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Optical and magneto-optical activity of cytochrome *bd* from *Geobacillus thermodenitrificans*

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ABSTRACT

Cytochromes *bd* are terminal oxidases in the respiratory chains of many prokaryotic organisms. They reduce O_2 to $2H_2O$ at the expense of electrons extracted from quinol. The oxidases can be divided into two subfamilies, L and S, based on the presence of either a long or a short hydrophilic connection between transmembrane helices 6 and 7 in subunit I designated as 'Q-loop'. The L-subfamily members, e.g. the enzyme from *Escherichia coli*, are relatively well-studied and were shown to generate proton-motive force. The S-subfamily comprises the majority of cytochromes *bd* including the enzyme from *Geobacillus thermodenitrificans* but is very poor studied. We compared the properties of cytochromes *bd* from *G. thermodenitrificans* and *E. coli* at room temperature using a combination of absorption, CD and MCD spectroscopy. The *G. thermodenitrificans* enzyme does contain the high-spin heme b_{HS} ("b_{595}") despite the fact that its characteristic Q_{00}-band ("α"-band) at 595 nm is not seen in the absorption spectra; stoichiometry of hemes b_{LS}, b_{HS} and *d* per the enzyme complex is suggested to be 1:1:1. At 1 mM CO, 20–25% of ferrous heme b_{HS} in the *G. thermodenitrificans* oxidase binds the ligand, while in case of the *E. coli* enzyme such a reaction is minor. In the *G. thermodenitrificans* oxidase, the excitonic interaction between ferrous hemes b_{HS} and *d* decreased as compared to that in the *E. coli bd*. The latter may suggest that the two enzymes differ in the distance between heme *d* and heme b_{HS} and/or in the angle between their porphyrin planes.

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1. Introduction

Cytochrome *bd* constitutes a terminal part of the respiratory chain of many prokaryotes including pathogens [1–3]. This oxidoreductase catalyzes reduction of molecular oxygen to water by electrons taken from quinol [4–6]. The enzyme is thought to contribute to mechanisms of nitrosative stress tolerance [3,7–12], hydrogen peroxide detoxification [13–15] and may be responsible for many other vital physiological functions (reviewed by [2,3]).

The cytochrome *bd* family can be divided into two subfamilies, L and S. The classification is based on the size of the hydrophilic region of subunit I which connects transmembrane helices 6 and 7 (so-called 'Q-loop') [2,16,17]. The enzymes of the L-subfamily containing an insert in the C-terminal portion of 'Q-loop' have thereby a '**l**ong Q-loop'. The enzymes of the S-subfamily have a '**s**hort Q-loop'. Most of previous studies have focused on the two members of the L-subfamily, the oxidases from *Escherichia coli*[2] (reviewed by [18]) and *Azotobacter vinelandii* [19–30]. These cytochromes *bd* consist of two different subunits, I and II, which carry three hemes, b_{LS} (previously called "b_{558}")[3], b_{HS} (previously called "b_{595}"), and *d*. The low-spin hexacoordinate heme b_{LS} located within subunit I near a quinol-binding site has His186/Met393 axial ligation [31,32] and mediates electron transfer from quinol to hemes b_{HS} and *d* [33]. The high-spin pentacoordinate heme b_{HS} has His19 of subunit I as the axial ligand. The role of heme b_{HS} is still a matter of debate. Heme b_{HS} facilitates electron transfer from heme b_{LS} to heme *d* [34,35] or to heme *d*-bound oxygen intermediates [36]. Since heme b_{HS} is high spin, it is also tempting to consider its involvement in the O_2 reduction. A number of observations indicate that in the *E. coli* cytochrome *bd* heme b_{HS} and heme *d* can form a common di-heme O_2-reducing site [28,36–44]. For instance, heme b_{HS} might serve as a binding site for hydroxide produced from heme *d*-bound oxygen upon the reductive O–O bond fission [36]. However, some data on the *A. vinelandii* enzyme do

Abbreviations: CD, circular dichroism; MCD, magnetic circular dichroism; Ox, 'air-oxidized' species; R, dithionite-reduced species; RCO, dithionite-reduced CO-bound species

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[2] *E. coli* has the two *bd*-type oxidases, *bd*-I and *bd*-II. Unless otherwise stated, we refer to cytochrome *bd*-I throughout the manuscript.

[3] Since hemes *b* do not always have the same absorption peaks, the two hemes *b* in cytochrome *bd*, previously called "b_{558}" and "b_{595}", are identified throughout the manuscript as the low-spin (b_{LS}) and the high-spin (b_{HS}) heme, respectively.

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not support the existence of the dihemic center. In particular, no spin coupling between hemes b_{HS} and *d* was observed [23]. Also, ligation of heme *d* to CO or cyanide did not alter the redox potential of heme b_{HS} [4]. Thus, an alternative viewpoint is that a "functional" di-heme site in a *bd*-type oxidase does not exist at all. As noted by one of the reviewers, although it is obvious that there is some spectroscopic evidence for heme b_{HS}/d interaction, even the estimated center-to-center distance of 10 Å [36] says it is not a "functional" di-metal site, such as that of heme-copper oxidases, where the heme-Cu distances are ca. 4.5 Å, which allows, for example, the heterolytic splitting of the dioxygen molecule, so that each oxygen atom may bind to each metal; other reasons may explain the interactions observed for cytochromes *bd*, such as a simple "stacked-like" conformation of the two hemes, i.e., a physical proximity of the porphyrin rings without involving functionally the heme irons. Another hypothesis is that heme b_{HS} can serve as a second site capable of reacting with O_2 [45,46].

