Spontaneous spectral changes of the reduced cytochrome *bd*

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Abstract Reduction of the membrane-bound cytochrome *bd* from *Bacillus subtilis*, *Escherichia coli* and *Azotobacter vinelandii* as well as of the purified enzyme from *E. coli* was followed by secondary absorption changes on a time scale of tens of minutes. The difference absorption spectra of these changes resembled those induced by CO binding with heme d^{2+} indicating interaction of the heme with an endogenous π-acceptor ligand. The spontaneous spectral changes were prevented and reversed by CO binding with the reduced cytochrome *bd*. Bonding of heme *d* iron to an endogenous protein ligand at the sixth axial position upon reduction is proposed and several possible mechanisms of such a process are considered.

Key words: Cytochrome *bd*; Heme-ligand interaction; Absorption spectrum; *Bacillus subtilis*; *Escherichia coli*; *Azotobacter vinelandii*

1. Introduction

Quinol oxidases of the *bd* type (cytochromes *bd*) are widely found in bacteria [1–4]. Cytochromes *bd* show no sequence homology to heme/copper terminal oxidases [5,6], do not pump protons [7], and contain no copper [8,9]. The two different subunits of the enzyme carry three metal redox groups: hemes b_{558}, b_{595} and *d* [10]. The low-spin heme b_{558} located in subunit I is likely to accept electrons from quinol [11]. The high-spin hemes *d* and b_{595} are believed to form a di-heme oxygen-reducing center analogous to the heme/copper center of other oxidases [12,13]. The catalytic cycle of cytochrome *bd* has not been resolved yet (see [14] for discussion).

The heme *d* component of the oxidase was shown to have some specific features. Being assigned a chlorine-type structure [15], it exhibits a relatively weak Soret absorption band [16]. The heme iron is believed to be high-spin [17,18], although low-spin EPR signals associated with heme *d* have been reported [19], and is presumably pentacoordinated [17,18]. However, the axial ligand has not been identified so far and is unlikely to be a histidine imidazole or a cysteine [20,21]. Like other high-spin hemoproteins, heme *d* shows facile reactions with exogenous ligands when oxidized [1,22–24] and reduced [8,9,4,24–27]. Surprisingly, it has been found to retain the high-spin pentacoordinated state upon ligand binding [17,18]. This may point to a labile coordination sphere of heme *d* iron.

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Abbreviations: CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; EDTA, ethylenediaminetetraacetate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid; NIR, near infrared; NSMP, nutrient broth sporulation medium with phosphate

Most of the earlier studies on cytochrome *bd* were carried out on the enzymes from *Escherichia coli* and *Azotobacter vinelandii*, and it is only recently that first investigations into *bd*-oxidase of Gram-positive bacteria have appeared (see [1] for references). In our collaborative research with the Microbiology Department of Lund University, a number of *Bacillus subtilis* strains lacking terminal oxidases other than cytochrome *bd* have been constructed which greatly facilitate spectrophotometric studies of cytochrome *bd* in membrane preparations. In the course of these studies we encountered peculiar behavior of membrane-bound cytochrome *bd* that displayed spontaneous spectral changes upon reduction. These changes were subsequently revealed in cytochromes *bd* from other bacteria as well.

2. Materials and methods

2.1. Strains and preparations

B. subtilis strain LUH-27 (constructed in the laboratory of Dr. L. Hederstedt, University of Lund) is depleted in the structural genes of the aa_3 and caa_3 terminal oxidases and in succinate:menaquinone oxidoreductase. Cells were grown to the stationary phase in NSMP medium [28] enriched with 0.5% yeast extract and 0.5% glucose. Membranes were obtained according to [28]. *E. coli* strain GO-102/pFH101 (kindly provided by Dr. Robert B. Gennis, University of Illinois at Urbana-Champaign) is deficient in *bo* oxidase and overproduces cytochrome *bd*. Cell growth and preparation of the membranes were performed as previously [29]. Cytochrome *bd* was isolated as described in [8]. *A. vinelandii* strain MK-8 (a kind gift of Dr. Robert K. Poole, King's College, London) overproduces cytochrome *bd*. Cells were grown in Burk's medium [30] and disrupted by French press to obtain the membranes.

