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Purification and characterization of the succinate dehydrogenase complex and CO-reactive *b*-type cytochromes from the facultative alkaliphile *Bacillus firmus* OF4

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Abstract

The presence of a cytochrome *bo*-type terminal oxidase in *Bacillus firmus* OF4 had been suggested from the effects of CO on the spectra of reduced membrane cytochromes (Hicks, D.B., Plass, R.J. and Quirk, P.G. (1991) J. Bacteriol. 173, 5010–5016). In that study the CO-binding *b*-type cytochrome was partially purified by anion exchange chromatography. No further purification was attempted but later HPLC analysis indicated the absence of significant heme O in the *B. firmus* OF4 membranes. The current work shows that the partially purified cytochrome *b* is actually composed of three different *b*-type cytochromes which can be separated and purified by a combination of ion-exchange, hydroxyapatite and gel filtration chromatographies. Two of the cytochromes were CO-reactive but lacked the characteristic multisubunit composition of known terminal oxidases. Neither purified cytochrome catalyzed quinol or ferrocytochrome *c* oxidation. The more abundant CO-reactive *b*-type cytochrome (cytochrome b_{560}) had an apparent molecular mass of 10 kDa, whereas the other, more minor component (cytochrome b_{558}), was partially purified and showed two bands of 23 and 17 kDa on SDS-PAGE. The functions of the cytochromes b_{560} and b_{558} remain unknown but together they account for the spectrum originally attributed to cytochrome *bo*. The third, non-CO reactive, cytochrome *b* was associated with substantial succinate dehydrogenase activity and was purified as a three subunit succinate dehydrogenase complex with high specific activity (17.7 μmol/min/mg). Limited N-terminal sequence of each subunit demonstrated marked similarity to the complex from *Bacillus subtilis*. The cytochrome *b* of the alkaliphile enzyme was reduced about 50% by succinate compared to the level of reduction achieved by dithionite. The enzyme reacted with both napthoquinones and benzoquinones. The results presented indicate that *Bacillus firmus* OF4 contains a succinate dehydrogenase complex with very similar properties to the enzyme from *Bacillus subtilis*, but does not contain a cytochrome *o*-type terminal oxidase under the growth conditions studied.

Keywords: Alkaliphile; Cytochrome; Succinate dehydrogenase

1. Introduction

The respiratory chains of bacteria have been extensively studied due to both the multiplicity of respiratory complexes and their relative structural simplicity when compared to their mitochondrial counterparts [1]. The respiratory chains of facultative alkaliphilic bacteria are of further interest due to the ability of these organisms to grow at both near neutral pH (7.5) and at very alkaline pH (up to 11) [2]. Interest in the respiratory chain of facultative alkaliphiles results from several observations. One is the growth pH-dependent differences observed in the expression of particular cytochromes [3,4]. Another is the successful growth at high pH despite an apparently very low bulk Δp [2]. A third is that at the high pH region of growth, ATP synthesis in vitro can be coupled to respiration but cannot be energized by artificial membrane potentials, whereas at the neutral pH both modes of energization are successful [5].

The facultative alkaliphile *Bacillus firmus* OF4, like many other bacteria, appears to express more than one terminal oxidase. Spectral analysis of *B. firmus* OF4 membranes suggested the presence of at least three terminal oxidases, cytochrome caa_3, cytochrome *bd*, and cytochrome *bo* [6]. The cytochrome caa_3 has been purified and the structural genes cloned and sequenced [7]. North-