Hemes b_{LS} and b_{HS} are protohemes IX, whereas heme *d* is a chlorin. The high-spin heme *d* is the site where O_2 is bound, activated and converted into $2H_2O$. Its axial ligand has not yet been determined but a highly conserved Glu99 of subunit I might be a candidate [47,48]. Cytochromes *bd* from *E. coli* and *A. vinelandii* generate the proton motive force by transmembrane charge separation yielding the H^+/e^- ratio of 1 [27,30,42,43,49–52].

Although most of the cytochromes *bd* belong to the S-subfamily, e.g., the enzyme from *Geobacillus thermodenitrificans* K1041 (formerly *Bacillus stearothermophilus* K1041) [16,53,54], they are very poorly studied. Recently cytochrome *bd* from *G. thermodenitrificans* was isolated and purified [16,53]. Compared with cytochromes *bd* from *E. coli* and *A. vinelandii*, the *G. thermodenitrificans* one shows lower molecular weights of the two subunits, shorter wavelength of the absorption maximum of the heme *d* Q_y-band ("α"-band) and lower quinol oxidase activity, although the latter can be significantly enhanced by preincubation with menaquinone-2 [16]. Another peculiar observation is that the absorption spectra of the purified enzyme in the fully reduced state do not show the Q_{00}-band ("α"-band) peak at 595 nm and the B-band ("γ"-band) shoulder at 439 nm [16] which are characteristic of ferrous heme b_{HS} [36,40,55]. A similar note was made for cytochrome *bd* from *Bacillus firmus* OF4 [56] but not for the *Corynebacterium glutamicum bd* that reveals spectral properties similar to the *E. coli* ones [57]. It has to be noted that at the moment it is not known at what extent the unusual spectroscopic properties of the *G. thermodenitrificans* enzyme may be extrapolated to the S-subfamily members in general. The lack of the heme b_{HS} spectral features in the *G. thermodenitrificans* preparations is intriguing and prompted us to examine heme composition by absorption, circular dichroism (CD) and magnetic circular dichroism (MCD) spectroscopy. The combination of these methods allows for the analysis of the spin and redox state of heme centers, the nature of heme axial ligands, as well as possible heme–heme and heme–ligand interactions. The MCD is particularly useful as it allows observing quantitatively separate signals of the low-spin heme *b* (b_{LS}) and the high-spin heme *b* (b_{HS}). The distinct MCD responses of the different hemes render it possible to quantitatively register the ligand interaction with heme b_{HS}. The validity of the MCD to the quantitative assay of the heme centers in cytochrome *bd* was demonstrated earlier with the *E. coli* enzyme [39].

2. Materials and methods

2.1. Chemicals

Carbon monoxide (CO) was from Sigma-Aldrich. DEAE-Toyopearl and hydroxyapatite were from Toso (Tokyo) and Bio-Rad (Hercules), respectively. *n*-Nonanoyl N-methylglucamide (MEGA9) and *n*-decanoyl N-methylglucamide (MEGA10) were from Doujin (Kumamoto). Other basic chemicals were from Sigma-Aldrich, Merck, and Fluka.

2.2. Cytochrome bd from G. thermodenitrificans

Recombinant cells of *G. thermodenitrificans* K1041 were constructed to overproduce cytochrome *bd* oxidase. The transformable strain K1041 was kindly given by Dr. I. Narumi [58]. An expression plasmid for this bacterium, pSTE12 [59], was used to construct the plasmids. The plasmids for overproduction of cytochrome *bd* were constructed as below. The *cbdAB* genes encoding *G. thermodenitrificans* cytochrome *bd* were excised from the plasmid pUC-BD [16], which includes the whole *cbdAB* operon and its authentic promoter region, by *Hin*dIII and *Eco*RI. The 3.3-kb fragment was ligated into pSTE12 vector digested by *Hin*dIII and *Eco*RI at the multi-cloning sites, and this plasmid was designated as pSTE-*cbdAB*. The plasmid pSTE-*cbdAB* was transferred into *G. thermodenitrificans* K1041 by electroporation (Bio-Rad Gene Pulser) as described previously [60]. Preculture in a test tube containing 2 × LB medium (2.0% w/v peptone, 1.0% w/v yeast extract, 1.0% w/v NaCl, pH 6.5) with 2 μg/ml tetracycline was carried out overnight at 48 °C. A 6-ml aliquot was inoculated to a 500 ml culture medium consisting of 2 × LB containing 2 μg/ml tetracycline in a baffled flask (1 l), and the culture was carried out under microaerobic growth conditions with moderate shaking (100 rpm) until stationary growth phase. Under these growth conditions, the expression of cytochrome *bd* was stimulated while that of cytochrome caa_3 was suppressed. In a previous work [16], about 0.06 mg of cytochrome *bd* per liter of culture was obtained from the K17 mutant strain. In this work, the value rose to about 0.8 mg/l. The amounts of cytochrome *d* in membrane preparations are increased much by the transformation, as estimated with **R**-*minus*-**Ox** difference spectra. The average values for K1041/pSTE12 control membranes and K1041/pSTE-*cbdAB* recombinant membranes are 0.0161 and 0.210 nmol/mg of protein, respectively.