2.2. Assays

Spectrophotometric measurements were performed in an Aminco-SLM DW-2000 dual wavelength/split beam instrument. For anaerobic assays, a gas-tight cell (Hellma) was used and the reaction mixture was degassed with a vacuum pump. The concentration of cytochrome *bd* was estimated from the difference spectra (dithionite reduced minus air-oxidized) using a provisional molar extinction of 17 mM^{-1} cm^{-1} for ΔA at 628 minus 655 nm. Protein was measured by the method of Lowry et al. [31].

3. Results

3.1. Spontaneous spectral changes of the reduced cytochrome bd

Addition of dithionite to membranes of *B. subtilis* LUH-27 brings about reduction of the cytochromes in about 5 min. The resulting difference absorption spectrum (Fig. 1, **a**) is dominated by cytochrome *bd*, as other cytochrome components of the respiratory chain are absent in the mutant.

Upon incubation, the reduced membranes reveal further absorption changes on a time scale of tens of minutes. The difference spectrum of these changes (Fig. 1, **b**) in lineshape and size resembles the spectral shift of heme *d* induced by binding of the ligands such as CO, O_2 or NO with the reduced

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cytochrome *bd* from *E. coli* or *A. vinelandii* [4,8,9,27,24]. Accordingly, this observation may indicate slow binding of some ligand (L) with heme d^{2+} following reduction.

Taking into account the extremely high affinity of heme *d* for CO and NO and the unfortunately high air pollution in Moscow, our immediate concern was that it could be atmospheric CO or NO reacting slowly with the reduced cytochrome *bd*. However, experiments with the vacuum degassed reaction mixture gave the same results. Our second guess was that the ligand could be an impurity in dithionite. To test this possibility, anaerobic reduction of the membranes with 2 mM NADH instead of dithionite was performed (Fig. 1, **c**) and found to be followed by essentially the same spontaneous absorption changes. Also replacing other major components of the reaction medium (see legend to Fig. 1) did not eliminate the effect. Finally, similar spectral changes could be observed with a partially purified cytochrome *bd* from *B. subtilis* indicating that L is not a lipid-soluble component of the membranes.

3.2. Relationships between the spontaneous and CO-induced spectral changes of cytochrome bd

The effect of CO on the absorption spectrum of the dithionite-reduced *B. subtilis* membranes (Fig. 2, **a**,**e**) is similar, but not identical to the spontaneous changes (Fig. 2, **b**). First, the CO-induced difference spectrum reveals an additional trough

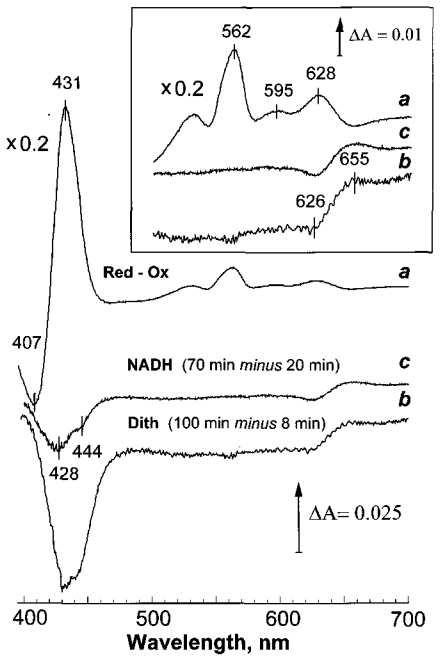

Fig. 1. Spontaneous absorption changes of the membrane-bound cytochrome *bd* from *B. subtilis*. *B. subtilis* membranes (~2 mg/ml) in 100 mM MOPS, 0.2 mM EDTA (pH 7.0), 18°C. **a**: Reduced by dithionite (8 min incubation) minus air-oxidized, the spectrum is scaled down 5-fold. **b**: 100 min minus 8 min after addition of dithionite. **c**: 70 min minus 20 min after addition of NADH. Reduction by NADH was carried out anaerobically in a vacuum degassed cell. Final concentrations: dithionite, 5 mM; NADH, 2 mM. The spectra have been normalized to 1 μM of cytochrome *bd*. Results similar to those in **b** have been reproduced in two more reaction buffers containing 20 mM HEPES, 5 mM $MgSO_4$, 25 mM K_2SO_4 (pH 7.5), or 50 mM CHES, 50 mM HEPES, 0.5 mM EDTA (pH 8.0). The inset shows enlarged visible parts of the spectra.