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ern analysis demonstrated that the mRNA levels for cytochrome caa_3 were 2- to 3-fold higher in cells grown at pH 10.5 than at pH 7.5, a result consistent with membrane redox spectra. Cytochrome *bd*, on the other hand, was found to be absent in exponentially growing cells at pH 10.5 according to both redox spectra and Western blots using heterologous antibodies, although present in stationary cells at both pH 10.5 and 7.5. The existence of cytochrome *bo* was inferred on the basis of the similarity of the reduced + CO minus reduced difference spectra in the Soret region of membranes to the *Eschericia coli* cytochrome *bo*. An *o*-type terminal oxidase had previously been purified from a different alkaliphile, *Bacillus* YN-2000 [8]. This oxidase was novel in that it contained hemes A, C and O (cytochrome aco_3). Interestingly, this cytochrome *o* appears to be the only terminal oxidase in YN-2000. Of particular interest regarding the putative *B. firmus* OF4 cytochrome *bo* was the finding that its concentration was somewhat higher in the highly alkaline grown cells compared to cells grown at pH 7.5 [9]. However, the apparent lack of heme O subsequently found by HPLC analysis of membranes [10] made the presence of a cytochrome *o* questionable and indicated a need for further study.

In order to further characterize the respiratory chain of the alkaliphilic bacterium, we attempted to purify the pH-regulated cytochrome that accounted for the *o*-like spectrum. Previous partial purification had identified a CO-binding cytochrome *b* from ion exchange chromatography. Results of the work presented here show that this cytochrome is actually composed of three different *b*-type cytochromes, two of which react with CO and one of which is associated with the succinate dehydrogenase complex. Although the CO-binding cytochromes have spectral properties similar to a cytochrome *o*, neither appears to be a terminal oxidase. They are both relatively small proteins and do not catalyze oxygen reduction. Purification and characterization of the third cytochrome *b* showed that *Bacillus firmus* OF4 contains a succinate dehydrogenase with similar properties to that from *Bacillus subtilis*.

2. Materials and methods

2.1. Growth of cells and preparation of membranes

Cells of *Bacillus firmus* OF4 were grown at pH 10.5 and membranes isolated essentially as described previously [11]. Membranes were washed in 50 mM Tris-Cl (pH 8), 1 mM EDTA, 5 mM *p*-aminobenzamidine, 0.2 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation, the membranes were resuspended to 10 mg/ml protein in 50 mM Tris-Cl (pH 8), 1 mM EDTA, 0.1 mM PMSF, 20% glycerol.

2.2. Purification of succinate dehydrogenase (SDH)

Membrane proteins of *Bacillus firmus* OF4 were extracted with dodecyl maltoside (DM, 1.5 g/g protein) at 4°C for 45 min with occasional mixing. After centrifugation (200 000 × *g* × 60 min), the supernatant was diluted with 1 volume of DEAE buffer A (50 mM Tris-Cl (pH 8) (4°C), 10% glycerol, 1 mM EDTA, 0.1 mM PMSF, 0.05% DM) and loaded onto a DEAE-Sepharose CL-6B column (2.6 cm × 20 cm) equilibrated in buffer A. After washing in buffer A, a 1000 ml gradient from 0 to 0.4 M NaCl in buffer A was applied. A red-colored fraction eluted from this gradient at 100 mM NaCl. Although apparently homogenous in terms of spectra, this peak was found to consist of two distinct fractions: an earlier non-SDH active, CO-binding component, and a later SDH-active component. The SDH-active fractions were pooled and applied directly to a hydroxyapatite (HPT) column (2.6 cm × 12 cm) equilibrated in buffer B (as DEAE buffer A except 10 mM NaP_i (pH 7.5) in place of 50 mM Tris-Cl). The column was washed in buffer B and then in the same buffer containing 200 mM NaP_i (pH 7.5). The SDH activity was eluted with 400 mM NaP_i in buffer B at room temperature. The active fractions were diluted to below 200 mM NaP_i and then concentrated at 4°C under N_2 via an amicon PM-30 membrane. The sample (1.5 ml) was applied to a Sephacryl S-300HR gel filtration column (1 cm × 90 cm) equilibrated in buffer A containing 100 mM NaCl. SDH active fractions from the gel filtration column were subjected to SDS-PAGE and pure fractions were pooled and stored at −80°C.