Cells were then harvested and membranes were prepared as described previously [61]. Cytochrome *bd* was purified as described previously with some modifications [53]. In brief, the membrane fraction was treated with 2% (w/v) sodium cholate to remove peripheral membrane proteins and intrinsic membrane proteins were solubilized with 1% (w/v) of a 1:1 mixture of MEGA9 and MEGA10 (MEGA9 + 10). Cytochrome *bd* oxidase was purified with two-step column chromatography (DEAE-Toyopearl and hydroxyapatite) in the presence of 0.5% (w/v) MEGA9 + 10. Three lines of analyses indicate the absence of contaminants, such as cytochrome caa_3, in the recombinant cytochrome *bd* sample. Firstly, SDS-PAGE analysis indicates that the purified sample shows only three protein bands due to subunit I, subunit II and a small amount of combined I + II (Fig. 1). Subunits I and II are partially associated even in the presence of SDS, as reported for the wild-type cytochrome *bd* [53]. Secondly, no absorption signals due to heme A or C were detected in **R**-*minus*-**Ox** different spectra of pyridine hemochromogen, indicating that the purified sample contains no detectable cytochrome caa_3 oxidase or cytochrome *c*-551, as in the wild-type enzyme. Thirdly, the purified sample has a quinol oxidase activity (the k_{cat} value is 11.74 s^{-1}) comparable to that of the wild-type cytochrome *bd* (the value is 8.00 s^{-1}, reported in Ref. [16]), whereas it contains extremely low cytochrome *c* oxidase due to cytochrome caa_3, if any. Cytochrome caa_3 has a high *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) oxidase activity, the k_{cat} value is 336 s^{-1} as reported previously, while the value of the present purified cytochrome *bd* sample was 0.145 s^{-1}, indicating that the contaminant cytochrome caa_3 is less than 0.04%. In contract to cytochrome *bd* from *E. coli*, which shows a relatively high TMPD oxidase activity, cytochrome *bd* from *G. thermodenitrificans* does very low one, as reported for the wild-type enzyme [53].

An aspect about the necessity of *cydCD* or *cbdCD* genes is worth a comment. In the case of *C. glutamicum*, a high GC Gram-positive bacterium, the presence of *cydDC* genes is necessary in the expression plasmid for the over-expression of *cydAB*, as shown in an earlier report [62]. On the contrary, in *G. thermodenitrificans*, a low GC Gram-positive bacterium, it was not necessary for *cbdCD* genes to coexist with *cbdAB* in the same plasmid, as for *cydAB*/*cydCD* of *E. coli* (e.g. [63]). Even though the

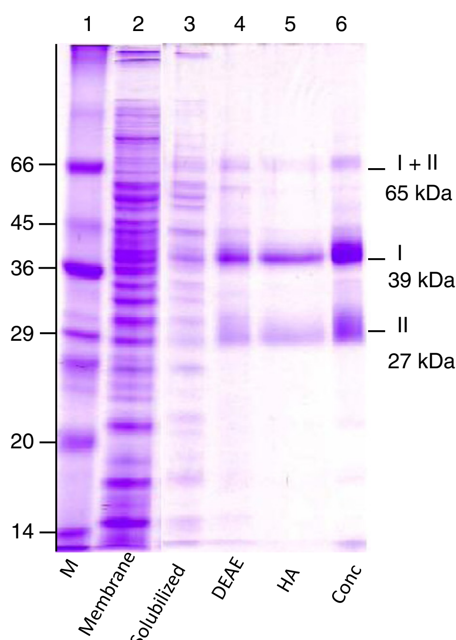

Fig. 1. SDS-PAGE analysis of the purified cytochrome *bd* from *G. thermodenitrificans*. Lane 1, standard proteins whose molecular weights are indicated in the left; lane 2, isolated membranes (35 μg); lane 3, proteins extracted with MEGA9 + 10 (10 μg); lane 4, the peak fractions from DEAE-Toyopearl column (5 μg); lane 5, the peak fractions from hydroxyapatite column (5 μg); lane 6, the purified sample concentrated with an ultrafilter (10 μg). The bars in the right indicate the positions of protein bands: I + II, associated subunits I and II; I, subunit I; II, subunit II.

cydCD/*cbdCD* genes might be essential for maturation of cytochrome *bd* in all of these organisms, their presence in the genomes is enough for *G. thermodenitrificans* and *E. coli*. The reason of this difference is not known, but may be related to the structural difference in the *cyd* operons; *cydCD* are located in the same operon with *cydAB* in the *C. glutamicum* genome, whereas their counterparts are far separated in the genomes of *G. thermodenitrificans* and *E. coli*.

2.3. Cytochrome bd from E. coli

The *E. coli* cytochrome *bd*, used for comparison, was isolated from strain GO 105/pTK1 as described in Refs. [64,65]. The strain lacks the bo_3-type oxidase and overexpresses cytochrome *bd* [32].

