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Cyanide-reactive sites in cytochrome *bd* complex from *E. coli*

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Cyanide reacts with cytochrome *bd* from *E. coli* in an 'aerobically oxidized' state (mainly, an oxygenated complex b_{558}^{3+} b_{595}^{3+} d^{2+}-O_2), bringing about (i) decomposition of the heme d^{2+} oxycomplex (decay of the 648 nm absorption band) and (ii) extensive red shift in the Soret region accompanied by minor changes in the visible range assigned to ferric heme b_{595}. MCD spectra show that the Soret red shift is associated with heme b_{595}^{3+} high- to low-spin transition. This is the first unambiguous demonstration that heme b_{595} can bind exogenous ligands. No reaction of cyanide with b_{558} is observed. In about 70% of the enzyme which forms the cyano complex, the spin-state transition of b_{595} decay of heme *d* oxycomplex match each other kinetically (k_{eff} ca. 0.002 s^{-1} at 50 mM KCN, pH 8.1, 25°C). This points to an interaction between the two hemes. The concerted binding of cyanide to d^{3+} and b_{595}^{3+}, perhaps as a bridging ligand, is probably rate-limited by d^{2+} oxycomplex autoxidation. In the remaining 30% of the isolated *bd*, there is a rapid phase of cyanide-induced b_{595} spin-state transition which can be tentatively assigned to that proportion of the enzyme in which heme *d* is initially in the ferric rather than ferrous-oxy form.

Cytochrome *bd*; Magnetic circular dichroism; Cyanide binding; Bacterial oxidases; Heme–heme interactions; *E. coli*

1. INTRODUCTION

The respiratory chain of *Escherichia coli* contains two major terminal oxidases: cytochrome *bo* and cytochrome *bd* (reviewed in [1,2]). Both enzymes catalyse oxidation of ubiquinol (or menaquinol) by molecular oxygen coupled to the generation of a protonmotive force across the bacterial cytoplasmic membrane [2].

While cytochrome *bo* is a typical member of a superfamily of oxidases containing a heme/copper binuclear oxygen-reducing centre and operating as the proton pumps [3], cytochrome *bd* has no copper [4,5] and, although generating a $\Delta\psi$ [5–7], does not pump protons [7,8]. It probably represents an example of a redox loop-type electrogenic terminal oxidase [9], although it has also been suggested that cytochrome *bd* can pump Na^+ ions [10].

The enzyme is composed of two subunits, 57 and 43 kDa, which carry three metal containing redox centres: low-spin heme b_{558} and two high-spin hemes b_{595} and *d*, of which the latter is believed to react with oxygen [1,2].

Cytochrome *bd* reveals a very high affinity for oxygen and surprisingly low sensitivity to inhibition by cyanide (see [2,11] for refs.), which points to a unique structure of the oxygen-activating centre of the enzyme.

Ligand binding studies is a classical approach towards elucidation of the structure–function relationships in the active centres of hemoproteins. Although there are a number of papers reporting the reaction of cyanide and other ligands with cytochrome *bd* followed by optical or EPR spectroscopy or activity measurements (reviewed in [2,11], see also [12]), the emerging picture is far from clear. Here we report on the cyanide reaction with the cytochrome *bd* complex monitored by optical and MCD spectroscopy.

Earlier spectrophotometric studies on KCN reacting with cytochrome *bd* were carried out with membrane vesicles from *Azotobacter vinelandii* [13,14] and *E. coli* [15] and were confined to measurements in the α-band. These studies revealed a decay of the peak at 650 nm induced by the inhibitor and were interpreted by that time as the disappearance of the free ferric cytochrome *d* [13–15]. This is reinterpreted now as the decay of the ferrous heme *d* oxycomplex [2].

The high-spin heme b_{595} could be a second ligand-binding site in cytochrome *bd* [2,11]. No effect of cyanide on the absorption spectrum of b_{595} (a_1) was found in [14]. On the other hand, evidence for the EPR spectrum of b_{595} affected by KCN was published [12]. Here we show that cyanide brings about large optical spectral changes of heme b_{595} associated with a high- to low-spin transition of the cytochrome.

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Abbreviations: MCD, magnetic circular dichroism.