2.3. Purification of CO-binding components (b_{558} and b_{560})

The CO-binding fractions which eluted prior to the SDH activity from the ion-exchange column were applied to a hydroxyapatite column. The column dimensions and buffer components were as described for the SDH purification. The column was washed in buffer B and then successively in buffer B with P_i concentrations of 200 mM, 400 mM and 800 mM. Two *b*-type cytochromes were eluted from this column, both of which bound CO. At 400 mM NaP_i a CO-binding cytochrome *b* eluted which had a reduced a peak at 558 nm. At 800 mM NaP_i, the second, more abundant CO-binding cytochrome eluted, whose α peak in the reduced form was at 560 nm. The two cytochromes were diluted to below 200 mM NaP_i and concentrated under N_2 at 4°C via an amicon PM-30 membrane. The cytochromes were further purified by Sephacryl S-300HR chromatography in the same manner as described for the succinate dehydrogenase. Fractions from the gel filtration were assayed for absorbance at 413 nm and then subjected to SDS-PAGE.

2.4. Protein assays

Protein was estimated by the procedure of Lowry et al. [12] using bovine serum albumin as standard.

2.5. Enzyme activity measurements

Succinate dehydrogenase activity was measured at room temperature by following reduction of the artificial electron acceptor 2,6-dichloroindophenol (DCPIP) at 600 nm in the presence of phenazine methosulfate (PMS) as described previously [13]. The reaction mixture contained 50 mM Tricine NaOH (pH 8), 10 mM succinate. During early stages of purification assays were performed in the presence of 1 mM NaCN. Prior to assay, the enzyme was preincubated in the cuvette for 5 min with succinate. Assays were initiated by addition of PMS.

Succinate:quinone oxidoreductase activity was measured by monitoring spectral changes in the ultra-violet region associated with quinone reduction. The wavelengths and extinction coefficients used were those described by Lemma et al. [14]. The reactions were performed in a 3-ml cuvette containing 50 mM Tricine (pH 8), 10 mM succinate and 200 μM quinone, made anaerobic by bubbling with N_2 for 15 min. Assays were initiated by addition of the enzyme. Quinone stocks were made by dissolving the appropriate quinone in methanol. When indicated, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) was present at a final concentration of 10 μM.

Quinol oxidase assays were performed in a Clark-type oxygen electrode using the coupled assay described by Lemma et al. [15]. The 3 ml reaction vessel contained 50 mM sodium phosphate (pH 6.5), 1 mM EDTA, 0.05% dodecyl maltoside, 0.6 mg/ml DT-diaphorase (Sigma), 0.3 mM quinone and 0.6 mM NADH. After NADH addition the rate of auto-oxidation was measured and the reaction was then started by addition of the sample. The background auto-oxidation rate was subtracted from all reaction rates measured.

2.6. N-Terminal sequence and SDS-PAGE

Protein samples were analyzed by SDS-10% T polyacrylamide gels using the Tricine buffer system of Shagger and von Jagow [16]. Samples were incubated in sample buffer for 15 min at room temperature prior to electrophoresis. Gels were stained either with Coomassie G-250 [17] or with silver [18] as indicated. For N-terminal sequence, the protein samples were transferred to a PVDF membrane (Bio-Rad) and stained with Coomassie R-250, as described previously [8]. N-terminal sequencing was performed by the Mount Sinai School of Medicine Protein Core Laboratory using a Proton Instruments Model 2090E automated gas phase sequencer.

2.7. Visible spectroscopy

Spectra were performed using a Perkin-Elmer 557 dual-wavelength spectrophotometer. Scans were recorded at 120 nm/min with a 2 mm slit width. Samples were diluted in 100 mM NaP_i (pH 7.5), 0.05% DM and divided between two 1-ml cuvettes. Difference spectra were recorded 5 min after addition of reductants (succinate, 10 mM final; dithionite, a few grains) to the sample cuvette. CO spectra were obtained by gently bubbling the dithionite-reduced sample with CO for 2 min. Spectra were recorded until the full extent of the spectral change had been achieved (10–20 min). Quantitation of heme B was performed after pyridine hemochrome difference (reduced versus oxidized) spectra in 10% pyridine/0.1 M NaOH. An extinction co-efficient for heme B of 23.98 mM^{-1} cm^{-1} (557 nm minus 540 nm) was used [19].

