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Cyanide binding to different redox states of the cytochrome caa_3 complex from *Bacillus subtilis*; a member of the cytochrome oxidase super-family of enzymes

Mahmoud Assempour, Bruce C. Hill *

Department of Biochemistry, Queen's University, Kingston, ON K7L 3N6 Canada

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Abstract

Cytochrome caa_3 from *Bacillus subtilis* is a member of the cytochrome oxidase family. The enzyme has a cytochrome *c* domain fused as a c-terminal extension into the structure of the oxidase subunit II. Analysis of the visible spectrum of cytochrome caa_3 by pyridine hemochromogen assay reveals an unusually intense α-band extinction for the cytochrome *c* domain relative to mitochondrial cytochrome *c*. When cyanide is added to oxidized cytochrome caa_3 it binds to cytochromes a_3 and *c*, and induces autoreduction of cytochrome *a*. Binding of cyanide to cytochrome *c* and autoreduction of cytochrome *a* are prevented if the reaction is done at pH 6.4 in the presence of ferricyanide. The reaction is then composed only of cyanide binding to cytochrome a_3, but is still kinetically complex with three cyanide concentration dependent rates. The resting bacterial oxidase cannot be converted fully into 'fast' or 'pulsed' forms as can the mitochondrial enzyme. It is concluded that the cyanide bound states of the caa_3 oxidase are similar to the mammalian oxidase. However, the kinetics of their formation, particularly with reference to the oxidized, resting caa_3, is distinct from the mitochondrial oxidase. This difference is ascribed to structural differences in the region of the binuclear centre of caa_3.

Keywords: Cytochrome caa_3; Cytochrome *c* oxidase; Cyanide; Reduction; Inhibition; (*Bacillus subtilis*)

1. Introduction

Cytochrome *c* oxidase is the terminal enzyme in the respiratory chain of all eukaryotes and many prokaryotes [1]. This membrane-bound protein catalyses the transfer of electrons from ferrocytochrome *c* to molecular oxygen resulting in the formation of water. The free energy of this redox reaction is utilized by the enzyme to translocate protons across the inner mitochondrial membrane, or plasma membrane in bacteria, resulting in an electrochemical proton gradient [2], which is later used in the synthesis of ATP or other energy-requiring processes [3].

Eukaryotic cytochrome *c* oxidases all contain three

Abbreviations: TMPD; *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

* Corresponding author. Fax: (1) (613) 545-2497; E-mail: hillb@post.queensu.ca

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mitochondrial-encoded subunits (I, II, and III) and up to ten nuclear-encoded subunits [4]. Prokaryotic enzymes contain 3–4 subunits. All contain homologs of subunit I, and most also contain homologs of subunits II and III [5]. Subunit I, the functional core of the enzyme, has three redox centres [6,7]; two heme A molecules, called cytochrome *a* and a_3, and one copper atom, Cu_B. Cu_B and cytochrome a_3 are about 4.5 Å apart and constitute a binuclear centre where oxygen and inhibitors such as cyanide bind to the enzyme. Two atoms of copper are bound to a conserved sequence of subunit II to form a redox centre called Cu_A.

Bacillus subtilis has four terminal oxidases [8]. One of the oxidases, cytochrome caa_3, has cytochrome *c* attached covalently as an approximately 100 amino acid extension to the C-terminal end of subunit II [9]. The cytochrome *c* domain shows sequence similarity to mitochondrial cytochrome *c*; 37% of the amino acid residues are identical compared to horse cytochrome *c* [10]. Cytochrome caa_3 has been purified from plasma membranes of *B. subtilis* [11,12]. Only subunits I and II are observed in the purified enzyme, indicating that subunits III and IV are loosely bound to the enzyme.

Cyanide is a potent inhibitor of cytochrome *c* oxidase [13]. Studies done on bovine heart cytochrome *c* oxidase suggest that cyanide binds in a 1:1 stoichiometry [14], and initially binds to Cu_B from which it passes to cytochrome a_3 to prevent binding of the substrate oxygen [15]. This 'ligand shuttle' mechanism, from Cu_B to cytochrome a_3, is proposed also for binding the inhibitor CO [16] and the substrate O_2 [17]. Cyanide binds very slowly with high affinity to the fully oxidized enzyme [14] and relatively fast with low affinity to fully reduced oxidase [18]. However, it binds both very fast and tightly to partially reduced oxidase that is generated during turnover [19].

We have examined the reaction of cyanide with different redox states of *B. subtilis* cytochrome caa_3. The kinetics of cyanide binding to reduced and partially reduced *B. subtilis* cytochrome caa_3 resembles that of the bovine oxidase. However, cyanide binding to oxidized cytochrome caa_3 appears to be more complicated than that of the mammalian oxidase and probably reflects a different conformation around the binuclear centre.

2. Materials and methods

Potassium cyanide, pyridine, sodium dithionite and potassium ferricyanide were obtained from BDH Chemical Company, Edmonton, Alberta, Canada. Ascorbic acid, TMPD and horse cytochrome *c* (type VI) were obtained from Sigma Chemical, St. Louis, MO, USA.

The enzyme was purified according to the method of Henning et al. [11]. Pulsed cytochrome caa_3 was prepared by anaerobic, ascorbate reduction of the enzyme in the presence of catalase followed by subsequent oxidation of the enzyme and removal of excess ascorbate by passage of the sample through a column of Sephadex G-25 equilibrated with 50 mM sodium phosphate, pH 6.4, 1 mg/ml *n*-dodecylmaltoside, and 1 mM EDTA. Bovine cytochrome *c* oxidase was purified according to the method of Kuboyama et al. [20]. For *B. subtilis* cytochrome caa_3, the concentration of heme groups was calculated from reduced minus oxidized difference spectra using extinction coefficients of 30 $mM^{-1} \cdot cm^{-1}$ for cytochrome *c* at 550–540 nm and 26 $mM^{-1} \cdot cm^{-1}$ at 604–630 nm for cytochrome aa_3. The extinction coefficients used for the pyridine hemochrome minus hemichrome difference spectrum were 25.02 $mM^{-1} \cdot cm^{-1}$ at 588–620 nm for heme A and 21.85 $mM^{-1} \cdot cm^{-1}$ at 550–540 nm for heme C [21]. The concentration of bovine cytochrome *c* oxidase was determined using an extinction coefficient of 27 $mM^{-1} \cdot cm^{-1}$ at 604–630 nm for the reduced minus oxidized difference spectrum [22]. The concentration of horse cytochrome *c* was measured using an extinction coefficient of 21.2 $mM^{-1} \cdot cm^{-1}$ at 550–540 nm for the reduced-oxidized difference spectrum [23].