2.4. Heme analysis

The heme *b* contents of both enzymes were determined by the pyridine hemochromogen assay using the value of $\Delta\varepsilon_{556.5-540} = 23.98\ \text{mM}^{-1}\,\text{cm}^{-1}$ [66]. The amount of heme *b* extracted from cytochrome *bd* was compared to the heme *d* content. Since the pyridine hemochrome of heme *d* is unstable [67], the heme *d* content for *G. thermodenitrificans* and *E. coli* was determined from the dithionite-reduced-*minus*-'air-oxidized' difference absorption spectra using the values of $\Delta\varepsilon_{618-583} = 16\ \text{mM}^{-1}\,\text{cm}^{-1}$ and $\Delta\varepsilon_{628-607} = 10.8\ \text{mM}^{-1}\,\text{cm}^{-1}$ [39], respectively. Both values correspond to the value of $\Delta\varepsilon_{561-580} = 21\ \text{mM}^{-1}\,\text{cm}^{-1}$ [38] used to estimate the content of heme b_{LS} of each enzyme. Independently, the heme *d* content was measured by the method based on the intensity of the dithionite-reduced absolute CD spectrum in the 600–700-nm range [36,42] and the same result was obtained. The amounts of the high-spin and the low-spin hemes *b* were determined from the dithionite-reduced absolute MCD spectra [39].

2.5. Spectroscopy

Absorption spectra were recorded using an Aminco-SLM DW-2000 UV–vis spectrophotometer (SLM Instruments) upgraded by A.M.A. CD spectra were recorded with a Jobin Yvon Mark V auto-dichrograph upgraded by A.M.A. MCD spectra were monitored with a dichrograph equipped with a permanent magnet (effective magnetic field ~0.7 T for a 1 cm cell). For each MCD spectrum, the spectra recorded in the direct and reverse magnetic field directions were subtracted from each other to eliminate the CD contribution. The measurements were performed at room temperature in the incubation medium containing either 100 mM Na/phosphate buffer, 0.5 mM EDTA, pH 6.1, and 0.06% *n*-dodecyl-β-D-maltoside (for cytochrome *bd* from *G. thermodenitrificans*), or 100 mM Na/phosphate buffer, 0.5 mM EDTA, pH 7.0, and sodium *N*-lauroylsarcosinate (for cytochrome *bd* from *E. coli*).

2.6. Spectral analysis

Data processing and simulation of the spectra were performed with the aid of the Graphwork software developed by A.M.A.

3. Results and discussion

3.1. Absorption spectra

The absolute absorption spectrum of cytochrome *bd* from *G. thermodenitrificans* in the 'air-oxidized' (**Ox**) state shows a major band with a maximum at 412 nm (ε_{412}~308 $\text{mM}^{-1}\,\text{cm}^{-1}$) in the Soret region (Fig. 2A) and minor bands at ~530, 580, 640, and 710 nm in the visible (Fig. 2B). The Soret band position suggests that *b*-type hemes are in the ferric state. The 640-nm band is broader and much smaller in magnitude than its *E. coli* counterpart at 646 nm (inset in Fig. 2B) that points to the lack of significant contribution of oxy-ferrous heme *d* to the **Ox** state. This is at variance with cytochromes *bd* from *E. coli* and *A. vinelandii* in which oxy-ferrous species dominates in the 'air-oxidized' enzyme preparations [29,68–71]. The reason may be lower O_2 affinity of ferrous heme *d* from the *G. thermodenitrificans* enzyme as compared to the *E. coli* or *A. vinelandii* ones. The **Ox** state spectrum (Fig. 2B) does not show a band at about 680 nm characteristic of the ferryl heme *d* species in the *bd* enzymes from *E. coli* and *A. vinelandii* [8,68,72–74]. The 710-nm band may be assigned to a charge transfer band of the ferric high-spin heme *d*. Thus, one may assume that heme *d* is in the ferric (**Ox**) state.

The **R** state spectrum shows the two peculiar features. First, there is no peak at 595 nm characteristic of the heme b_{HS} Q_{00}-band (Fig. 2B). Second, there is a significant blue shift of the maximum of the ferrous heme *d* Q_y-band as compared to the *E. coli* one, 619 versus 630 nm (cf. the **R** spectra in the main panel and inset of Fig. 2B). This can also be well seen in the **R**-*minus*-**Ox** difference spectra (Fig. 2C, main panel and inset). At the same time, the maxima at 427, 530, and 562 nm typical of the B-, Q_n-, and Q_{00}-bands (i.e., "γ"-, "β"-, and "α"-bands) of heme b_{LS} respectively are present (Fig. 2A, B, C). The blue shift of the heme *d* Q_y-band might be explained by the differences of the heme environments between the two enzymes.

Addition of 1 mM CO to the **R** enzyme brings about a red shift of the heme *d* Q_y-band (Fig. 2B, D) pointing to the formation of the ferrous heme *d*-CO adduct. A concomitant increase in absorbance around 530 nm (Fig. 2D) is likely due to either the heme *d*-CO Q_{xn}- or Q_x-band (i.e., "β"- or "α"-band). As shown in Fig. 2D, CO-induced changes in the Soret region are different between the two enzymes. In cytochrome *bd* from *G. thermodenitrificans*, CO binds not only to heme *d*, but also to part of the high-spin heme *b* as evidenced by a maximum at 419 nm and a minimum at 436 nm in a difference spectrum [75]. This is not the case for the *E. coli* enzyme in which under the same conditions no significant binding of CO to the high-spin heme b_{HS} is observed [28,39,40,65].