2. MATERIALS AND METHODS

Membranes were prepared from the *E. coli* strain GO-102/pFH cells (lacking cyt *bo*; cyt *bd* overexpressed). After sonication and DNAse

treatment, cytochrome *bd* was isolated and partially purified essentially according to [4] but omitting the final hydroxylapatite chromatography step.

Absorption changes were measured in a computer-controlled Aminco-SLM DW-2000 spectrophotometer. MCD measurements were made in a computer-interfaced dichrograph constructed by Dr. A. Arutjunjan equipped with an electromagnet (magnetic field, ca. 1 T for a 1 cm cell). For each MCD spectrum, the traces recorded in the direct and reversed magnetic field directions were subtracted from each other to eliminate contribution of CD.

Concentration of cytochrome *bd* was determined from the dithionite-reduced *minus* air-oxidized difference spectra using the values of $\Delta\varepsilon_{628-648}$ = 18.8 $mM^{-1} \cdot cm^{-1}$ or $\Delta\varepsilon_{560-580}$ = 14.8 $mM^{-1} \cdot cm^{-1}$ [5].

3. RESULTS

Addition of 50 mM KCN to the air-oxidized cytochrome *bd* brings about the time-dependent absorption changes shown in Fig. 1.

In the visible region (B), a trough develops at 648 nm in accordance with [13–15] indicating decomposition of the heme *d* oxycomplex and transition of *d* to the ferric state. This is accompanied by a rather complex pattern of absorption increase in the 537–627 range.

The Soret band changes of cytochrome *bd* induced by cyanide, not described previously, are quite extensive (Fig. 1A). A size of the difference spectrum (peak-to-trough) can reach ca. 150 $mM^{-1} \cdot cm^{-1}$ after completion which implies a very large red shift of the γ-peak.

It can be seen from Fig. 1 that a substantial part of the absorption changes in the Soret takes place before any significant development of the trough at 648 nm. This is better illustrated by dual wavelength kinetics measurements (Fig. 2).

Except for a small initial jump, the decrease of absorbance at 646 nm is monophasic (a). The same major exponent is observed in the Soret (b) but, in addition, there is a rapid phase of ΔA increase contributing about $^1/_3$ to the overall process. It is noteworthy, that parallel experiments with membrane vesicle-bound *bd* reveal only the slow component of the reaction (k_{eff} = 0.002–0.003 s^{-1} at 50 mM KCN and pH 8) both in the Soret and 650 nm bands (not shown).

The difference spectrum of the rapid phase is better resolved at lower concentrations of KCN because of a decreased contribution of the slow phase to the initial absorption changes (Fig. 3). In the Soret (A), a somewhat asymmetric curve is observed with a rather broad minimum at 400–410 nm and λ_{max} varying from 434 to 437 nm in different preparations. In the visible (B), there is a shallow trough at 640 nm ($\Delta\varepsilon$ ca. 1 $mM^{-1} \cdot cm^{-1}$) which is clearly blue-shifted as compared to the minimum at 648 nm of the final difference spectrum. The trough is accompanied by a small broad peak at 561 nm. Altogether these changes would be typical of a high- to low-spin transition of a ferric heme b_{595}, including a loss of a charge transfer band at 640 nm.

The amplitude of the rapid phase saturates with cyanide concentration with an apparent K_d of 4 mM at pH 8 (Fig. 2, inset).

The slow phase of the changes can hardly be seen at cyanide concentrations below 5–10 min mM. In the visible range (Fig. 3B) it is dominated by a trough at 648 nm (heme *d* oxycomplex decay). This is accompanied by further development of the red shift in the Soret with a slightly altered lineshape (Fig. 3A). Presumably, the second phase corresponds to concerted optimal changes of both *d* and b_{595}.