3. Results and discussion

3.1. Protein purification

Previous analysis of the *B. firmus* OF4 terminal oxidases showed that they could be separated by ion exchange chromatography [6]. In that study, the cytochrome *bo* candidate eluted as a red colored fraction at low ionic strength. In this study we extracted the membrane proteins with dodecyl maltoside and then subjected the extract to ion exchange chromatography. As expected, a major red colored fraction eluted at low salt concentration. Careful analysis of this peak, however, indicated that it was not composed of a single component. The initial part of the

Table 1
Purification of *Bacillus firmus* OF4 succinate dehydrogenase

Purification stage	Total protein (mg)	Total activity (units) [a]	Specific activity (units/mg)	Yield (%)
1. Membranes	1232	428	0.4	100
2. Dodecyl maltoside	767.6	465	0.6	109
3. DEAE	62.0	352	5.7	82.2
4. Hydroxyapatite	15.2	222	14.6	52.0
5. Sephacryl S-300	6.2	109	17.7	25.5

A sample of succinate dehydrogenase at each stage of purification was analyzed for activity and protein. Activity was assayed at room temperature as described in Section 2.

[a] μmol DCPIP reduced/min.

peak contained the expected CO-binding cytochrome *b*. However, the main part of the peak contained a cytochrome *b* which was not CO-reactive and which was associated with substantial succinate dehydrogenase (SDH) activity. SDH activity had previously been measured in membranes of *Bacillus firmus* OF4 [10] but no further characterization was performed. In order to further analyze the respiratory chain of the alkaliphile, we pursued the purification of both the CO-reactive cytochrome *b* and the cytochrome *b* associated with the SDH activity.

3.2. Succinate dehydrogenase

After ion-exchange chromatography the succinate dehydrogenase was purified by hydroxyapatite and gel filtration chromatography. The specific activity increased with each step of the purification and the final product had a specific activity of 17.7 μmol/min/mg (Table 1), with an enrichment of over 50-fold. The enzyme is activated upon detergent solubilization and this results in the apparent increase in yield observed after extraction. This phenomenon was also observed for the SDH from *E. coli* [20] and *B. subtilis* [21]. The specific activity of the purified succinate dehydrogenase is similar to that of the enzyme from *B. subtilis* (22.5, [21]), *Desulfolobus elongatus* (11.2, [22]) and *E. coli* (31, [20]), but is lower than the bovine heart enzyme which has a specific activity of 50 μmol/min/mg at 38°C [23].

The protein profile of the SDH fractions at each stage of purification is shown in Fig. 1. The purified complex consists of three subunits with apparent molecular mass of 64.5, 28.5 and 16 kDa. A doublet band is occasionally seen below the largest subunit. This probably results from proteolysis of this subunit since samples left for a prolonged period at room temperature show increased levels

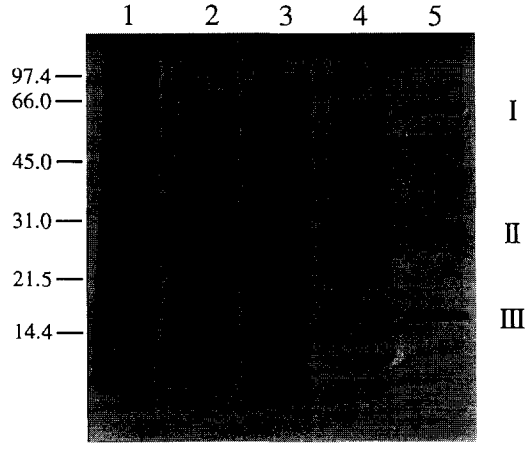

Fig. 1. SDS-10% T PAGE of succinate dehydrogenase purification. Samples of succinate dehydrogenase at each stage of purification were analyzed by SDS-PAGE. Lane 1, 50 μg membranes; lane 2, 50 μg dodecyl maltoside extract; lane 3, 20 μg DEAE fraction; lane 4, 10 μg hydroxyapatite fraction; lane 5, 5 μg Sephacryl S-300 fraction. The gel was stained with Coomassie G-250.