Spectroscopic measurements and analysis were made on a Hewlett-Packard 8452A diode array equipped with a water-jacketed cuvette holder and circulating water bath. Cyanide binding to the oxidized oxidases was initiated by quickly mixing a few microliters of freshly prepared 1.0 M KCN with sample solution. Spectra were taken immediately following addition of cyanide at 5-s intervals for 300 s and 1-min intervals after that for several hours. The fully reduced complex of *B. subtilis* cytochrome caa_3 was prepared as described by Hill and Marmor [18]. Time-resolved reduction of cyanide-inhibited *B. subtilis* caa_3 was carried out by adding the indicated

reductant and recording spectra using the diode array spectrophotometer.

3. Results

Absolute spectra of the oxidized and reduced states of the cytochrome caa_3 complex are shown in Fig. 1A. The absolute spectrum of the oxidized enzyme shows two distinct contributions in the Soret region at 410 nm from ferricytochrome *c* and at 420 nm from oxidized cytochrome aa_3. when the enzyme is reduced intense absorbance bands appear at 416 nm, 520 nm and 550 nm due to ferrocytochrome *c* and at 444 nm and 604 nm due to reduced cytochrome aa_3. The inset shows the difference spectrum between the reduced and oxidized states of the caa_3 complex (Fig. 1B). Due to the unusually high absorbance seen for the α-band of the cytochrome *c* component relative to the cytochrome aa_3 component we performed a pyridine hemochromogen assay of purified cytochrome caa_3. The reduced-oxidized difference spectrum in pyridine is compared to the native enzyme in Fig. 1C. On this basis the reduced-oxidized extinction coefficient for the cytochrome *c* domain at 550–540 nm is determined to be 30 $mM^{-1} \cdot cm^{-1}$ and that for cytochrome aa_3 26 $mM^{-1} \cdot cm^{-1}$ at 603–630 nm. The extinction for the cytochrome *c* band is high relative to that of 21 $mM^{-1} \cdot cm^{-1}$ found for many species of cytochrome *c* (e.g., [23]) whereas the value obtained for the cytochrome aa_3 band is in good agreement with that reported for bovine heart cytochrome oxidase.

When cyanide is added to oxidized cytochrome caa_3 a spectral shift ensues with difference maxima at 432 nm and 580 nm (see Fig. 2A). The absorbance intensity at 432 nm appears to reach a maximum value 300 s after addition of cyanide, and upon longer incubation the 432 nm band shifts to 440 nm with a slight increase in intensity. In the visible region the initial absorbance change is at 580 nm with an increase after 300 s at 604 nm as well as a small increase at 550 nm. The later spectral changes are due to processes other than binding of cyanide to cytochrome a_3. Fig. 2B shows the difference spectrum taken 1 h after addition of cyanide to oxidized cytochrome caa_3 minus that taken after 300 s. This spectrum is similar in form to the reduced minus oxidized difference spectrum shown in Fig. 1B. The 1 h minus 300 s spectrum has bands at 444 nm and at 604 nm due to reduction of cytochrome *a*. There is also a band at 416 nm, but this is not accompanied by bands at 520 and 550 nm, and thus cannot be ascribed to reduction of cytochrome *c*. These latter spectral features are due to cyanide binding to the cytochrome *c* domain of caa_3 and are similar to those observed for cyanide binding to soluble, mitochondrial cytochrome *c* [24].

These results demonstrate that cyanide binds not only to ferricytochrome a_3, but also to the cytochrome *c* centre of cytochrome caa_3. Furthermore, the cytochrome *a* component of the enzyme undergoes autoreduction upon addition of cyanide. Because we wished to examine the binding of cyanide to cytochrome a_3 without interference from these other reactions, conditions were sought in which binding of cyanide to cytochrome *c* and autoreduction of cytochrome *a* were minimal. Reduction of cytochrome *a* is prevented by pre-incubation of the oxidase with ferricyanide. Cyanide binding to cytochrome *c* of caa_3 was found to be negligible at pH 6.4 and maximal at pH 8.4. The extent of binding of cyanide to cytochrome *c* was observed by its effect on the reducibility of this cytochrome. Cyanide is known to block the reduction of mitochondrial ferricytochrome *c* by ascorbate plus TMPD, but not by the more powerful reductant dithionite [24]. Fig. 3 describes the ascorbate-TMPD and dithionite reducibility of cytochrome caa_3 incubated with 5 mM KCN for 1 day at pH 6.4 and pH 8.4. The reaction of cyanide with cytochrome a_3 is complete after this time, so turnover of the enzyme is blocked. Fig. 3A shows a time course measured at 550–540 nm to assess the reduction of cytochrome *c* in buffer at pH 8.4. Ascorbate plus TMPD added at time zero produces a rapid initial, but partial reduction of cytochrome *c*. Fig. 3B shows difference, spectra constructed at distinct points during the reduction time course (see the figure legend for details). The initial jump in absorbance corresponds to reduction of both cytochromes *c* and *a* as indicated by prominent peaks at 550 and 604 nm, respectively. There is a slow rise in absorbance at 550–540 nm following the initial jump due to further reduction of cytochrome *c*, now limited by the rate of dissociation of cyanide from ferricytochrome *c*. At about 1200 s dithionite was

