide concentrations, may be dependent on the molecular state of the enzyme preparation used; 'aged' preparations (stored at −10° for several days) gave values as low as 0.6 $M^{-1} \cdot sec^{-1}$ for this rate constant. Such changes may be correlated with conformational changes in the oxidase and emphasize the need to store the enzyme samples at liquid-nitrogen temperature if consistent properties are to be seen.

A possible interpretation of the observed rates of cyanide binding is discussed below.

Effect of azide on the cyanide binding reaction

To determine whether (a) there is a spectroscopically invisible binding of cyanide to the ferric enzyme that precedes the change at 432 nm (suggested by the kinetics of Eqn. 1, as discussed below), and (b) competition occurs between cyanide and azide for a common binding site (as in other ferric haemoproteins), experiments were carried out in which azide was added following cyanide, and *vice versa*. Surpri-

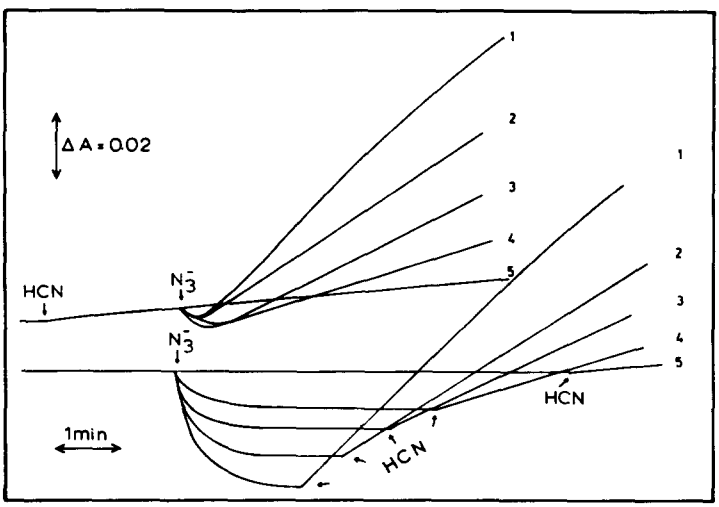

Fig. 12. Effect of azide on the rate of formation of cyanide–cytochrome aa_3 at 432 nm. 20 μM cytochrome aa_3 and 0.33 mM cyanide diluted as described in METHODS. Cyanide and azide were added as indicated. 1, 500 μM azide; 2, 125 μM azide; 3, 50 μM azide; 4, 25 μM azide; 5, no azide.

singly, the addition of azide was found to accelerate sharply the binding of cyanide, the opposite of the effect expected on the assumption of competition for the a_3 haem.

Fig. 12 illustrates the action of various concentrations of azide on the cyanide reaction, adding the azide either after (top traces) or before (bottom traces) the cyanide. The azide-induced spectroscopic change at 432 nm is in the opposite direction to that produced by cyanide[23]. Fig. 12 shows that (i) the rate of the cyanide reaction is a function of azide concentration; (ii) the binding of azide seems to precede the reaction with cyanide (*cf.* the initial declines in the upper set of traces); (iii) the increased rate of reaction with cyanide continues after all the absorbance change originally induced by azide has been abolished (*cf.* the lower set of traces).

The total change induced by cyanide is about 10 times that of azide at 432 nm. Fig. 13 illustrates the changes in difference spectrum of the oxidized enzyme on first adding azide and then cyanide. The isosbestic points in the Soret region for the cyanide and azide complexes are at 423 and 425 nm, respectively, with respect to the orginal