Heme *d* contributes little to the Soret band of cytochrome *bd* [2,11], while the low-spin b_{558} would not be expected to react readily with exogenous ligands. Accordingly, it is reasonable to assign the KCN-induced

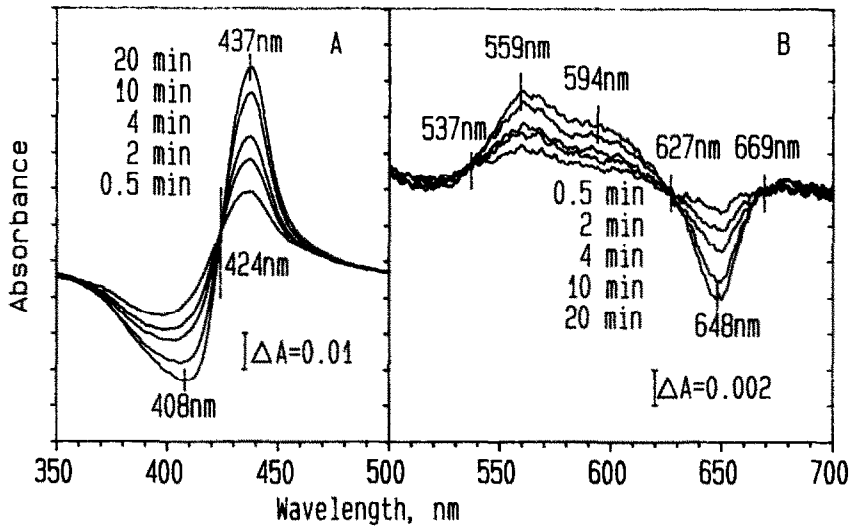

Fig. 1. Absorption changes induced by cyanide binding with cytochrome *bd* in the Soret (A) and visible (B) region. The sample and reference cells contained 0.72 μM cytochrome *bd* complex in the basic medium (50 mM HEPES/50 mM CAPS buffer, 0.025% sodium *N*-lauroyl-sarcosinate), pH 8.1. 50 mM neutralized KCN has been added to the sample (equal volume of the buffer to the reference). The difference spectra shown in the figure have been recorded 0.5, 2, 4, 10 and 20 min after the addition.

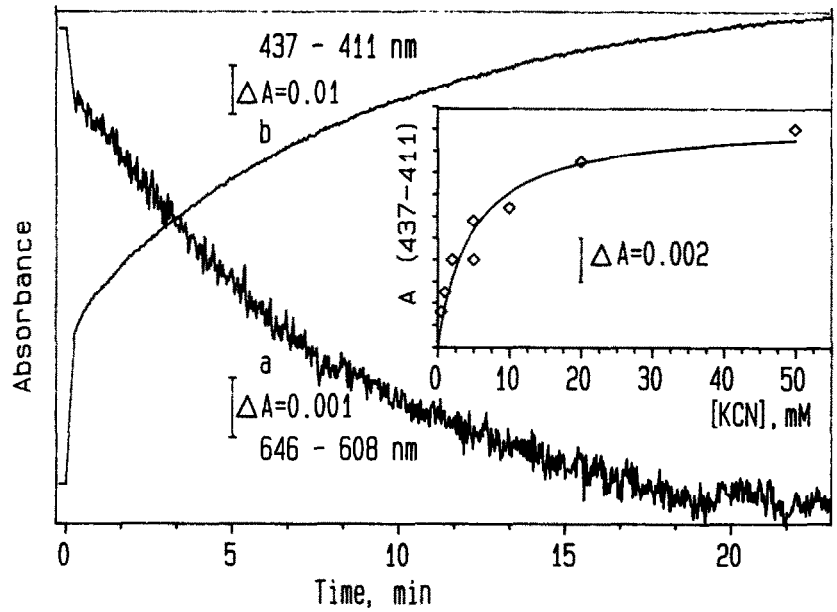

Fig. 2. Kinetics of the KCN-induced absorption changes. Basic conditions, as in Fig. 1. At the zero time 50 mM KCN has been added. Curve fitting gives two exponents for the Soret trace with rate constants and contributions of 0.086 s^{-1} (33%) and 0.0020 s^{-1} (67%); except for the small initial jump, the 646–608 nm trace is fitted by 1 exponent with k = 0.0019 s^{-1}. Inset: Concentration dependence of the rapid phase of KCN binding with heme b_{595}^{3+}. Computer-resolved amplitude of the rapid phase of the KCN-induced absorption changes measured at 437 minus 411 nm has been plotted as a function of cyanide concentration. The points are fitted by a curve for ligand binding with a K_d of 4 mM.

Soret band red shift to heme b_{595}. This has been directly confirmed by MCD measurements.