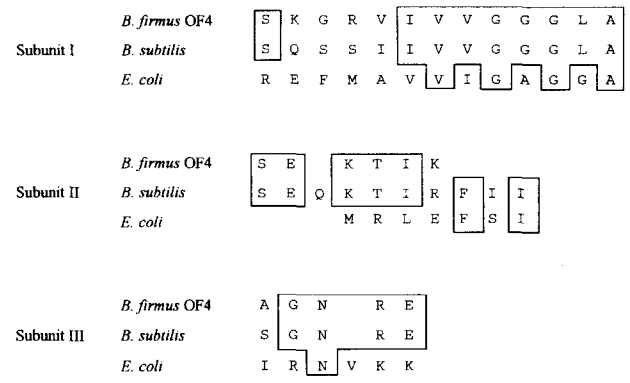

Fig. 2. N-Terminal sequence of selected succinate dehydrogenases. A comparison of the N-terminal sequence of *B. firmus* OF4 SDH with the sequence from *B. subtilis* [26,27] and *E. coli* [40,41] is shown.

of these bands. Proteolytic susceptibility of subunit I has also been observed for the SDH from *E. coli* [20]. The three subunit composition of the *B. firmus* OF4 enzyme resembles that from *B. subtilis* which contains three subunits of apparent molecular mass 65, 28, and 19 kDa [21]. The enzymes from bovine heart, *E. coli* and *Paracoccus denitrificans* are purified with an additional fourth subunit which runs close to that of the third on SDS-PAGE.

Succinate dehydrogenase is found to be highly conserved amongst the different organisms from which it has been studied [24]. An FAD molecule is generally associated with the largest subunit (flavoprotein (Fp) subunit). The intermediate subunit is thought to contain three Fe-S clusters (iron-containing (Ip) subunit) and the smaller subunit contains the B-heme, when present. The Fp and Ip subunits are water-soluble and are thought to be anchored to the membrane by the smaller hydrophobic subunit. Given the highly conserved nature of this respiratory complex we would anticipate a similar structure and distribution of redox centers in the alkaliphile enzyme. Limited N-terminal sequence of the SDH subunits indicated a high degree of similarity with the enzyme from *B. subtilis* (Fig. 2). The enzyme from this organism has been characterized both genetically and biochemically. The genes have been cloned and exist in an operon, *sdhCAB*, encoding the cytochrome *b*, Fp, and Ip subunits respectively in a 1:1:1 stoichiometry [25,26].

The visible spectrum of the purified *B. firmus* OF4 enzyme is shown in Fig. 3. Succinate reduces the heme B component of the enzyme by about 50% of the level achieved by dithionite. The dithionite reduced enzyme does not bind CO. The heme B content among SDH enzymes is known to vary and, as a result, its role in the enzyme mechanism remains unclear [24]. Based on protein estimates by the Lowry method [12], pyridine hemochrome spectra of the SDH indicated one heme B per complex. In *B. subtilis*, the same approach used above also indicated one heme B per complex [27]. However, subsequent quantitation of the 2Fe-2S signals in the epr spectrum indicated

that the conventional protein assays (Lowry and BCA) had overestimated the protein content several-fold [21]. Based on the epr experiments a heme B content of two per complex was concluded. Redox potentiometry indicated two heme components, one high potential and one low potential [22]. It is clear from the studies in *B. subtilis* that more detailed analyses of the *B. firmus* OF4 succinate dehydrogenase are required before making a definitive statement regarding the number of heme B equivalents in the enzyme. The SDH from *E. coli* contains only one heme B per complex and it is succinate-reducible [20]. The *P. denitrificans* enzyme also contains one heme B but is not reduced by succinate [28]. In contrast, the succinate dehydrogenase from the thermoacidophile *Sulfolobus acidocaldarius* contains no detectable heme B [29].