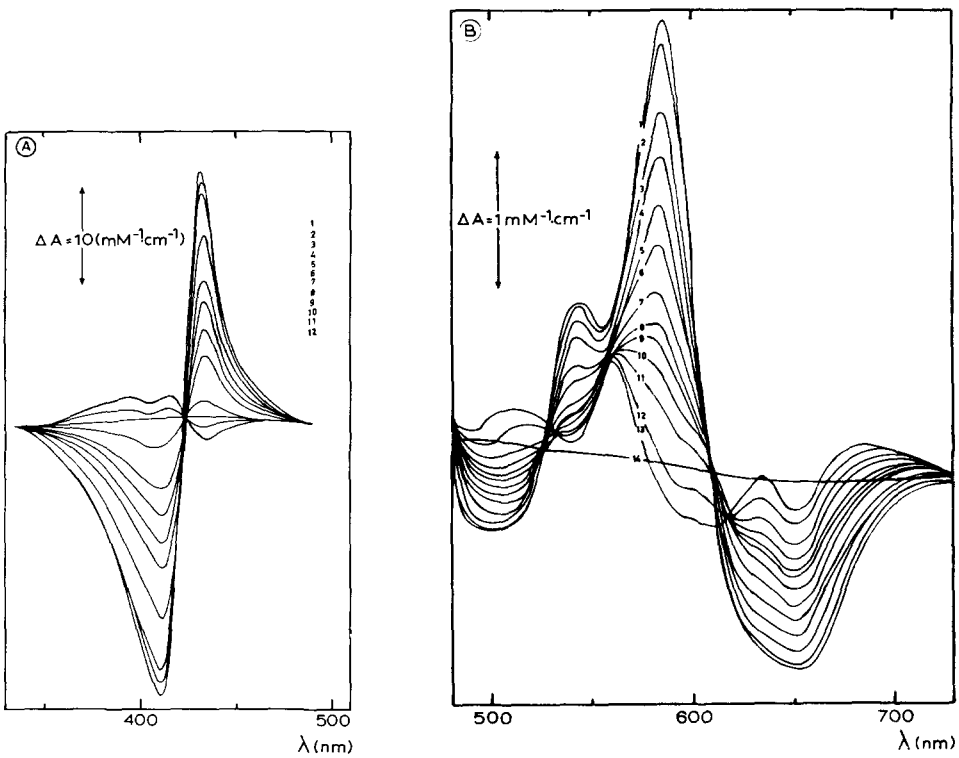

Fig. 13. Effect of azide on the formation of cyanide–cytochrome aa_3. The time course was measured as in Fig. 3. A. Soret region, 6 μM cytochrome aa_3 and 0.1 mM cyanide. Difference spectrum 12, recorded 5 min after addition of 0.2 mM azide; 11, scanning initiated immediately after addition of cyanide; 10, without azide and cyanide; 9, after 1.5 min; 8, after 3.0 min; 7, after 4.5 min; 6, after 7.5 min; 5, after 15 min; 4, after 30 min; 3, after 1 h; 2, after 2 h; 1, after 4 h. B. Visible region, 23 μM cytochrome aa_3 and 0.1 mM cyanide. Difference spectrum 14, without cyanide; 13, recorded 5 min after addition of 0.2 mM azide; 12, scanning initiated immediately after addition of cyanide; 11, after 2.5 min; 10, after 5.0 min; 9, after 7.5 min; 8, after 10 min; 7, after 15 min; 6, after 20 min; 5, after 30 min; 4, after 45 min; 3, after 1 h; 2, after 2 h; 1, after 4 h.