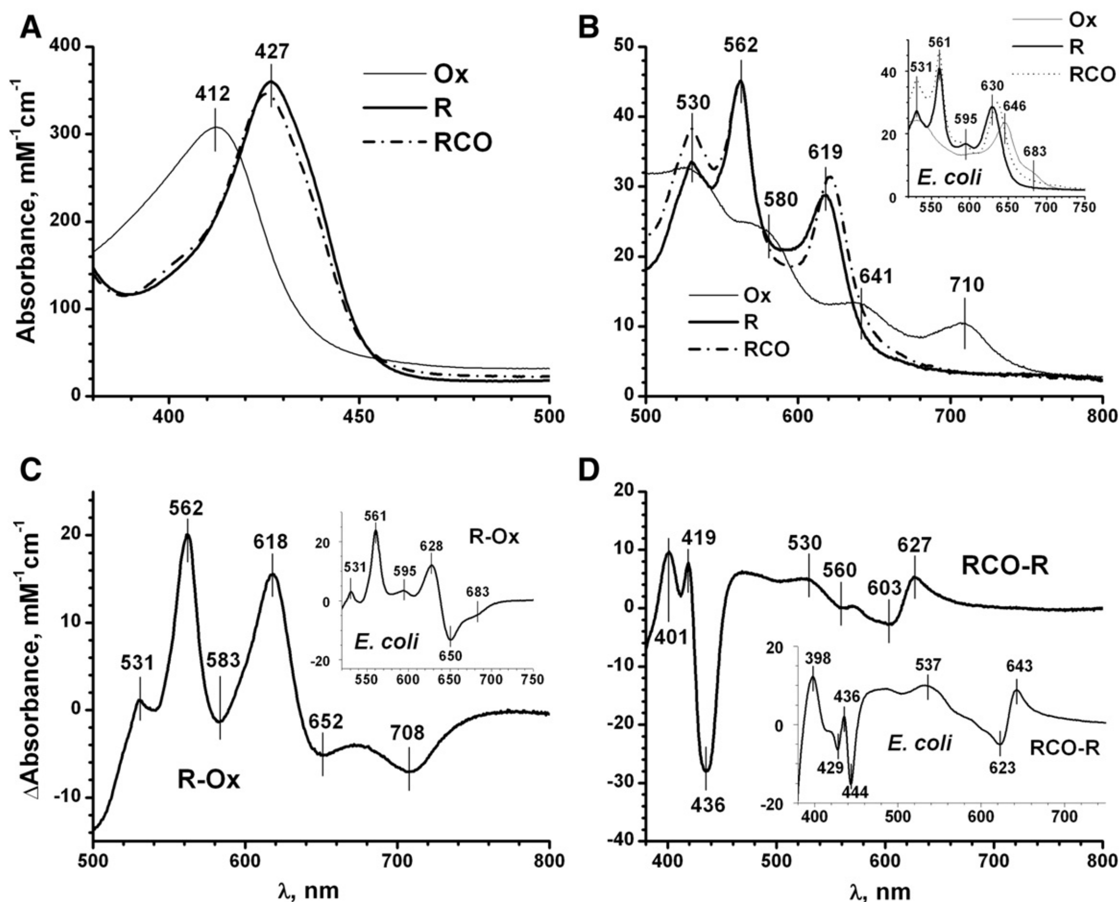

Fig. 2. Absorption spectra. *Main panels*: cytochrome *bd* from *G. thermodenitrificans* (3.6 μM), absolute spectra of the 'air-oxidized' (**Ox**), dithionite-reduced (**R**) and dithionite-reduced CO-bound (**RCO**) states in the Soret (A) and visible regions (B); difference spectra, **R**-*minus*-**Ox** (C), and **RCO**-*minus*-**R** (D). *Insets*: cytochrome *bd* from *E. coli* (4.8 μM), absolute spectra of **Ox**, **R**, and **RCO** states in the visible region (B); difference spectra, **R**-*minus*-**Ox** (C), and **RCO**-*minus*-**R** (D). CO, 1 mM. For conditions, see Materials and methods.

3.2. Heme stoichiometry

The pyridine hemochromogen analysis was performed and the amount of heme B extracted from the oxidase was compared to the specific content of heme *d*. The heme *d* content was measured by the two independent methods, as described in the Materials and methods section. The data show that, for the *G. thermodenitrificans* and *E. coli* enzymes, the heme b_{LS}/heme *d* ratio is 1.0, and the total heme *b*/heme *d* ratio is 2.0. The high-spin heme *b* (b_{HS}) is present in the *G. thermodenitrificans* cytochrome *bd*. Thus the *G. thermodenitrificans* oxidase carries three hemes, b_{LS}, b_{HS} and *d*, which are in a 1:1:1 stoichiometry per the enzyme complex.

3.3. MCD spectra

The MCD spectrum of the **Ox** enzyme is dominated by a derivative-shaped signal in the Soret region typical of a low-spin heme *b* with a maximum at 414 nm, a minimum at 429 nm, and the zero-crossing point at 421 nm (Fig. 3A). This signal is very similar to that in the *E. coli* oxidase [39] suggesting the presence of the low-spin ferric heme b_{LS} in the 'as-isolated' cytochrome *bd*. Other hemes are not expected to contribute much to this region. In the visible region (Fig. 3B), there are a number of small signals, a negative band at 576 nm being the most prominent, but they are difficult to interpret to date.