The MCD spectrum of the *bd* complex, as isolated, is dominated in the Soret band by a typical A-term signal centered at 419 nm and is obviously one of the low-spin ferric heme b_{558} (Fig. 4a). The high-spin ferric b_{595} and the low-spin ferrous d^{2+}-O_2 are not expected to contribute significantly to the MCD Soret spectrum at room temperature [16–18].

KCN brings about extensive changes in the Soret MCD spectrum (trace b) inducing an additional A-term signal, now centered at 434 nm (trace c). This is consistent with a conversion of a ferric high-spin heme *b* to the low-spin cyanide-ligated state as modelled by a

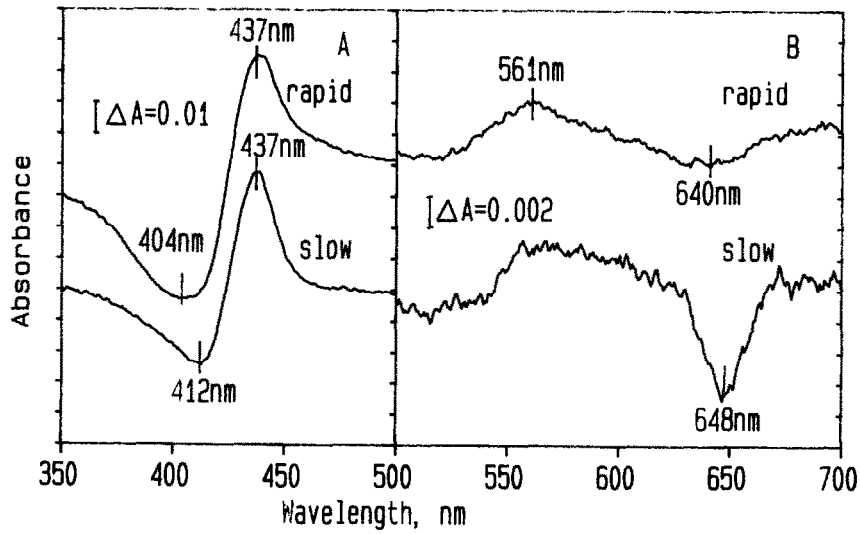

Fig. 3. Difference spectra of the rapid and slow phases of cyanide-induced absorption changes. Basic conditions, as in Fig. 1, but [KCN] = 10 mM and pH 8.7. (Rapid phase): from the experimental spectrum recorded within 2 min after KCN addition contribution of the slow phase was subtracted. The latter was determined on the basis of rate constant and contribution of the slow phase measured separately under the same conditions in a dual-wavelength kinetic mode (cf. Fig. 2) and taking the lineshape of the slow changes from late phase of the reaction at 50 mM KCN. (Slow phase): 30 min minus 20 min after KCN addition.