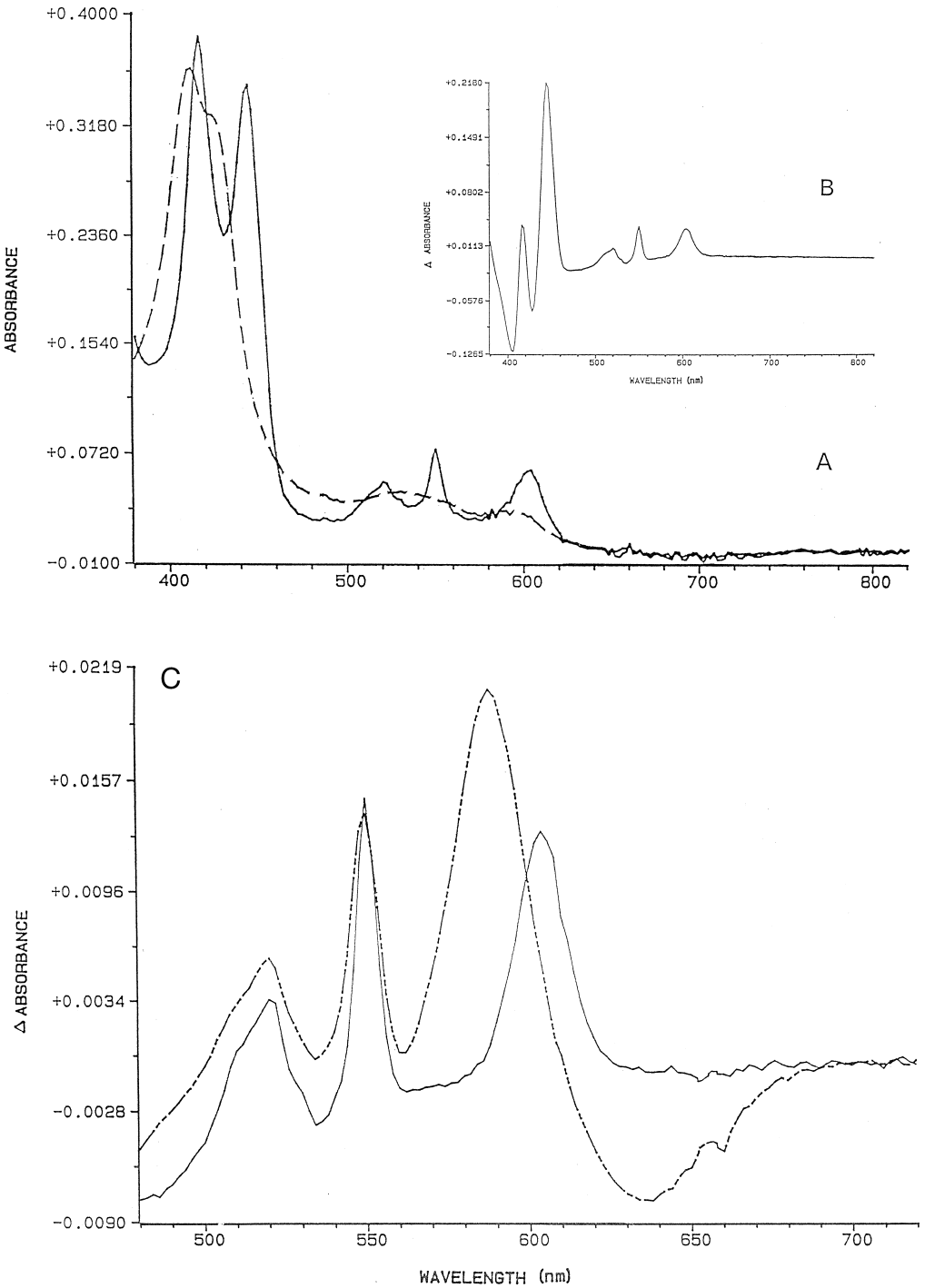


Fig. 1. UV-visible spectral properties of *Bacillus subtilis* cytochrome caa_3. A: oxidized (— — —) and fully reduced (————); B: reduced minus oxidized. The enzyme concentration was 2.31 μM in 10 mM Tris-MOPS, pH 7.4, containing 1 mg/ml *n*-dodecylmaltoside and it was reduced with 2 mg sodium dithionite. C: pyridine hemochromagen assay of *B. subtilis* cytochrome caa_3. The pyridine hemochrome minus hemichrome spectrum (— - - —) was obtained by taking spectra before and after addition of 2 mg sodium dithionite to a solution containing 0.53 μM enzyme, 100 mM NaOH, 20% pyridine, and 100 μM $K_3Fe(CN)_6$. The reduced minus oxidized difference spectrum (————) was obtained by reduction 0.53 μM enzyme with 2 mg of sodium dithionite in the same buffer as above.

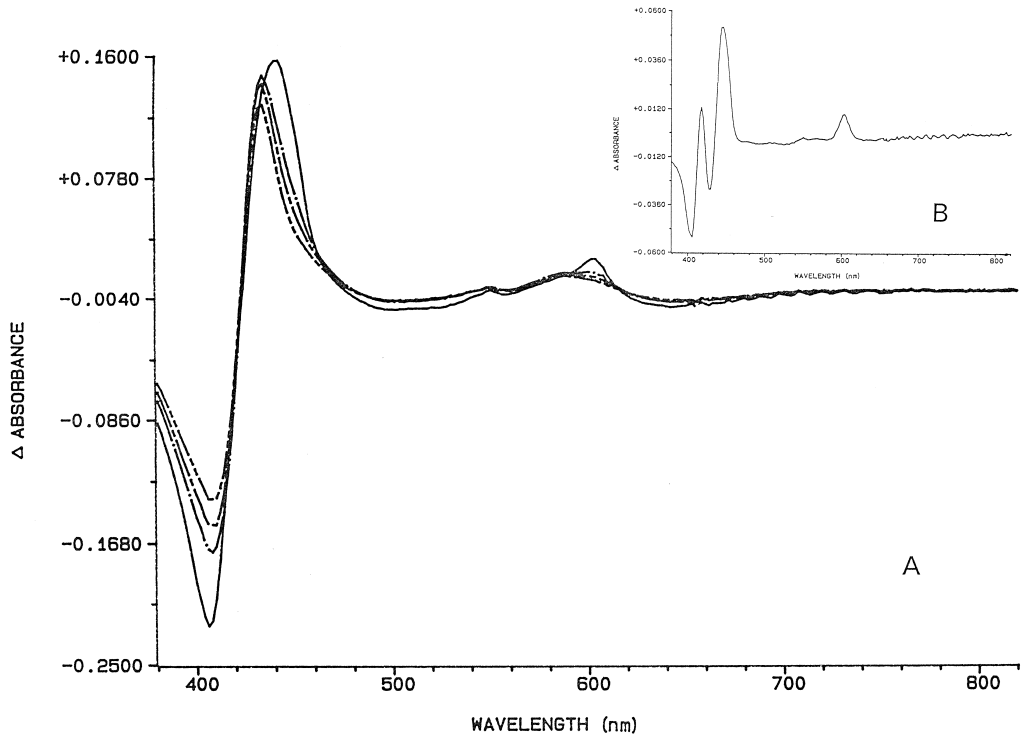


Fig. 2. Time-resolved difference spectra of oxidized *B. subtilis* cytochrome caa_3 plus cyanide. A: the difference spectra of oxidized plus cyanide minus oxidized enzyme taken at different times after addition of cyanide. KCN is 5 mM and spectra recorded at 50 s (— - - —), 100 s (— - —), 300 s (– · –) and 1 h (————) after cyanide addition. B: the difference spectrum between oxidized plus cyanide taken at 1 h after addition of cyanide and that taken 300 s after addition of cyanide. The buffer is 50 mM Tris pH 8.0 with 1 mg/ml *n*-dodecylmaltoside and 1 mM EDTA.