The MCD spectrum of the **R** enzyme shows an intense band characteristic of a low-spin hexacoordinate ferrous heme *b*: a derivative-shaped *A*-term signal centered at 562 nm with a maximum at 558 nm, a minimum at 566 nm accompanied by a well-resolved vibronic structure in the 500–550 nm region. (Fig. 3D). This signal can be definitely assigned to the Q_{00}-band of the low-spin ferrous heme b_{LS}. In addition, there are two signals typical of a high-spin pentacoordinate ferrous heme *b*: (i) an intense positive asymmetric signal in the Soret region with a maximum at 438 nm (Fig. 3C) and (ii) a small negative band at about 600 nm in the visible (Fig. 3D). The position and intensity of these signals are very similar to those in the *E. coli* enzyme ([39], see also Fig. 3F) that contains heme b_{HS}. Hence, the *G. thermodenitrificans* enzyme also contains the high-spin pentacoordinate heme *b*. The reason why the 595-nm band of the high-spin heme *b* (b_{HS}) is not seen in the absorption spectra may be (i) a short-wavelength shift of the heme *d* Q_y-band and (ii) broadening of the heme b_{LS} Q_{00}-band which thus overlaps with the weak heme b_{HS} Q_{00}-band.

Contributions of the ferrous hemes b_{LS} and *d* to the entire MCD Soret band are not expected to be high. It has to be noted that although the *A*-term signals of the two enzymes are similar, they are not identical; the peak-to-trough molar intensity of the *A*-term signal in *G. thermodenitrificans* is smaller than that in *E. coli*, 250 versus 400 M^{-1} cm^{-1} T^{-1} (Fig. 3F). This might suggest that there is less than one heme b_{LS} per the *G. thermodenitrificans* cytochrome *bd* complex. However, when the areas of these spectra were compared, they appeared to be virtually the same (not shown). The *G. thermodenitrificans* signal is broadened as compared to the *E. coli* one. Thus, in *G. thermodenitrificans*, there is one heme b_{LS} per the enzyme complex, in agreement with the absorption spectroscopy data.

The addition of 1 mM CO to the **R** enzyme causes marked changes in the MCD spectrum. In particular, the Soret signal becomes more narrow and smaller in magnitude (Fig. 3C), and the difference spectrum shows a maximum at 415 nm and a deep minimum at 430 nm, as well as a local minimum at 441 nm (Fig. 3E). Also, there are changes in the visible

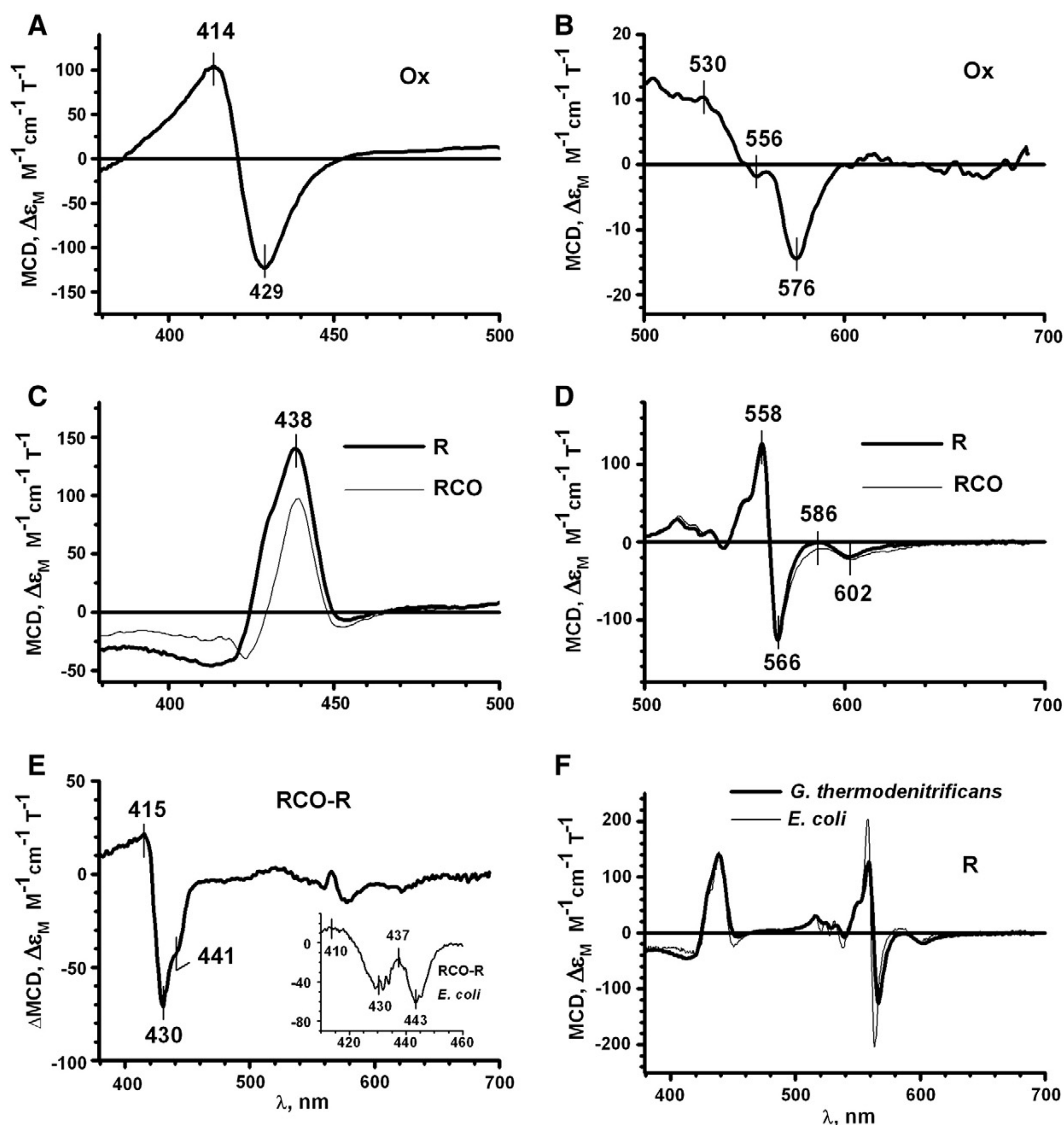

Fig. 3. MCD spectra. Cytochrome *bd* from *G. thermodenitrificans* as **Ox** (A, B); **R** and **RCO** (C, D). E: difference between **RCO** and **R** for the *bd* enzyme from *G. thermodenitrificans* (main panel) and *E. coli* (inset). F: comparison of spectra of cytochromes *bd* from *G. thermodenitrificans* and *E. coli* in the **R** state.