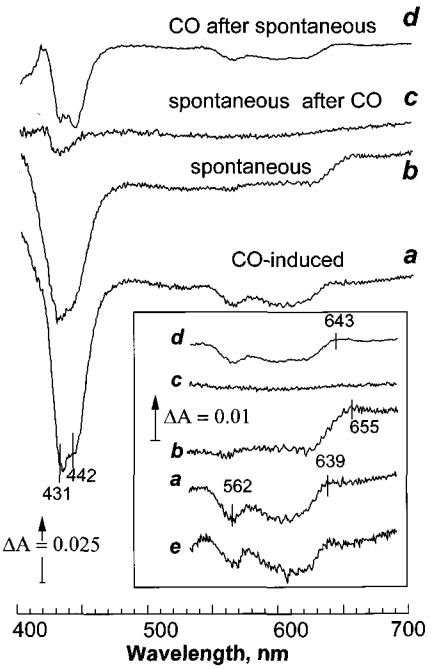

Fig. 2. Relationships between the spontaneous and CO-induced spectral changes of the reduced cytochrome *bd* from *B. subtilis*. Basic conditions as in Fig. 1. The following difference spectra are shown: **a**: treated with CO after 100 min incubation with dithionite minus the initial dithionite-reduced state (8 min); **b**: 100 min minus 8 min with dithionite; **c**: as **b**, but bubbled with CO before addition of dithionite; **d**: CO-induced changes in the sample incubated for 100 min with dithionite; **e**: reduced for 8 min by dithionite under atmosphere of CO minus the same in the absence of CO, only the visible part of the spectrum is shown (see the inset). Inset: enlarged visible parts of the spectra.

at ca. 562 nm; such a trough has often been observed for cytochrome *bd* from other bacteria [4,24,9,32] and is usually ascribed to a minor reaction of CO with the low-spin heme b_{558}. Second, there is a well developed trough around 600–605 nm. Such a trough has been observed in the case of *A. vinelandii* [24] but is much less pronounced in *E. coli* enzyme [9] and may indicate CO binding with the high-spin heme b_{595} in *B. subtilis* cytochrome *bd*. The reaction of CO with the heme(s) *b* is likely to account for the larger trough in the Soret induced by CO as compared to the spontaneous changes. Third, the heme *d*-associated derivative-shaped feature around 630 nm is clearly blue-shifted in the CO-induced difference spectrum relative to that of spontaneous changes (cf. spectra **a** and **b** in Fig. 2, inset).

If reduced cytochrome *bd* is first converted to a CO adduct and then allowed to stand, the time-dependent spectral changes are essentially prevented (Fig. 2, **c**), except for a minor trough at 431 nm complete in the first 15 min. In turn, measuring the response to CO after prolonged standing of the reduced sample (i.e. after completion of the spontaneous changes) may result in a significant underestimation of the ligand-induced changes in the Soret band (Fig. 2, **d**) due to the similarity of the spectral perturbations of heme *d* induced by the ligand and by incubation. It has to be emphasized that the CO-induced difference spectra versus the *initial* reduced state are the same regardless of whether CO was added after completion of the spontaneous changes or right at the time of the reduction (cf. spectra **a** and **e** in Fig. 2, inset). Apparently,

the spontaneous and CO-induced changes are not additive and the final spectrum of the CO complex is the same irrespective of the history of the sample. This allows us to suggest that CO not only prevents, but also reverts the spontaneous spectral shift of cytochrome *bd*. Conceivably, this would be the case if CO competes with the putative ligand L for binding with ferrous heme *d* and is able to displace it from the complex.

3.3. Dynamics of the spontaneous spectral changes

Recording difference spectra successively makes it possible to see that in *B. subtilis* membranes, the spontaneous changes in the Soret and visible bands are nearly synchronous and approach saturation in about 2 h (Fig. 3, **a**). The kinetics of the effect measured in more detail (e.g. Fig. 3, inset) is fitted by two exponents. The rates of both phases increase with temperature (τ_1 and τ_2 values of 12 ± 0.4 min and 81 ± 6 min at 23°C, and 5.2 ± 0.06 min and 39 ± 0.4 min at 33°C); such a moderate acceleration (2.3-fold per 10°C) argues against global protein rearrangements being involved in the process. The computer-resolved contributions of the rapid and slow phases are about 35% and 65%, respectively, but a significant part of the rapid phase is lost within the 8 min allocated for completion of reduction.