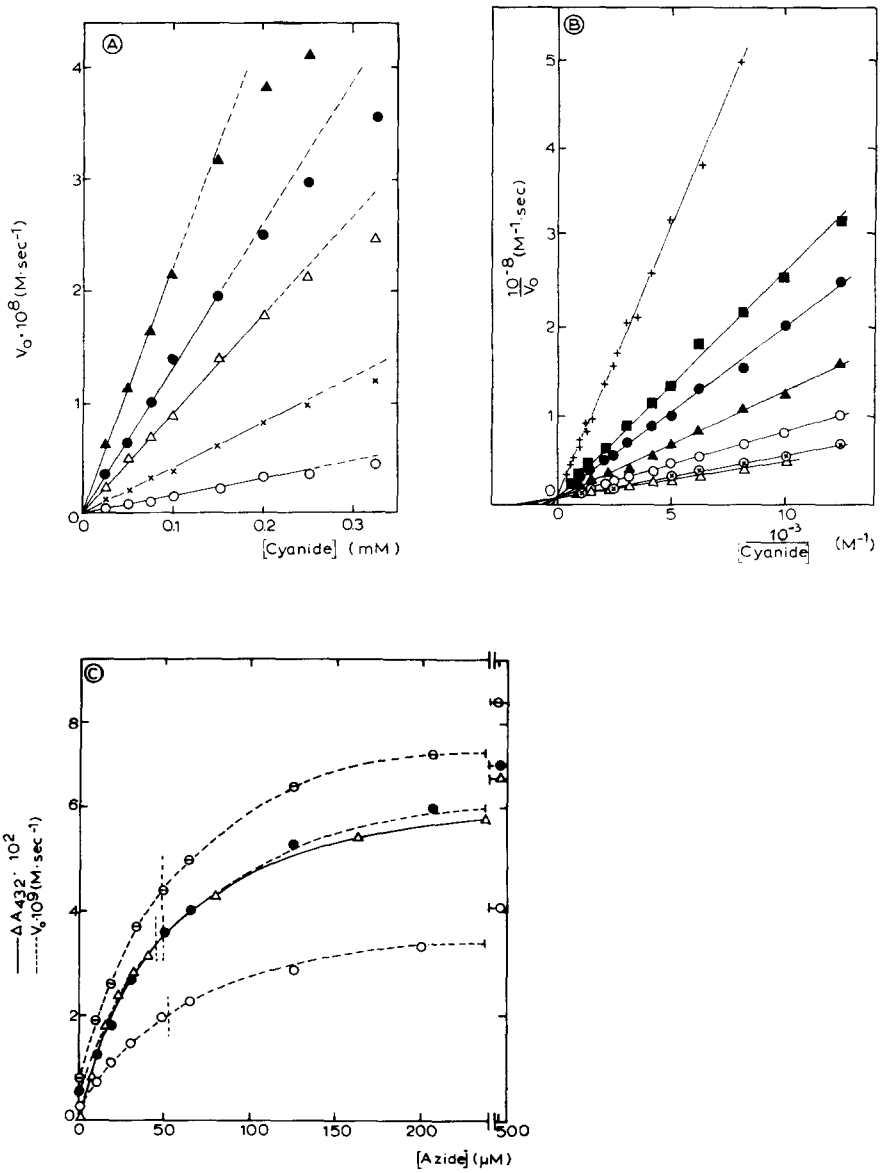

Fig. 14. Effect of azide on the initial rate of cyanide–cytochrome aa_3 formation. Cyanide was added 5–15 min after addition of azide to 9.5 μM cytochrome aa_3. For the determination of azide-induced spectral shifts, 15.6 μM cytochrome aa_3 was diluted in 100 mM phosphate (pH 7.4) and 0.5% Tween 80 as described in METHODS. All rates are corrected for the spectral effect of azide. A. Low cyanide concentrations. ○—○, no azide; ×—×, 10 μM azide; △—△, 30 μM azide; ●—●, 60 μM azide; ▲—▲, 500 μM azide. B. High cyanide concentrations. △—△, 500 μM azide; ⊗—⊗, 200 μM azide; ○—○, 60 μM azide; ▲—▲, 30 μM azide; ●—●, 20 μM azide; ■—■, 10 μM azide; +—+, without azide. C. Effect of azide concentration on the initial rate and on the spectral shift. ○---○, 0.1 mM cyanide; ●---●, 0.16 mM cyanide; ⊖---⊖, 0.24 mM cyanide; △—△, absorbance change at 432 nm measured 10 min after addition of azide. The dotted vertical lines show the position of the half maximal effect.

oxidized enzyme. Observations at these wavelengths, undertaken in the search for ternary intermediates in the reaction of cyanide with azide–ferric oxidase, were thus difficult to interpret, due to the small changes involved in a spectroscopic region of steep changes in ΔA with $\Delta\lambda$ (Fig. 13A). As indicated in the figure, however, any such intermediates must have very few spectroscopic differences from the original azide or final cyanide complexes. Fig. 13B shows that this is also true for the transition from azide–oxidase (λ_{max} 558 nm) to cyanide–oxidase (λ_{max} 585 nm) in the visible region (although the slight shifts in isosbestic points in this case indicate a possibility that the change induced by azide has disappeared before the final cyanide spectrum has been completed).

The final cyanide spectrum was the same in presence and absence of azide (*cf.* Figs. 3 and 13), and azide had no effect when added to a sample of cyanide-treated oxidase[1], unlike the reaction which occurs between azide and the 'oxygenated' enzyme[23].