region. All these changes are clearly different from those observed in the *E. coli* enzyme (inset in Fig. 3E) and mainly reflect CO binding with a high-spin pentacoordinate heme *b*. At the same time, the ligand does not decrease to any substantial extent the *A*-term signal originated from heme b_{LS} (Fig. 3D). Modeling using the **RCO**-*minus*-**R** MCD spectrum of horseradish peroxidase shows that the MCD difference spectrum, shown in Fig. 3E, corresponds to the disappearance of about 20–25% of the high-spin unliganded ferrous heme b_{HS} with its concomitant conversion to the low-spin CO derivative (not shown). This is in contrast to the *bd*-enzymes from *E. coli* and *A. vinelandii* in which binding of CO at the same concentration, or other ligand, such as NO, O_2, cyanide, hydrogen peroxide, to heme b_{HS} appeared to be minor if any [7,23,25,28,39,65,73,74,76,77]. The difference in the CO binding under the same conditions might suggest longer center-to-center distance between heme *d* and heme b_{HS} in the *G. thermodenitrificans* enzyme as compared to the *E. coli* one.

3.4. CD spectra

The CD spectrum of the **Ox** enzyme is characterized by a small positive band at 586 nm in the visible, and an intense signal in the Soret with a minimum at 422 nm, a zero-crossing point at 409 nm and a maximum near 385 nm (Fig. 4A). The lack of the far-red CD band around 645 nm indicates clearly that the oxy-ferrous heme *d* species does not populate in the 'air-oxidized' state of cytochrome *bd* from *G. thermodenitrificans*, in agreement with the absorption spectroscopy data.

The CD spectrum of the **R** cytochrome *bd* in the far red region shows a maximum at 619 nm (Fig. 4B) that matches the position of the absorption peak of ferrous heme *d* (Fig. 2B). In the Soret region, there is a strong asymmetric signal with a prominent minimum at 440 nm, a zero-crossing point at 427 nm and a maximum at 413 nm (Fig. 4B). The CD signal is dominated by contribution from heme *d*, while the optical activities of heme b_{LS} and heme b_{HS} are likely to be low [36]. The

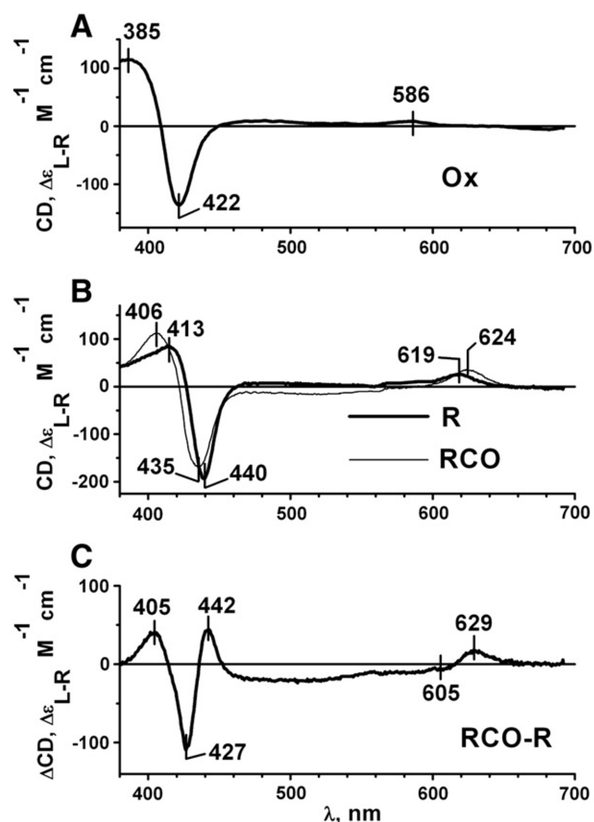

Fig. 4. CD spectra. Cytochrome *bd* from *G. thermodenitrificans* as **Ox** (A); **R** and **RCO** (B). C: difference between **RCO** and **R**.

high ellipticity of heme *d* is thought to be due to decreased symmetry of the chlorin macrocycle and strong splitting of the B_{00} and Q_{00} transitions into the *x* and *y* components [78,79]. In contrast, in the protohemes b_{LS} and b_{HS} those transitions are nearly degenerate [36]. The CD Soret spectrum of cytochrome *bd* from *G. thermodenitrificans* in the **R** state is very similar in line shape and position to the *E. coli* one [36], however its peak-to-trough intensity is substantially lower (~280 versus ~400 $M^{-1}\ cm^{-1}$, respectively). The difference can be due to significant attenuation of the excitonic interaction between heme *d* and heme b_{HS} in the *G. thermodenitrificans* oxidase as compared to the *E. coli* one. The reduced excitonic coupling in the *G. thermodenitrificans bd* may reflect a difference in the distance between heme *d* and heme b_{HS} and/or in the angle between their porphyrin planes for the two enzymes.