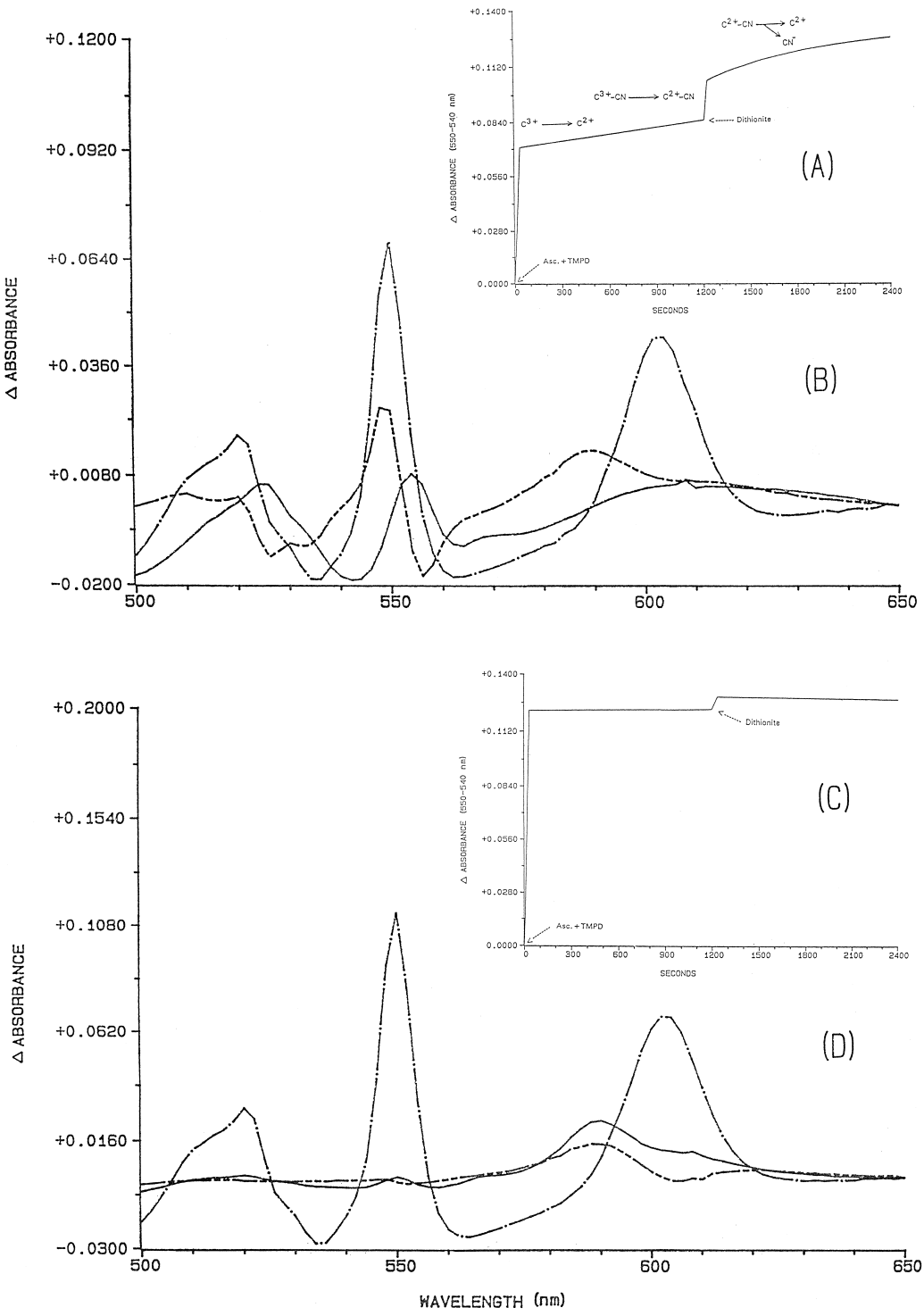
added and there was a second rapid phase of cytochrome *c* reduction. when a difference spectrum is constructed of the initial product of dithionite reduction minus the product of reduction by ascorbate plus TMPD the α-band is at 555 nm indicative of formation of ferrocytochrome *c*-CN [24]. Upon further incubation with dithionite the absorbance at 550 nm continues to increase. when a difference spectrum is constructed between the final dithionite reduced minus the initial dithionite reduced species it is apparent that the slow increase is due to a shift of the α-band from 555 nm back to 550 nm. We assign this process to the dissociation of cyanide from ferrocytochrome *c*. In addition we observe a band at 588 nm that develops in the presence of dithionite due to the formation of ferrocytochrome $a_3 - \mathrm{CN}$ [18].

Fig. 3 panels C and D show the reducibility of the enzyme by ascorbate-TMPD and dithionite following 24 h incubation with cyanide at pH 6.4. Panel C shows the time course of reduction of cytochrome *c* and begins with an immediate absorbance increase following addition of ascorbate plus TMPD. Spectra taken following this initial phase minus the oxidized sample reveals almost complete reduction of cytochrome *c* and cytochrome *a*. There is a small amount of further reduction of cytochrome *c* upon addition of dithionite, but there is no shift in the spectrum to 555 nm. Again there is evidence of formation of ferrocytochrome $a_3 - \mathrm{CN}$ as indicated by the band at 588 nm.

Lowering the pH to 6.4 diminishes greatly the extent of cyanide binding to the cytochrome *c* domain. Cyanide ligation to the cytochrome *c* domain approaches 45% after 24 h incubation at pH 8.4 and only 5% after 24 h at pH 6.4. Since the experiments were performed in sealed cuvettes and the extent of formation of ferrocytochrome a_3 is similar at the two pH's, we do not think that the decrease in cyanide binding to cytochrome *c* is merely due to the loss of cyanide from solution. If CN^- is the species that

binds to the cytochrome *c* domain, as it is with free, mitochondrial cytochrome *c* [25] then the decrease in reactivity is due largely to the decreased concentration of the CN^- species at pH 6.4.

Fig. 4 illustrates difference spectra taken at various times after addition of cyanide to cytochrome caa_3 at pH 6.4 in the presence of potassium ferricyanide. The spectra exhibit only the development of a cytochrome