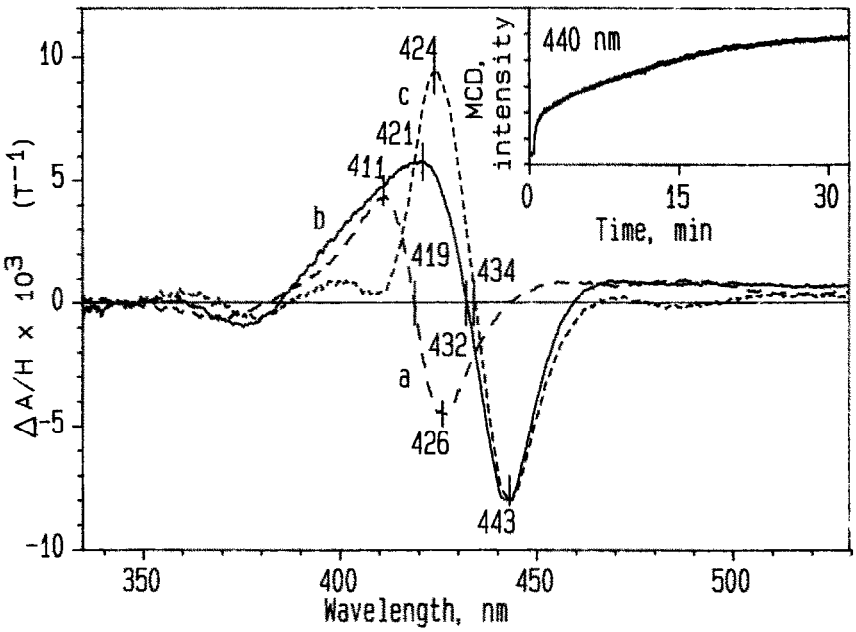

Fig. 4. Effect of cyanide binding on the MCD characteristics of the *bd* cytochrome. Basic conditions, as in Fig. 1, but [*bd*] = 2.7 μM. (a) Complex *bd* as isolated; (b) 20 min after addition of 50 mM cyanide; (c) b minus a. Inset: Kinetics of the cyanide-induced MCD changes in the Soret band (appearance of an A-term signal of low-spin heme b_{595}^{3+}).

$Mb^{3+} \rightarrow Mb^{3+}$-CN transition (not shown). Notably, the spectrum of the low-spin heme b_{558} is not perturbed, showing that the latter does not react with the ligand. Also the visible MCD spectra (not included) do not reveal any reduction of b_{558} under these conditions.

Importantly, the KCN-induced MCD changes show the same biphasic kinetics as the γ-band absorption changes (Fig. 4, inset).

4. DISCUSSION

We confirm previous reports [13–15] that cyanide reaction with air-oxidized cytochrome *bd* results in a loss of the heme *d* oxycomplex and in oxidation of heme *d*. However, in contrast to earlier conclusions [14], our measurements of the cyanide-induced absorption and MCD changes in the Soret band show clearly that in the isolated *bd* complex, the ligand affects heme b_{595} as well, the latter converted to the low-spin state. This is probably the first unambiguous demonstration of b_{595} being able to react with exogenous ligands.

It is then most interesting, that the decay of heme *d* oxycomplex and the spin state transition of b_{595} are coupled in a major part of the isolated cytochrome *bd*, observed as the slow phase of the reaction. This indicates some kind of heme–heme interaction between b_{595} and *d*.

It is to be noted that the optical changes associated with the *bd* reaction with KCN, as observed here or by others, cannot be taken as proof of direct cyanide binding to heme *d* since a signature of ferric heme *d*, free or ligand-bound, in the absorption spectrum of complex *bd* is not known. However, various reductants, including dithionite, added to the KCN-preincubated enzyme fail to reduce rapidly heme *d* (data not shown, cf. [13,15]). This favours the d^{3+}-CN adduct formed, although an indirect effect of cyanide binding to b_{595} on the reducibility of heme *d* cannot be fully excluded. Assuming that KCN does bind to d^{3+}, the following alternatives may be considered.

(i) There are two molecules of cyanide which bind to both b_{595} and *d*, ligation at one site facilitating (kinetically or thermodynamically) binding at the other, which results in a concerted reaction.

(ii) All the spectral changes are induced by binding of a single molecule of cyanide to *bd*, e.g. cyanide could bind simultaneously to b_{595}^{3+} and d^{3+} as a bridging ligand like in the a_3/Cu_B centre of aa_3-type oxidases ([19,20] and refs. therein). A less likely possibility, which however cannot be fully excluded, is that cyanide binds to heme *d* only, which entails b_{595} transition to a low-spin state mediated by a conformational change of the protein and binding of a protein-derived 6th axial ligand to heme iron of b_{595}.

The simplest interpretation of the slow phase, consistent with both (i) and (ii), is that the presence of an oxy-ligand at heme *d* prevents cyanide binding to either b_{595} or *d* hemes. A simple steric hindrance might explain the effect of heme *d* bound ligand on b_{595} reactivity since the two hemes are likely to be in close proximity as implied by CO migration from heme *d* to heme b_{595} at low temperatures following photolysis [21]. Autoxidation of the oxycomplex (e.g. its spontaneous dissociation to d^{3+} and O_2^-) could limit the rate of cyanide bind-

ing with b_{595}, *d* or both. The rapid phase of cyanide reaction observed in the isolated complex *bd* could then be due to a fraction of the enzyme in which heme *d* is initially in the ferric rather than oxygenated state; this fraction is likely to be very small in bacterial membranes due to a steady leak of reducing equivalents. Notably, partial conversion of the oxygenated *bd* complex to the oxidized state with the aid of various oxidants results in an increased contribution of the rapid phase of b_{595} reaction with cyanide (I. Krasnoselskaya, unpublished).

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