Addition of CO to the **R** enzyme causes a red shift of the far-red CD band, whereas the Soret CD spectrum shifts to the blue with concomitant changes in intensity of the extrema (Fig. 4B, C). The fact that the CO-induced spectrum in the Soret region, specifically, the magnitude and position of its intense negative band at 435 nm, is very similar to that of the E445A *E. coli* mutant in which the excitonic interaction is abolished (cf. the spectra from Fig. 4B of this work and Fig. 2A of Ref [36]) allows us to conclude that the CO binding to the *G. thermodenitrificans* enzyme also annuls such interaction.

3.5. Is histidine a sixth ligand to heme b_{LS}?

The MCD vibronic structure of a ferrous low-spin hexacoordinate heme can serve as a fingerprint for the heme iron axial ligation [80,81]. In particular, it allows one to distinguish between a His–Met and a His–His coordination [82]. Surprisingly, a detailed comparison of the well-resolved MCD vibronic structures for the **R** enzymes from *G. thermodenitrificans* and *E. coli* in the 500–550-nm region (i.e. the Q_n-band) shows the clear difference (Fig. 5A). The difference might only be due to broadening of the *A*-term MCD signal of ferrous heme b_{LS} in the *G. thermodenitrificans* cytochrome *bd*. However the modeling shows that at least the two-fold broadening would be required, whereas the band is broadened 1.2-fold maximally (not shown). Hence, the broadening is not sufficient to account for the difference. The low-spin hexacoordinate heme b_{LS} in cytochrome *bd* from *E. coli* is known to have a His–Met axial ligation [31,32]. Indeed, the line shape of its MCD vibronic structure is very similar to that of cytochrome *c* which is a typical hemoprotein with His–Met coordinated heme (Fig. 5B). On the contrary, the line shape of MCD vibronic structure of cytochrome *bd* from *G. thermodenitrificans* differs from those of the *E. coli* oxidase or cytochrome *c* but very close to that of cytochrome b_5 which has a bis-histidinyl axial coordination (Fig. 5B). One can therefore assume that heme b_{LS} in cytochrome *bd* from *G. thermodenitrificans* might have a His–His axial ligation. Although the 438-nm MCD signal dominated by the high-spin heme b_{HS} is very similar in the enzymes, the *E. coli* oxidase shows a small but detectable negative feature at 450 nm that reflects the contribution of a His–Met coordinated low-spin heme. This feature is clearly absent in the *G. thermodenitrificans* enzyme (Fig. 3F). This might be one more piece of evidence for a bis-histidinyl axial ligation of heme b_{LS} in the *G. thermodenitrificans bd*. Another feature related to a Met–His coordination of heme iron would be a small absorption band (charge transfer band) at 695 nm in the visible spectra of ferrihemes (although it is not always observed [83]). This band, however, is not seen even in the oxidized spectra of the *E. coli* enzyme since it is overlapped with the bands of hemes b_{HS} and *d* [31]. The amino acid sequence analysis of the *G. thermodenitrificans* cytochrome *bd* provides no clear answer. On the one hand, a conserved Met325 of subunit I could be the sixth ligand [16]. On the other hand, one of the histidines of subunit I, such as His319 that is conserved among *Bacillus* and *Geobacillus* species, might also be a candidate. An argument against the possibility of the existence of a bis-his ligation raised by one of the reviewers is that it would be really weird if two homologous enzymes (the *G. thermodenitrificans* one and, e.g., the *E. coli* one), would have distinct heme ligation having taken into account the huge effect

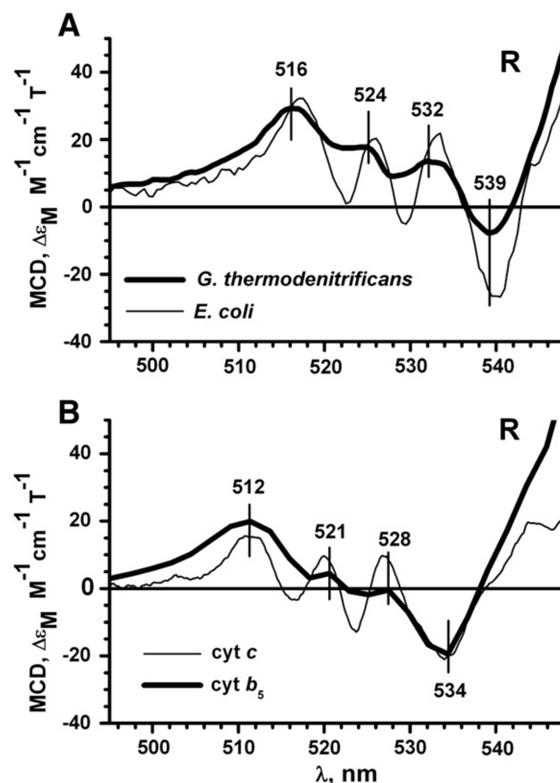

Fig. 5. Comparison of MCD vibronic structures. A: cytochromes *bd* from *G. thermodenitrificans* and *E. coli*. B: cytochromes *c* and b_5.

on the redox properties of a heme upon substitution of a heme axial ligand. Further work is required to address this issue.

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