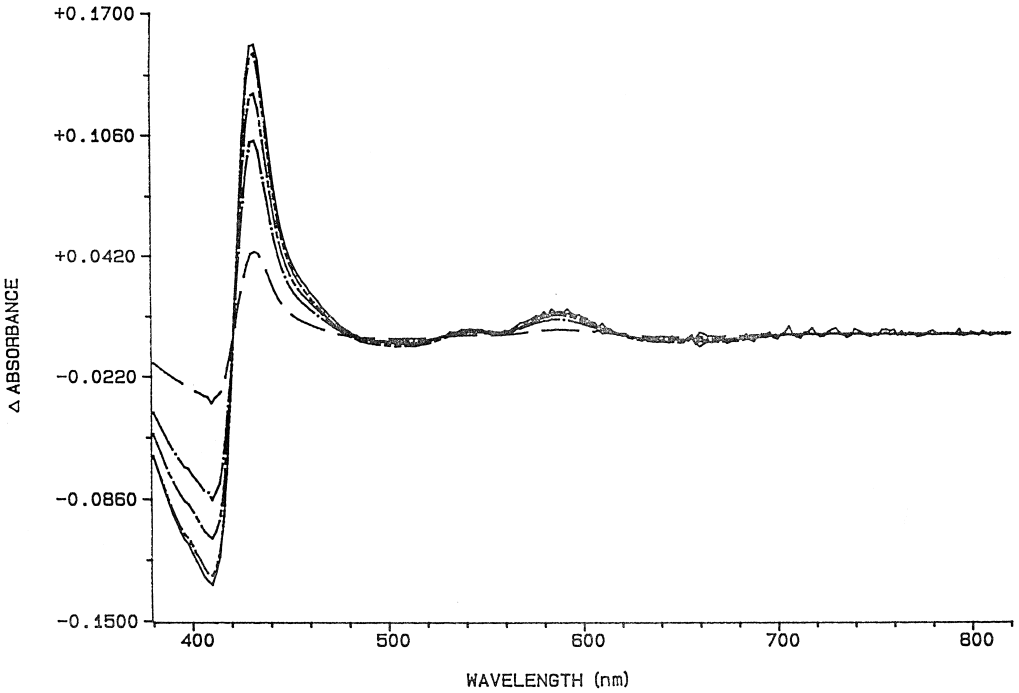


Fig. 4. Time course of cyanide binding to cytochrome a_3. A: spectra were taken following addition of 5 mM cyanide to 5 μM caa_3 in buffer containing 200 mM sodium phosphate, pH 6.4, 1 mg/ml *n*-dodecylmaltoside, 1 mM EDTA, and 200 μM potassium ferricyanide. Spectra were recorded 10 s (— — —), 50 s (– · –), 100 s (— - —), 300 s (— - - —) and 1 h (————) after cyanide addition.

a_3 − cyanide complex with a set of isosbestic points at 422, 480, 562, and 628 nm that are maintained over the full course of the reaction. There is now no evidence of cyanide ligation to cytochrome *c* or reduction of cytochrome *a*, even after prolonged incubation. The extinction coefficient for cyanide binding to oxidized cytochrome caa_3 is 57 $mM^{-1} \cdot cm^{-1}$ at 432–412 nm and is close to the value of 60 $mM^{-1} \cdot cm^{-1}$ reported for the complete reaction of bovine heart cytochrome *c* oxidase with cyanide [14,26].

The binding of cyanide is observed to be kinetically complex under all the conditions examined here. The time course monitored at 432–410 nm for the formation of ferricytochrome a_3 − CN is composed of three exponential terms. The observed rates for each phase are proportional to cyanide as illustrated in Fig. 5. Table 1 shows the effect of different solution conditions on the reaction kinetics for cyanide binding to cytochrome a_3. The presence of ferricyanide slows the rate of the fastest phase and may indicate the presence of partially reduced states of the

Fig. 3. Reduction of *B. subtilis* cytochrome caa_3 by ascorbate plus TMPD and dithionite after one day incubation with cyanide at pH 8.4 and 6.4. A: time course of reduction of the cyanide inhibited enzyme in 200 mM sodium phosphate pH 8.4, 1 mM EDTA with 1 mg/ml *n*-dodecylmaltoside, 5 μM enzyme and 5 mM KCN. B: difference spectrum between: (– · –) ascorbate plus TMPD reduced and oxidized (t_{1200}–t_0); (2) (————) immediate dithionite reduced and ascorbate plus TMPD reduced (t_{1220}–t_{1200}); (3) (— - - —) final dithionite reduced and immediate dithionite reduced (t_{2400}–t_{1220}). C: time course of reduction of the cyanide inhibited enzyme in 200 mM sodium phosphate, pH 6.4, 1 mM EDTA, and 1 mg/ml *n*-dodecylmaltoside. D: difference spectrum between: (1) (– · –) ascorbate plus TMPD reduced and oxidized (t_{1200}–t_0); (2) (————) immediate dithionite reduced and ascorbate plus TMPD reduced (t_{1220}–t_{1200}); (3) (— - - —) final dithionite reduced and immediate dithionite reduced (t_{2400}–t_{1220}). The enzyme was reduced by 1.25 mM ascorbate plus 0.25 mM TMPD followed by 2 mg of sodium dithionite.

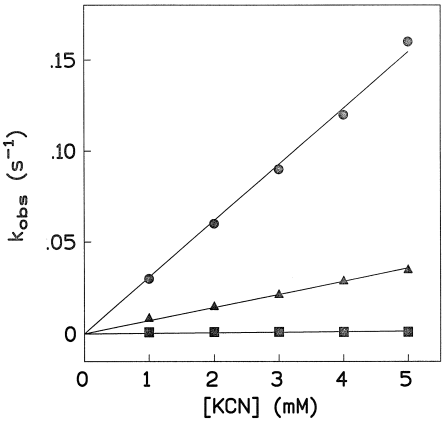


Fig. 5. Cyanide concentration dependence of the three phases observed for formation of cytochrome a_3-CN. The observed rates were derived from triple exponential fits to the time courses of absorbance change at 432–412 nm. The three phases are designated fast (●), intermediate (▲) and slow (■). The enzyme concentration was 5 μM and the buffer was 10 mM Tris-MOPS pH 7.4 with 1 mM EDTA and 1 mg/ml of *n*-doecylmaltoside. The slopes of each line are reported as bimolecular rate constants in Table 1.

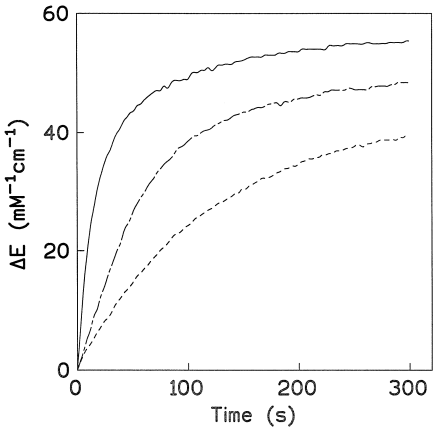


Fig. 6. Kinetics of cyanide binding to *B. subtilis* caa_3 and the mammalian *c:*aa_3 complex. Time courses of cyanide binding to: (---) bovine heart cytochrome *c* oxidase alone (8.5 μM), (—·—) bovine heart cytochrome *c* oxidase (7.4 μM) plus horse cytochrome *c* (7.4 μM), and (————) *B. subtilis* cytochrome caa_3 (5 μM). The curves have been plotted as extinction coefficient changes as measured at 432–410 nm. The concentration of cyanide in the reaction was 5 mM and the buffer was 10 mM Tris/MOPS, pH 7.4 with 1 mM EDTA and 1 mg/ml *n*-dodecylmaltoside.

enzyme as such forms of the bovine enzyme are found to have increased reactivity with cyanide. However, incubation of the resting, oxidized *B. subtilis* enzyme with ferricyanide does not generate a spectral difference as reported for bovine oxidase [27]. The effect of subjecting the enzyme to a cycle of reduction and oxidation or 'pulsing' is also documented in Table 1. The amount of the reaction occurring in the fast phase is increased but the rate is the same implying that there is an appreciable amount of the 'pulsed conformer' in the resting preparation. However, under no conditions tested is caa_3 converted to a form with a single phase of cyanide binding as seen for the bovine enzyme [28].