The kinetics of the initial cyanide reaction in presence of azide are summarized in Fig. 14. As shown in Fig. 14A, the rate of spectroscopic change at low cyanide concentrations remains proportional to cyanide concentration but with an increasing apparent second-order rate constant as the azide concentration is increased. At higher cyanide concentrations, however, the effect of azide is much less, and Fig. 14B suggests that it is acting to decrease the apparent K'_a for cyanide (Eqn. 1) (that is, the amount of cyanide required to achieve half maximal rates of formation of the cyanide complex) without modifying k_{max} (previously found to be 0.018 sec^{-1} under these conditions). If the effect of azide at low cyanide concentrations is plotted against the concentration of azide (Fig. 14C) it is seen that the amount of azide needed for half maximal effect is the same as the apparent K'_a for azide in this system (solid line in Fig. 14C) determined separately from the absorbance change at 432 nm.

It is hard to resist the conclusion that cyanide is indeed reacting more readily with the azide complex than with the free enzyme. At maximum azide concentrations

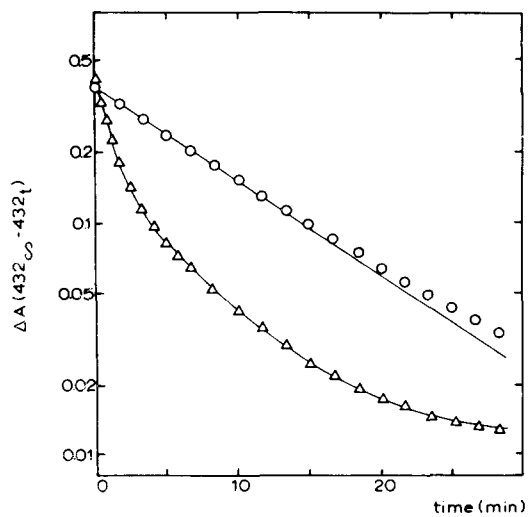

Fig. 15. Time course of cyanide-complex formation with ferric aa_3 in presence (△—△), and absence (○—○) of 0.5 mM azide. 1 mM cyanide and 13.6 μM enzyme diluted as described in METHODS.

the value of K'_a for cyanide (Eqn. 1) is decreased from 10 to 0.7 mM, in agreement with a maximum increase of the second order rate constant (Fig. 14A) from 1.8 $M^{-1} \cdot sec^{-1}$ to 25 $M^{-1} \cdot sec^{-1}$ in presence of azide. That the situation is not quite this simple is illustrated by the time course of Fig. 15. The deviation from first-order behaviour is much more marked in the presence of azide, with a final reaction rate (the last 10 % of binding sites) the same as, or less than, the rate with cyanide alone. The possible significance of this phenomenon is discussed below.

DISCUSSION

The spectroscopic properties of the cyanide complexes with ferric and ferrous enzymes are summarized in Table I, and compared with the corresponding complexes with azide and fluoride (ferric enzyme) and carbon monoxide (ferrous enzyme) previously reported[24–26].

The effect of cyanide is consistent with its acting to render the a_3 haem more low-spin, while that of fluoride suggests a more high-spin state has been induced; the effect of azide is similar to that of fluoride in the Soret region, but its induction of 558-nm and 678-nm bands in the visible region does not seem to be attributable to a simple high spin–low spin transition.

The complexity of the cyanide reaction with oxidized enzyme lies in its kinetic behaviour. Table II lists the kinetic parameters obtained for the binding of cyanide in the presence and absence of azide. Two models for this behaviour may be postulated, as in Eqns. 2 and 3.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI^* \qquad \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI \tag{2}$$

$$E \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E^* \qquad \begin{matrix} I \downarrow & k_1 \\ & \rightleftharpoons \\ I \uparrow & k_{-1} \end{matrix} EI \tag{3}$$

Both mechanisms give rise to an equation for the rate of formation of EI similar to Eqn. 1. In the first mechanism (Eqn. 2), K'_a is equal to $(k_{-1} + k_2)/k_1$ and k_{max} is given by k_2. In the second mechanism, K'_a is equal to $(k_{-2} + k_2)/k_1$ and k_{max} is given by k_2. The effect of azide in either model is to increase k_1, presumably by combining with E (Eqn. 2)or E^* (Eqn. 3).

