High-yield purification of cytochrome *aa*₃ and cytochrome *caa*₃ oxidases from *Bacillus subtilis* plasma membranes

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When grown in aerated shaking culture, *Bacillus subtilis* expresses two different haem A-containing terminal oxidases: cytochrome *aa*₃-quinol oxidase and cytochrome *caa*₃ oxidase. This paper describes a high-yield conventional procedure for purifying the two haem A-containing oxidases from the same aerobic culture of *Bacillus subtilis*. Yields of close to 40% of the total haem A are achieved and about 6 mg of each of the purified oxidases is obtained from 4 litres of liquid culture. Both of the purified

enzymes have two subunits, with apparent molecular masses of 71.6 kDa and 34.3 kDa for the cytochrome *caa*₃ oxidase, and 67.6 kDa and 37.2 kDa for *aa*₃-quinol oxidase. These features are in agreement with the sequence data for the corresponding structural genes in the *aa*₃ and *caa*₃ operons of *B. subtilis*. Some spectral and enzymic features of the two purified oxidases are reported that are consistent with the inclusion of both of these enzymes as members of the cytochrome oxidase superfamily.

INTRODUCTION

The cytochrome oxidase superfamily of enzymes encompasses a structurally diverse group of membrane protein complexes that range from the mammalian mitochondrial enzyme, which consists of 13 different polypeptides present in a 1:1 non-covalently associated complex, to the four-subunit cytochrome *bo*-ubiquinol oxidase of *Escherichia coli* [1,2]. One of the unifying themes of this family is the high sequence similarity found for the two core subunits which contain the prosthetic groups. *Bacillus subtilis* expresses two different members of this family, a cytochrome *caa*₃ oxidase and a cytochrome *aa*₃-quinol oxidase, and these have both been characterized at the gene level. The main distinguishing feature of the *caa*₃ oxidase is that it has a cytochrome *c* domain fused to subunit II, which is one of the highly conserved subunits found in other members of this family [3]. In contrast, the *aa*₃-oxidase does not oxidize cytochrome *c*, does not possess the highly conserved residues involved in ligation of the Cu_A centre found in other members of this family, and is proposed to be a quinol oxidase [4]. The DNA sequence of the *caa*₃ enzyme includes five closely spaced genes which encode four subunits and one putative assembly factor. The gene sequence of the cytochrome *aa*₃-quinol oxidase includes four genes which are thought to encode a four-subunit enzyme.

Purification of cytochrome *c* oxidase from *Bacillus subtilis*, reported originally by de Vrij et al. [5], describes an *aa*₃-type oxidase that can oxidize cytochrome *c*. However, subsequent genetic [3,4] and biochemical [6,7] studies have demonstrated that the cytochrome *c*-oxidizing activity of *Bacillus subtilis* plasma membranes is associated with the cytochrome *caa*₃ oxidase. The enzyme with the spectral features reported by de Vrij et al. [5] resembles more closely the cytochrome *aa*₃-quinol oxidase. A new procedure for the purification of both *aa*₃ and *caa*₃ oxidases from *Bacillus subtilis* has been reported, but details regarding the yield and purity of the products were not given [6]. Therefore we describe in this paper a procedure based on conventional low-pressure chromatography that gives a high yield of both haem A-

containing oxidases. The *aa*₃ and *caa*₃ oxidases have substrate specificities which classify the *aa*₃ enzyme as a quinol oxidase and the *caa*₃ enzyme as a cytochrome *c* oxidase, in agreement with the conclusion of Lauraeus et al. [6]. We believe that this purification protocol will be useful to groups that require a substantial amount of protein for studying the bioenergetic and electron transfer mechanisms of this family of enzymes.

EXPERIMENTAL

Plasma membranes were prepared from *Bacillus subtilis* grown on a super-rich medium containing 2.5% Bacto-tryptone (all concentrations w/v, unless otherwise noted), 2.0% yeast extract, 0.3% K₂HPO₄ and 3.0% succinate at pH 7.5. This medium was supplemented with 300 µl/l of a micronutrient solution containing 2.2% MnCl₂, 0.5% ZnSO₄·7H₂O, 0.5% H₃BO₃, 0.016% CuSO₄·5H₂O, 0.025% Na₂MoO₄·2H₂O, 0.46% Co(NO₃)₂·6H₂O and 0.5% (v/v) conc. H₂SO₄. The cultures were grown in 1 litre of medium in 2-litre Fernbach Flasks. The 1 litre cultures were inoculated with 25 ml starter cultures prepared from *Bacillus subtilis* DB 104 (courtesy of Dr. S.-L. Wong) freshly grown on plates of tryptose blood agar. The cultures were grown for 18 h at 37 °C in an orbital shaker at 250 rev./min. The cells were harvested by centrifugation for 5 min at 2600 g in a GS-3 rotor (Sorvall).

The following description is for 40 g wet weight of cells, obtained from 4 l of cell culture. The cells were washed by suspending in a volume of 600 ml of 50 mM KH₂PO₄, and then centrifuged at 2600 g in a GS-3 (Sorvall) rotor for 10 min. The washed cells were resuspended in 600 ml of lysing solution (50 mM Tris, 1 mM EDTA, 0.375 mg/ml lysozyme, 0.025 mg/ml RNase and 0.025 mg/ml DNase at pH 7.8) prior to addition of 1.2 g of anhydrous MgCl₂. The mixture was incubated at 45 °C while shaking at 125 rev./min for 45 min. Following this incubation, 56 ml of 250 mM EDTA, pH 7, was added to the

lysing solution and this was allowed to incubate for a further 3 min prior to the addition of 6.4 g of MgSO_4 . This was incubated for a further 15 min at 45 °C while shaking at 125 rev./min. The mixture was centrifuged for 45 min at 8600 *g* in a GS-3 rotor. The resulting pellet was homogenized in 320 ml of 50 mM Tris and 1 mM EDTA at pH 7.8 and the suspension was centrifuged for 45 min at 25200 *g* in an SS-34 rotor (Sorvall). The pellet was retained as the membrane preparation.