Fig. 6 compares time courses of binding of cyanide to bovine heart cytochrome *c* oxidase, a 1:1 complex of bovine oxidase and horse cytochrome *c*, and *B. subtilis* cytochrome caa_3. The reaction of cyanide with the bovine heart cytochrome *c* oxidase in the presence or absence of cytochrome *c* is biphasic. Pseudo-first order rates with 5 mM KCN are $8.35 \cdot 10^{-3}$ s^{-1} and $5.92 \cdot 10^{-4}$ s^{-1} in the absence of cytochrome *c* and $1.82 \cdot 10^{-2}$ s^{-1} and $4.52 \cdot 10^{-4}$

Table 1
Kinetics of binding of cyanide to oxidized *Bacillus subtilis* cytochrome caa_3

Buffer	Fast		Intermediate		Slow	
	$k(M^{-1} \cdot s^{-1})$	%	$k(M^{-1} \cdot s^{-1})$	%	$k\ (M^{1} \cdot s^{-1})$	%
200 mM NaH_2PO_4, pH 6.4	28	31	4	63	0.2	6
200 mM NaH_2PO_4, pH 6.4, 200 ELM ferricyanide						
Resting	17	38	3	58	0.2	4
Pulse	15	63	5	24	0.2	13
200 mM NaH_2PO_4, pH 7.4	17	42	6	51	0.2	7
200 mM NaH_2PO_4, pH 8.4	13	76	4	18	0.2	6
10 mM Tris-MOPS, pH 7.4	32	45	7	36	0.2	19
10 mM Tris-MOPS, pH 7.4, 200 μM ferricyanide	11	67	3	20	0.2	13

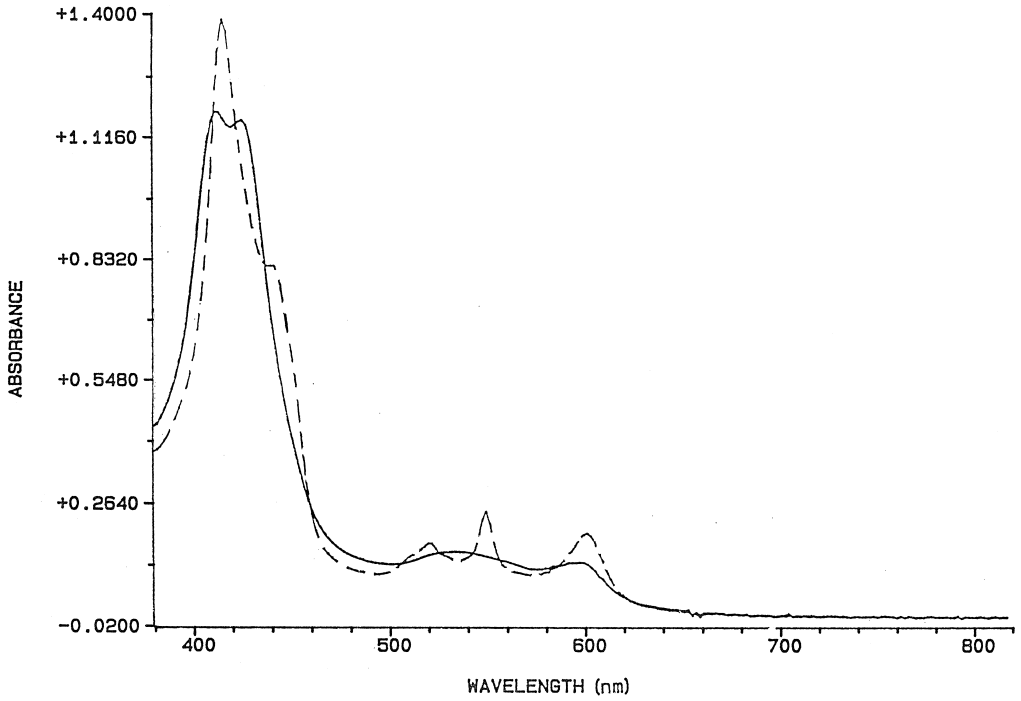


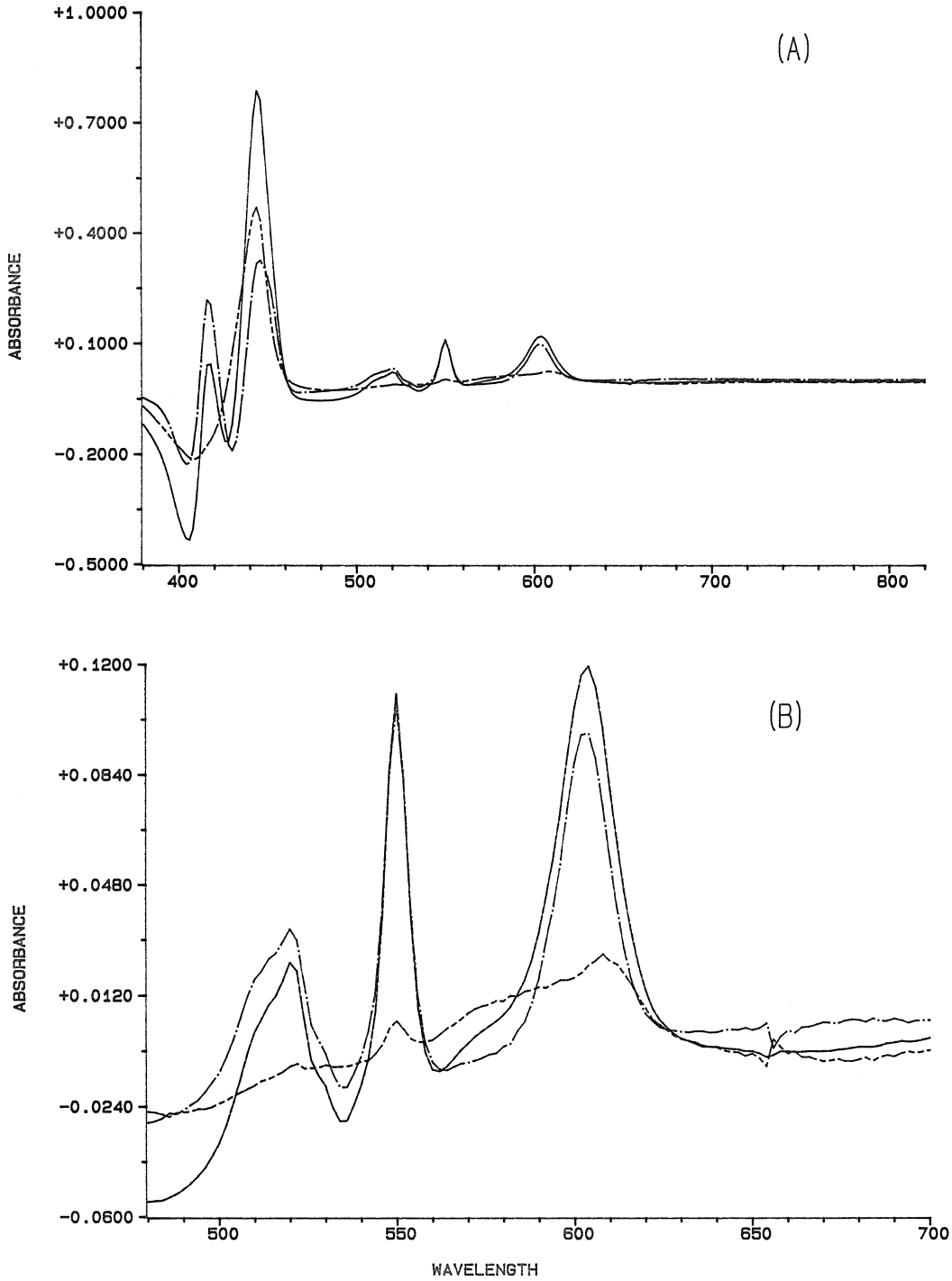
Fig. 7. Reduction of cyanide-inhibited *B. subtilis* cytochrome caa_3. The enzyme concentration was 5 μM in 10 mM Tris/MOPS, pH 7.4, 1 mM EDTA, 1 mg/ml *n*-dodecylmaltoside. Cyanide bound, oxidized (————) and cyanide, bound reduced (— — —) spectra are shown. For reduction ascorbate was 5 mM and TMPD was 0.2 mM.