The second mechanism requires an enzyme conformation, kinetically different from but spectroscopically identical to the original enzyme since only one set of isosbestic points on the baseline was observed during the formation of the complex. At present we tend to prefer the model of Eqn. 2, firstly because we are reluctant to postulate, in addition to the spectroscopically different species of ferric cytochrome aa_3 (*cf.* refs. 23, 27 and 28), a spectroscopically identical form and, secondly, since a great similarity exists between the binding reactions of cyanide and azide[29]. According to Eqn. 2, therefore, the observed rates and equilibria (Table II) give $(k_2 + k_{-1})/k_1 = 10^{-2}$ M, and $k_2 = 0.02$ sec^{-1}, for an overall equilibrium constant $k_{-1}k_{-2}/(k_1k_2 + k_1k_{-2})$ of 1 μM.

The model requires that the spectrum of the initial product, EI^*, be almost identical with that of the free enzyme, and that the spectrum given in Fig. 1 is in fact that of the second complex, EI. We may envisage that the initial binding reaction

TABLE I

SPECTROSCOPIC PROPERTIES OF LIGANDED CYTOCHROME aa_3

Wavelengths and extinction coefficients in parentheses represent minima. Millimolar extinction coefficients in terms of cytochrome aa_3 (*i.e.* twice the values computed on a total haem *a* basis).

Enzyme (redox state)		*HCN*		*Complex* with*					
				N_3^-		F^-		*CO*	
		λ (*nm*)	ΔA ($mM^{-1}\cdot cm^{-1}$)	λ (*nm*)	ΔA ($mM^{-1}\cdot cm^{-1}$)	λ (*nm*)	ΔA ($mM^{-1}\cdot cm^{-1}$)	λ (*nm*)	ΔA ($mM^{-1}\cdot cm^{-1}$)
Ferric	α	585	3.2	678	0.7	638	2.0	—	—
($a^{3+}a_3^{3+}$)	β	540	0.9	558	1.1	603	2.1	—	—
	γ	(411)	(−30)	415	2.8	400	5.5		
		432	28.0	(432)	(−3)	(435)	(−4.5)	—	—
Ferrous	α	587	14.4	—	—	—	—	590	6.0
($a^{2+}a_3^{2+}$)	β	540	2.3	—	—	—	—	545	4.2
	γ	(440)	(−24)	—	—	—	—	(444)	(−80)

References: this paper, MUIJSERS *et al.*[23,25] and VANNESTE[26].
* Difference spectrum (± ligand) for indicated complexes.

TABLE II

KINETIC PARAMETERS FOR REACTION OF CYANIDE WITH OXIDIZED ENZYME

100 mM phosphate (pH 7.4), 0.5% Tween 80, 22°.

Kinetic or equilibrium constant	*Enzyme form reacting*	
	$a^{3+}a_3^{3+}$	$a^{3+}a_3^{3+}HN_3$
* $K_D = (k_{-1}k_{-2})/(k_1(k_2+k_{-2}))$	1 μM	—
** $K'_a = (k_{-1}+k_2)/k_1$	10 mM	0.7 mM
k'_{on} (low [HCN]) $= (k_1k_2)/(k_{-1}+k_2)$	1.8 $M^{-1}\cdot sec^{-1}$	25 $M^{-1}\cdot sec^{-1}$
k'_{on} (high [HCN]) $= k_2$	0.018 sec^{-1}	0.018 sec^{-1}
*** k_{off}	$2\cdot10^{-6}$ sec^{-1}	—

* Dissociation constant for enzyme–cyanide complex.
** Concentration of cyanide giving half maximal rate of cyanide complex formation.
*** Estimated off constant (equal to $k_{on}\cdot K_D$).

(governed by k_{-1}/k_1) represents the penetration of cyanide into a protein crevice or otherwise restricted site, and that the second step (governed by k_2) monitors the binding to the haem iron. The effect of azide on k_{-1}/k_1 (Table II) may then be interpreted as a consequence of a conformational change induced by azide binding, that has 'opened up' the restricted site. The subsequent haem binding is independent of the ligand (H_2O, protein or azide) already at the sixth coordination position.