The reaction of the *B. firmus* OF4 enzyme with succinate occurred with a K_m of 300 μM. This value is higher than that found for the *E. coli* (71 μM [20]) and beef heart (20 μM [23]) enzymes, but lower than that for *S. acidocaldarius* (1.4 mM [29]). Malonate, a substrate analogue, acted as a competitive inhibitor of SDH activity with a K_i of 100 μM. This value is somewhat lower than that found for the *E. coli* enzyme (200 μM [19]), but both are substantially higher than the value of 0.25 μM obtained with the beef heart enzyme [22].

3.3. Succinate:quinone oxidoreductase activity

Complex II is associated with two distinct types of enzyme activity, succinate dehydrogenase and succinate:quinone oxidoreductase activity. The former activity can be performed by the water-soluble components of the complex alone (Fp and Ip). The latter activity which is more physiologically relevant, requires the additional presence of the small hydrophobic subunits.

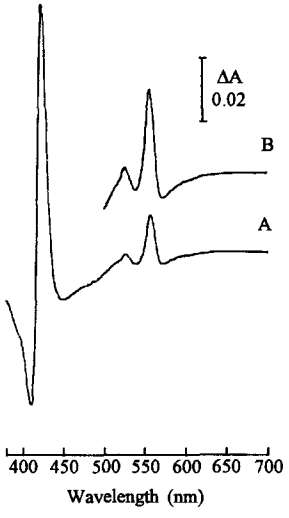

Fig. 3. Visible spectra of purified *B. firmus* OF4 succinate dehydrogenase. The visible spectrum of 150 μg/ml of SDH in 100 mM NaP_i (pH 7.5), 0.05% dodecyl maltoside was recorded at room temperature. Succinate-reduced minus oxidized spectrum (A) was obtained after addition of 10 mM succinate to the sample cuvette and incubation for 5 min. Dithionite (a few grains) was then added to the sample cuvette to obtain the dithionite-reduced minus oxidized spectrum (B).

Table 2
Succinate:quinone oxidoreductase activity

Acceptor	Specific activity [a]
1,4-Napthoquinone	0.8
1,4-Napthoquinone + HQNO	0.12
2-Methyl-1,4-napthoquinone (Menadione)	1.09
2,3-Dimethyl-1,4,-napthoquinone (DMN)	0
2,3-Dimethoxy-5-methyl-1,4-benzoquinone (Qo)	0.9
Qo + HQNO	0.13
2,3,5,6-Tetramethyl-1,4-benzoquinone (Duroquinone)	0.53

[a] μmol quinone reduced min^{-1} mg^{-1}.

The specificity of the succinate dehydrogenase for quinones was studied by assaying activity towards a number of commercially available hydrophilic quinone analogues (Table 2). The aerobic respiratory chains of mitochondria and gram-negative bacteria use ubiquinone (a benzoquinone) as the acceptor for electrons from SDH, whereas in *Bacillus* species, including alkaliphilic *Bacillus* [30], only menaquinone (a naphthoquinone) is found in the membranes [14]. No clear relationship was observed between SDH activity and structure of the quinone. Both naphthoquinones and benzoquinones were able to act as electron acceptors from the *B. firmus* OF4 SDH although the activities toward both were lower than those with PMS/DCPIP (Table 2). HQNO, an inhibitor of quinol requiring enzymes, inhibited the succinate:Q oxidoreductase activity about 85% when added to a final concentration of 10 μM. In *B. subtilis* a relationship was observed for the napthoquinones between increasing E_m and increasing activity [31]. A similar relationship was not apparent in the alkaliphile SDH. However like *B. subtilis*, DMN, the naphthoquinone most similar in structure and midpoint potential to menaquinone, was least active (Table 2).