3.4. Spontaneous spectral changes of cytochrome bd in E. coli and A. vinelandii

The spontaneous spectral shift of cytochrome *bd* can also be observed in the membranes from *E. coli* and *A. vinelandii*

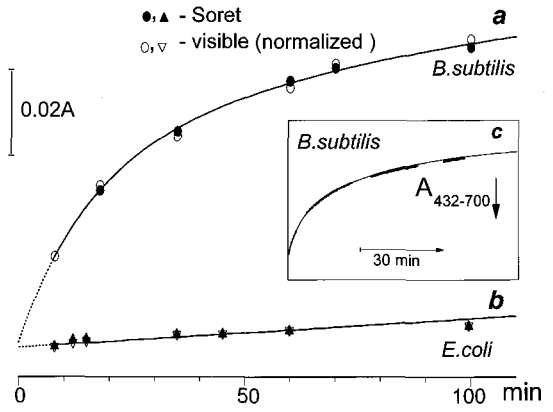

Fig. 3. Kinetics of the spontaneous spectral changes of membrane-bound cytochrome *bd*. The spectrophotometric cell contains membranes from *B. subtilis* (**a**,**c**) or *E. coli* (**b**) in the buffer indicated in Fig. 1. **a**,**b**: Experiments in a spectrum scan mode. At time zero, dithionite was added to the membranes and absolute spectra of the sample were recorded in the range 400–700 nm. The scan in 8 min after dithionite addition (full reduction) was taken as the baseline. Absorption changes in the visible $\Delta A_{655-626}$ are given normalized to the responses in the Soret band $\Delta A_{475-432}$ (expanded 5.3-fold for *B. subtilis*, and 3-fold for *E. coli*). t = 18°C. In **a**, a theoretical two-exponential curve is drawn through the points with characteristic times ($\tau_1 = 15.2$ min, $\tau_2 = 100$ min) as obtained from the kinetics mode experiments (inset) and extrapolated to 18°C. The curve extrapolates to time zero as shown by a dotted part of the line. In **b**, the small absorption changes were fitted simply by a straight line. Inset: The spontaneous absorption changes were followed in *B. subtilis* membranes ([*bd*] = 0.55 μM) at 432 nm vs. 700 nm reference in a kinetic dual-wavelength mode at 33°C. The gaps in the experimental points correspond to the time intervals at which the spectra of the sample were checked. A line drawn through the points is the best two-exponential fit with $\tau_1 = 5.1$ min and $\tau_2 = 39$ min.

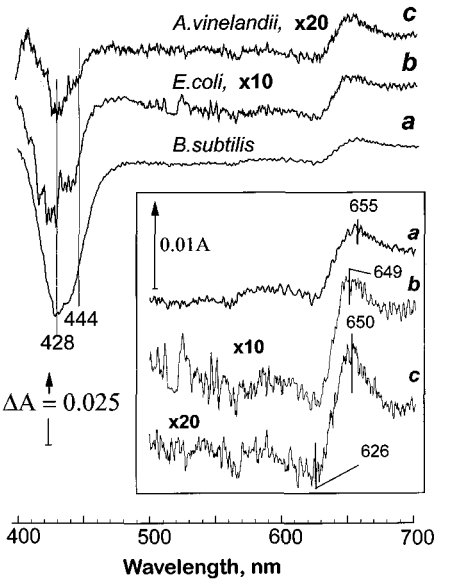

Fig. 4. Spontaneous spectral changes of the dithionite-reduced cytochrome *bd* in membranes from different bacteria. Basic conditions as in Fig. 1. **a**,**b**: 100 min minus 8 min after addition of dithionite; **c**: 100 min minus 15 min after addition of dithionite. Inset: enlarged visible region. The *E. coli* and *A. vinelandii* absorbance changes were expanded relative to those of *B. subtilis* as indicated.

(Fig. 4). The phenomenon is much less pronounced in these bacteria (traces **b**,**c**) as compared to *B. subtilis* membranes (trace **a**), which is presumably due to the slower rate of the changes (cf. the kinetics of curves **a** and **b** in Fig. 3). Purification of *E. coli* cytochrome *bd* dramatically accelerates the spontaneous spectral changes so that they reach saturation in about 1 h after the reduction ($\Delta\varepsilon_{432-475} \sim -30$ mM^{-1} cm^{-1}, $\Delta\varepsilon_{650-628} \sim 5$ mM^{-1} cm^{-1}, data not shown) approaching in size the perturbation observed in *B. subtilis* membranes.