s^{-1} in the presence of cytochrome *c*. In both cases with the eucaryotic oxidase, only the fast phase of the reaction is cyanide concentration dependent, whereas the rate of the slow phase is cyanide concentration independent.

Fig. 7 shows optical spectra of oxidized, cyanide-bound cytochrome caa_3 and ascorbate-TMPD reduced, cyanide-bound caa_3. These spectra show that cytochrome *c* and cytochrome *a* remain reducible, whereas the reduction of cytochrome a_3 is blocked in the presence of cyanide. The construction of reduced minus oxidized difference spectra for cytochrome *a* and cytochrome a_3 is illustrated in Fig. 8. The spectrum of cytochrome *a* being made up of the difference between partially reduced enzyme with cyanide bound to cytochrome a_3 minus oxidized, cyanide bound enzyme (i.e., the difference between the two absolute spectra in Fig. 7). The spectrum of cytochrome a_3 is constructed from the fully reduced, unliganded enzyme minus fully oxidized, minus the spectrum of cytochrome *a*. From these resolved difference spectra it is calculated that cytochrome a contributes 40% to the 444–460 nm absorbance intensity and 80% to the intensity at 604–630 nm. The spectrum of cytochrome a_3 in the visible region is quite different from cytochrome *a*. Cytochrome a_3 shows a pair of peaks at 608 nm and 580 nm. There is also a small peak at 550 nm due to a small difference in the state of reduction of cytochrome *c*.

The binding of cyanide to fully reduced cytochrome caa_3 is illustrated in Fig. 9. The difference spectrum between the cyanide complex of fully reduced cytochrome caa_3 and the fully reduced uninhibited enzyme is in panel A. The difference spectrum is very similar to that observed with bovine heart cytochrome *c* oxidase [18]. It has peaks at 452, 510, 544, 588 nm, and troughs at 412, 562, 608 nm. The results of a titration of fully reduced cytochrome caa_3 with cyanide are shown as a direct plot of absorbance change versus cyanide concentration (Fig. 9B). The solid line in this plot is a rectangular hyperbolic fit to the data and yields a K_d of 2 mM

and a maximal absorbance change of 0.057. The K_d represents about 8-fold weaker binding compared to that found previously for bovine heart oxidase [18]. The maximal absorbance obtained from Fig. 9B corresponds to an extinction coefficient of about 14 $mM^{-1} \cdot cm^{-1}$ and is consistent with formation of



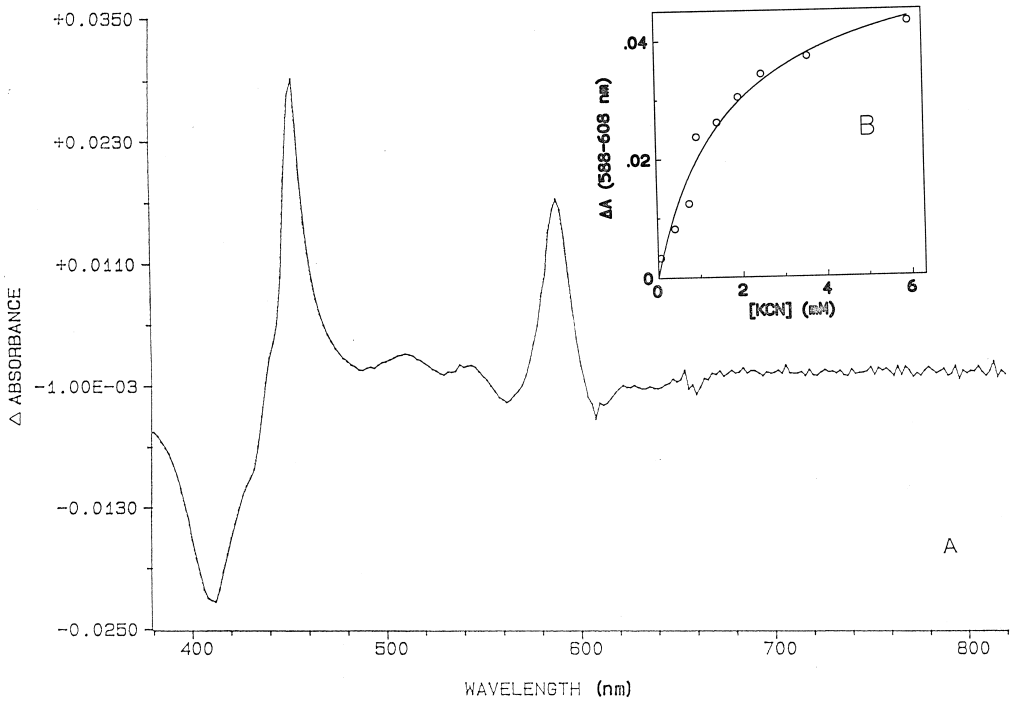


Fig. 9. Cyanide binding to fully reduced *B. subtilis* cytochrome caa_3. A: difference spectrum between the reduced cyanide-bound *B. subtilis* cytochrome caa_3 and the fully reduced unliganded oxidase. Cyanide, at 10 mM, was added to 5 μM reduced enzyme in 50 mM phosphate buffer. B: concentration dependence of cyanide binding to fully reduced *B. subtilis* cytochrome caa_3. The change in absorbance is taken from the magnitude of the peak and trough of the difference spectra and plotted as a function of cyanide concentration. The enzyme concentration was 4 ELM and the buffer was 50 mM sodium phosphate pH 7.0 with 1 mg/ml lauryl maltoside and 1 mM EDTA. The solid line is a fit to the equation, $y = A \cdot [\mathrm{KCN}]/B + [\mathrm{KCN}]$, where y is ΔA at each cyanide concentration, A is maximal ΔA and B is K_D.

ferrocytochrome $a_3 - \mathrm{CN}$ with a cyanide to heme ratio of one.