3.4. CO-binding cytochromes b

The CO-reactive component from the ion-exchange column was fractionated into two separate CO-binding *b*-type cytochromes by hydroxyapatite chromatography. The earlier eluting fraction (400 mM NaP_i) had an α peak in the reduced form at 558 nm (cytochrome b_{558}). The later eluting (800 mM NaP_i), and more abundant fraction had a reduced α peak at 560 nm (cytochrome b_{560}). Both cytochromes were purified further by gel filtration chromatography. Both cytochromes were insensitive to staining by Coomassie G-250 and could only be observed after silver staining was used. The b_{560} on SDS-PAGE appeared to be composed of a major band of approx. 10 kDa and a minor band of about 32 kDa (Fig. 4). Analysis of a number of different preparations in addition to the absorbance

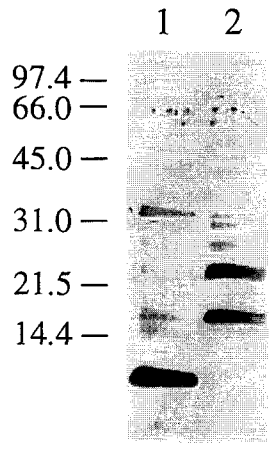

Fig. 4. SDS-10% T PAGE of purified CO-reactive *b*-type cytochromes. 0.5 mg of purified cytochrome b_{560} (lane 1) and cytochrome b_{558} (lane 2) were applied to SDS-PAGE. After electrophoresis, the proteins were visualized by silver staining.

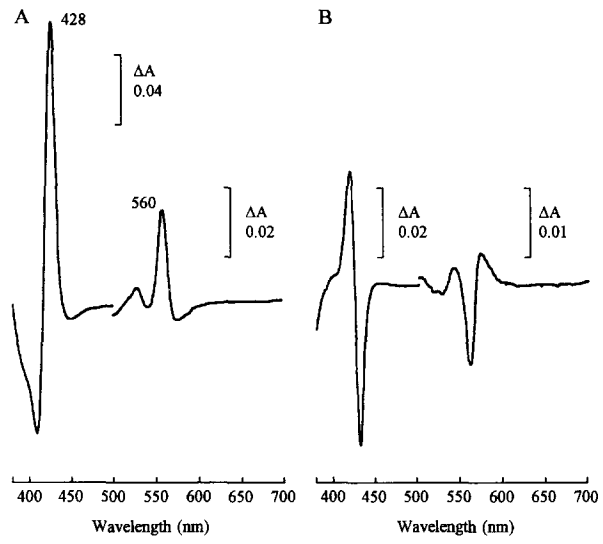

Fig. 5. Visible spectra of purified cytochrome b_{560}. The visible spectrum of 60 μg/ml of cytochrome b_{560} in 100 mM NaPi (pH 7.5), 0.05% dodecyl maltoside was recorded at room temperature. (A) Dithionite reduced minus oxidized. (B) Dithionite + CO minus Dithionite. CO spectra were obtained as described in Section 2.

profile from gel filtration indicates that the cytochrome is associated with the band at 10 kDa. The cytochrome b_{558} preparation was resolved into two polypeptides of 23 and 17 kDa. The low level of cytochrome b_{558} prevented further analysis and it was not possible to determine which polypeptide is associated with the cytochrome. Further attempts at purification including HPLC and repeat gel filtration did not result in increased purity. The results on SDS-PAGE suggested that neither protein was a member of the cytochrome *o* type terminal oxidase family. The cytochrome *bo* of *E. coli* is a member of the heme copper family of terminal oxidases and consists of five subunits ranging in size from 58 to 17 kDa [32]. The largest subunit (I) contains the binuclear center where O_2 is bound and reduced. Since neither cytochrome b_{558} or b_{560} contains a polypeptide similar in size to subunit I they are unlikely to be *bo*-type terminal oxidases. In fact, neither cytochrome showed quinol or cytochrome oxidase activity. Recent heme analysis of *B. firmus* OF4 indicated that no heme O was present in membranes [10]. From data presented above in conjunction with the recent heme analysis, we conclude that *B. firmus* OF4 does not contain a cytochrome *bo* under the growth conditions used. It is still possible that a cytochrome *bo* could be expressed under different growth conditions or after deletion of the other terminal oxidases. Recent mutagenesis work in *P. denitrificans* has shown that certain terminal oxidases are not expressed until the genes for all other terminal oxidases have been deleted. [33,34].