4. Discussion

The significance of the present observations for the spontaneous spectral shift of the reduced cytochrome *bd* is 3-fold.

First, the slow changes of heme *d* should be taken into consideration in the spectroscopy studies of cytochrome *bd*. Interference of the spontaneous spectral shift can be quite significant in the case of the membrane-bound cytochrome *bd* from *B. subtilis* or purified enzyme from *E. coli*. For instance in *B. subtilis*, the size of the CO-induced difference spectra in the Soret varied more than two-fold depending on the time elapsed between recording of the reduced spectrum and addition of CO (Fig. 2, **a**,**d**).

Second, our data reveal significant differences in behavior of the membrane-bound cytochrome *bd* in *B. subtilis* as compared to *E. coli* and *A. vinelandii*.

Third, the lineshape and magnitude of the spontaneous spectral changes of the reduced cytochrome *bd* are remarkably similar to the effects of the exogenous π-acceptor axial ligands. As reaction with impurities is not supported by control experiments, it is tempting to propose endogenous protein ligand binding to heme *d*.

Heme *d* iron has been reported to retain its pentacoordinated high-spin state even after binding of such strong ligands as cyanide (when oxidized) and O_2 (when reduced) [17]. A

plausible explanation for this unusual behavior may consist in a relatively weak axial bonding of heme *d* iron to the protein so that coordination of a strong ligand at the sixth (distal) axial position disrupts the fifth (proximal) ligand bond:

$$L_5 - Fe + L_{exo} \rightarrow L_5\ Fe - L_{exo} \qquad (I)$$

where L_{exo} denotes the exogenous ligand like CO or HCN. Taking into account the peculiar lability of the heme *d* iron coordination sphere in the cytochrome *bd* complex, a number of meaningful interpretations of the spontaneous spectral shift may be considered.

(1) In the 'air-oxidized' membranes, a significant part of heme *d* may be in the stable oxy-complex and oxo-ferryl forms, as is typical for instance of the *E. coli* cytochrome *bd* 'as isolated' [18]. If these forms are pentacoordinated [17], the bond between heme *d* iron and the protein axial ligand has to be broken in them. We propose that anaerobic reduction of the heme, converting the distal oxygenous ligands to water, generates transiently a tetracoordinated state (without axial ligands) followed by rebinding of the protein ligand at the fifth position:

$$L_5\ Fe^{2+}\text{-}O_2 \xrightarrow[H_2O]{\text{reduction}} L_5\ Fe^{2+} \longrightarrow L_5\text{-}Fe^{2+} \qquad (II\ a)$$

$$L_5\ Fe^{4+}{=}O \xrightarrow[H_2O]{\text{reduction}} L_5\ Fe^{2+} \longrightarrow L_5\text{-}Fe^{2+} \qquad (II\ b)$$

(2) In the free ferric cytochrome *bd*, the endogenous proximal protein ligand of heme *d* can be replaced slowly by a stronger ligand at the distal side upon reduction:

$$L_5\text{-}Fe^{3+}\ \ L_6 \longrightarrow L_5\text{-}Fe^{2+}\ \ L_6 \longrightarrow L_5\ \ Fe^{2+}\text{-}L_6. \qquad (III)$$

(3) Reduction initiates structural rearrangement of the enzyme resulting in substitution of the heme iron axial ligand on the same (proximal) side of the heme plane (reaction analogous to substitution of lysine for methionine in alkaline cytochrome *c* [33]).

(4) A 5- to 6-coordinated state transition of heme *d* iron upon reduction cannot be excluded; however, this should entail drastic changes in the characteristics of the heme that have not been detected so far by resonance Raman spectroscopy.

The possible presence of several forms of heme *d* in the 'air-oxidized' cytochrome *bd* (ferric, ferrous-oxy and ferryl-oxo) complicates the interpretation of the spontaneous changes and can account for their biphasic kinetics. Unfortunately, the redox state of the 'as isolated' membrane-bound cytochrome *bd* is not controlled as easily as with the purified cytochrome.

Resonance Raman studies of the purified cytochrome *bd* from *E. coli* could discriminate between the above possibilities and provide a direct insight into the mechanism of the spontaneous changes of heme *d*.

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