4. Discussion

Cyanide binding experiments show clearly that the ligand forms a complex with both oxidized and reduced as well as partially reduced *B. subtilis* cytochrome caa_3. Although we have not studied the kinetics of cyanide inhibition of caa_3 during turnover, we do observe that when cyanide is added to caa_3 during steady-state activity the onset of inhibition is instantaneous on the timescale (i.e., 2 s) of mixing used here (Assempour and Hill, unpublished observation). This is consistent with the rapid onset of inhibition observed previously for the mammalian enzyme [19]. The kinetics of cyanide binding to fully reduced

Fig. 8. Resolved difference spectra for cytochrome *a* and cytochrome a_3. The concentration of enzyme was 4.67 μM cytochrome caa_3 in 10 mM sodium phosphate pH 7.0 with 0.4 mg/ml n-dodecylmaltoside. The enzyme was initially incubated with 200 μM $K_3Fe(CN)_6$ for 1 h prior to addition of 5 mM KCN. The cyanide binding reaction was followed to completion prior to reduction with 5 mM sodium ascorbate plus 200 μM TMPD. The fully reduced state was generated on an identical sample of enzyme except in the absence of KCN. The spectrum of ferricyanide was subtracted to obtain the absolute oxidized spectra as reference. Panel A compares the UV-visible spectra of unliganded fully reduced minus oxidized (————), partially reduced, cyanide bound minus oxidized, cyanide bound (– · –, a^{2+}-a^{3+}) and fully reduced-oxidized, unliganded minus partially reduced-oxidized, cyanide bound (— - - —, a_3^{2+}-a_3^{3+}). Panel B shows the visible region on an enlarged scale with the same designations as above.

caa$_3$ also resembles those of bovine oxidase [18]. However, the kinetics of cyanide binding to oxidized *caa*$_3$ exhibits substantial differences compared to bovine cytochrome *c* oxidase. The major complications here are (i) the three rate constants representing the triphasic behaviour of the reaction are all cyanide concentration dependent and (ii) the enzyme cannot be converted into a form with biphasic or monophasic kinetics by incubation at either high or low pH, or by oxidation following reduction. This pattern of reaction is not derived from cyanide binding to cytochrome *c* or autoreduction of cytochrome a since it is observed under conditions where these side reactions are prevented. Furthermore, these side reactions are both seen with the mammalian *caa*$_3$ complex but they have no effect on the biphasic nature of the cyanide reaction with cytochrome a_3. The multiphasic behaviour of the enzyme toward cyanide may be due to a mixture of conformers that differ in their cyanide reactivity or alternatively, to a series of consecutive reactions initiated by cyanide binding to the cytochrome $a_3 - Cu_B$ binuclear centre [29]. The present results with the *caa*$_3$ oxidase favour the parallel reaction of different conformers since each shows cyanide-proportional reaction kinetics.

The fastest phase, which has a bimolecular rate constant of 17 $M^{-1} \cdot s^{-1}$ and can occupy up to 75% of the reaction time course, is 4–5-fold faster, than the value reported for the fast [27] or pulsed [30] conformers of mitochondrial cytochrome *c* oxidase. It is close to values reported for the rate of cyanide binding to peroxy and ferryl states of the binuclear centre [27,31]. However, we do not see any spectral evidence of these forms in our resting, oxidized preparations of cytochrome *caa*$_3$. It has also been reported that cytochrome *c* binding to the mammalian enzyme can promote fast reactivity to the cytochrome $a_3 - Cu_B$ centre and this can be mimicked by poly-L-lysine binding ([26], but cf. [27]). In the case of the *caa*$_3$ oxidase the association of cytochrome *c* is fixed covalently and its association appears to be unaffected by ionic strength [32]. Thus it may be that the physical presence of the cytochrome *c* domain contributes to the high reactivity of cytochrome a_3 with cyanide via an allosteric effect.

The different reactivity of cytochrome a_3 in the *caa*$_3$ complex is proposed ultimately to reflect a different environment around the cytochrome a_3 centre of the *Bacillus* enzyme as opposed to that of bovine heart oxidase. This is also supported by the different spectrum seen for the cytochrome a_3 centre in the *caa*$_3$ complex relative to that found for the bovine enzyme [33]. Although, these enzymes are homologous, the amino acid sequences between the different protein subunits are not identical and so it is feasible that there is a difference in the immediate environment of cytochrome a_3 which confers the differences in the visible spectrum, although still characteristic of a high spin heme, and the reactivity of the binuclear centre with cyanide. Detailed analysis of the subtle structural differences between members of the cytochrome oxidase family will require that more members of the family are the subject of high resolution structural analysis. Alternatively, comparative structures may be assembled by homology modelling but this approach awaits the availability of the coordinate sets for the bovine and Paracoccus oxidases.

It has been shown that cyanide binds the heme iron of horse cytochrome *c* and subsequently displaces the sulphur of methionine 80 normally bound to the heme iron [34]. However, the ligand does not bind to heme C in either cytochrome *caa*$_3$ or cytochrome *cao* from *Bacillus* PS3 [35,36]. Cyanide binding to the cytochrome *c* domain of *B. subtilis caa*$_3$ indicates that the stability of the axial ligation of the cytochrome *c* domain more closely resembles that of mitochondrial cytochrome *c* than the other bacterial *c* domains. However, the *c* domain of the *B. subtilis caa*$_3$ is distinguished from mitochondrial cytochrome *c* by a 40% increase in the intensity of the α-band extinction, whereas the reported extinction coefficients for the heme *c* domains of cytochrome *caa*$_3$ and cao from *Bacillus* PS3 are close to that of mitochondrial cytochrome *c*. The spectral difference we see in *B. subtilis caa*$_3$ is due largely to a slightly narrower bandwidth of this species relative to mitochondrial cytochrome *c* presumably due to a difference in the protein environment of the heme macrocycle.

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