The visible spectra of b_{560} and b_{558} are shown in Figs. 5 and 6 respectively. The cytochromes appear to be very similar, but they are distinct in the position of the α band and in the Soret/α ratio (b_{560}, 2.9; b_{558}, 3.2). As shown in Fig. 5B and Fig. 6B, both cytochromes react with CO, although the effect on the spectrum appears somewhat more pronounced on the cytochrome b_{560}.

Recently a number of CO-binding cytochromes *b* have been isolated from bacterial membranes including 18 kDa proteins from *B. subtilis* [35] and *Thermoplasma acidophilum* [36] and a 30 kDa protein from *S. acidocaldarius* [37]. None of these cytochromes has quinol oxidase activity, although the proteins from *T. acidophilum* and *S. acidocaldarius* were found to contain copper. The function

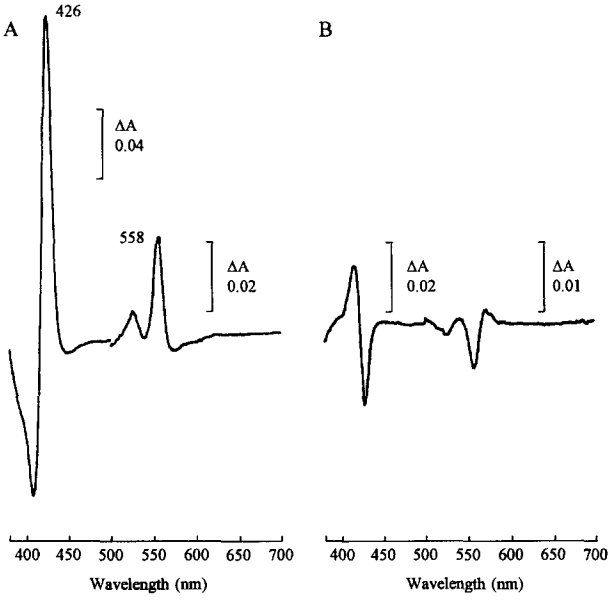

Fig. 6. Visible spectra of purified cytochrome b_{558}. The visible spectrum of 60 μg/ml of cytochrome b_{558} in 100 mM NaPi (pH 7.5), 0.05% dodecyl maltoside was recorded at room temperature. (A) Dithionite reduced minus oxidized. (B) Dithionite + CO minus Dithionite. CO spectra were obtained as described in Section 2.

of these proteins is unknown. The only small CO-binding cytochrome *b* for which functional information is available is the product of the *ctaA* gene in *B. subtilis*. Overexpression of this gene results in the presence of a CO-reactive cytochrome *b* in membranes. The purified protein (cytochrome b_{558}) runs at approx. 23 kDa on SDS-PAGE and, although spectra were not shown, reacts with carbon monoxide [38]. The *ctaA* and *ctaB* genes are required for heme A synthesis [39].

4. Conclusions

We have isolated three *b*-type cytochromes from alkaliphilic *Bacillus firmus* OF4. One of these is a succinate dehydrogenase and appears to be similar to the well-characterized enzyme from *B. subtilis*. Previously, enzyme assays of everted membrane vesicles from cells grown at pH 7.5 and 10.5 indicated that the SDH activity did not change with growth pH. Thus the SDH of *B. firmus* OF4, like NADH dehydrogenase and the F_1F_o-ATP synthase, is apparently not subject to growth pH regulation. On the other hand, quantitation of the CO difference spectrum of membranes did indicate that a cytochrome *b* component or components increased with growth pH. This signal, originally attributed to cytochrome *bo*, is shown here to be due to two low molecular weight cytochromes *b* devoid of terminal oxidase activity. The function of these CO-binding cytochromes has yet to be established.

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