The postulation of an intermediate bound state of the cyanide moiety in which the a_3 spectrum is unaffected is similar to the hypothesis of GIBSON AND GREENWOOD[30] for the reaction of oxygen with the fully reduced enzyme (although their rate constants, of 10^8 $M^{-1} \cdot sec^{-1}$ and $3 \cdot 10^4$ sec^{-1} are 10^7 times greater than those for cyanide and the oxidized enzyme). However, the reaction of cyanide with the reduced enzyme, for which the rate constants are listed in Table III, is clearly unaffected by the steric problems that confront the ligand reacting with the oxidized enzyme[1].

TABLE III

KINETIC PARAMETERS FOR REACTION OF CYANIDE WITH REDUCED ENZYME

100 mM Tris–H_2SO_4 buffer (pH 8.0), 0.5% potassium cholate, 10 mM $Na_2S_2O_4$, 25°.

Kinetic or equilibrium constant	*Estimated or calculated value*
K_D	100 μM
k_{on}	150 $M^{-1} \cdot sec^{-1}$
k_{off}	0.015 sec^{-1}

Table IV compares the effect of cyanide on the spectra of five haemoproteins - methaemoglobin, metmyoglobin, peroxidase, catalase and cytochrome aa_3. Except that the β-band of the cyanide–oxidase complex seems to be weaker than in the other haemoproteins, there is a remarkable similarity between the various spectral changes. We conclude that the main difference between the oxidase and the other enzymes lies in the accessibility of the haem and not in the structure of the final compound. However, in the competition for binding sites, the oxidase is unique in showing an *acceleration* of cyanide binding when in the form of the azide complex. Under the same conditions (100 mM phosphate (pH 7.4), 0.5% Tween 80), beef methaemoglobin gave a velocity constant of 160 $M^{-1} \cdot sec^{-1}$ for the reaction with cyanide[33]; addition of azide reduced the rate constant in proportion to the amount of azide complexed at low cyanide concentrations, while at higher cyanide concentrations the rate of cyanmethaemoglobin formation became independent of cyanide concentration, and presumably a measure of the slow dissociation of azide (about 0.03 sec^{-1}, *cf.* ref. 24).

The present value of approximately 1 μM for the binding of cyanide to the oxidized enzyme (Fig. 5B) is similar to the inhibitory K_i observed without prolonged incubation[9,10]. The difference lies in the much more rapid attainment of the inhibited state; this will be discussed in a following paper. The equilibrium constant is still one order of magnitude larger than the value of 100 nM obtained for the binding of cyanide under conditions of incubation with electron donor and air[1]. As discussed previously, the reduction of some component of the aa_3 system, probably cytochrome *a*, may promote the binding of cyanide to give the quasi-irreversible cyancytochrome

TABLE IV

SPECTROSCOPIC AND KINETIC COMPARISON OF CYANIDE COMPLEXES OF FIVE HAEMOPROTEINS

Haemoproteins were collected from various sources (*cf.* NICHOLLS[31], SAUNDERS *et al.*[32]). Values for pH 7.0–7.5, 20–25°. λ_{max} indicates position of maximum absorbance in the difference spectrum (enzyme + ligand *minus* enzyme).

Parameter	*MetHb*	*MetMb*	*Peroxidase*	*Catalase*	*Cytochrome* aa_3
(*a*) *Kinetic*					
k_{on} ($M^{-1} \cdot sec^{-1}$)	200	400	10^5	10^6	2
K_D (μM)	4.5	10	4	4	1
(*b*) *Spectroscopic*					
α-band: λ_{max}	575	570	581	580	585
ΔA ($mM^{-1} \cdot cm^{-1}$)	4.2	5.5	6.0	5.1	3.2
β-band: λ_{max}	543	543	540	553	540
ΔA ($mM^{-1} \cdot cm^{-1}$)	5.8	3.6	4.2	3.6	0.9
γ-band: λ_{max}	approx. 429	427	423	425	432
ΔA ($mM^{-1} \cdot cm^{-1}$)	approx. 80	65	64	55	28

aa_3 complex[2,3]. The complex described in the present paper, formed by prolonged aerobic incubation, is nevertheless probably the same species. Although it is thermodynamically unstable in the absence of free cyanide (Fig. 5B), it is kinetically stable enough ($k_{off} = 2 \cdot 10^{-6}$ sec^{-1}, *cf.* Table II) to permit its separation by column chromatography as described[1,3] and a half life of 100 h in the absence of cyanide is guaranteed.

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