The membrane pellet was initially homogenized in 120 ml of 50 mM Tris/HCl, 1 mM EDTA, 0.1 M NaCl and 1 % Tween-80 at pH 7.8. This homogenate was stirred at 4 °C for 2 h and then centrifuged at 25200 *g* for 30 min. The supernatant was discarded and the pellet was homogenized in 150 ml of buffer containing 5 % (v/v) Triton X-100, 50 mM Tris, 1 mM EDTA and 0.1 M NaCl at pH 7.8. The homogenate was incubated for 18 h with stirring at 4 °C and then centrifuged at 25200 *g* for 45 min to give a grey pellet and a reddish-brown supernatant. Further treatment of the pellet under identical conditions as described above produced no further extraction of cytochromes and about 5 % of the total protein extracted in the first Triton incubation.

The Triton X-100 extract was dialysed overnight at 4 °C against 2 l of 10 mM sodium phosphate buffer, pH 7.0, with 0.1 % Triton X-100. The sample was then loaded at a flow rate of 2 ml/min on to a Bio-Gel HTP hydroxyapatite column (2.5 cm × 35 cm) previously equilibrated with 10 mM sodium phosphate, pH 7.0, with 1 % Triton X-100. The column was eluted with 400 mM sodium phosphate, pH 7.0, with 1 % Triton X-100. Both haem A-containing oxidases eluted together and these fractions were retained for further purification.

The haem A-containing fractions from the hydroxyapatite column were pooled and dialysed overnight at 4 °C against 10 mM sodium phosphate, pH 7.0, with 0.1 % Triton X-100. The sample was loaded at 2 ml/min on to an S-Sepharose fast flow column (2.5 cm × 25 cm) equilibrated with 10 mM sodium phosphate, pH 7.0, with 1 % Triton X-100. The column was eluted with 10 mM sodium phosphate, 1 % Triton X-100 and 1 M NaCl, pH 7.0. The cytochrome *caa*₃ and cytochrome *aa*₃ oxidases were eluted separately from this column.

The *aa*₃ and *caa*₃ oxidases were both dialysed overnight at 4 °C against 50 mM Tris/HCl, pH 7.8, with 0.1 % Triton X-100. Each sample was then applied to a Q-Sepharose Fast Flow column (1.5 cm × 17 cm) equilibrated with 50 mM Tris/HCl, pH 7.8, containing 0.1 % Triton X-100. The column was eluted at 2 ml/min with 50 mM Tris/HCl, pH 7.8, with 1 % Triton X-100 and 1 M NaCl.

Cytochrome *c* oxidase from beef heart muscle mitochondria was prepared by the method of Kuboyama et al. [8], except that cholate was used throughout the entire purification. The purified oxidase was dissolved in phosphate buffer with 1 mg of lauryl maltoside per ml. The haem to protein ratio for the mitochondrial oxidase was 9.5 nmol of haem A per mg of protein.

Ascorbic acid, EDTA, sodium dithionite, KCN and NaCl were from BDH, Edmonton, Alberta, Canada. Tris, lysozyme (grade I from chicken egg white), deoxyribonuclease I, *NNN'*-tetramethyl-*p*-phenylenediamine (TMPD), Triton X-100, succinic acid, bichinchoninic acid, tetrachlorohydroquinone (TCHQ), asolectin and horse heart cytochrome *c* were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Bio-Gel HTP hydroxyapatite was from Bio-Rad Laboratories Ltd, Mississauga, ON, Canada. S-Sepharose Fast-Flow and Q-Sepharose Fast Flow resins were from Pharmacia, Baie d'Urfe, PQ, Canada.

Spectroscopic and kinetic measurements were made on either a Shimadzu UV-160 spectrophotometer or a Hewlett-Packard 8452A Diode Array spectrophotometer in a 1 cm cuvette at

20 °C. Molar absorption coefficients for the *B. subtilis* oxidases were determined from pyridine haemochrome spectra [9]. The reduced-minus-oxidized molar absorption coefficients obtained were: for cytochrome *c* (in the *caa*₃ oxidase), 30 mM⁻¹·cm⁻¹ at 550/540 nm; for cytochrome *aa*₃ (in the *caa*₃ oxidase), 26 mM⁻¹·cm⁻¹ at 604/630 nm; and for the *aa*₃-quinol oxidase, 28 mM⁻¹·cm⁻¹ at 600/630 nm. A value of 27 mM⁻¹·cm⁻¹ for the wavelength pair 604/630 nm was used for mitochondrial cytochrome *c* oxidase [10]. Protein concentrations were determined using a bichinchoninic acid assay [11].

Oxygen electrode measurements were made with a Yellow Springs O₂ electrode and Model 5300 Biological Oxygen Monitor. The assay buffer was 25 mM sodium phosphate, pH 7.0, with 0.5 mg/ml lauryl maltoside and 0.1 % asolectin. The two purified enzymes were diluted 5-fold into the assay buffer, containing 1.0 % asolectin, 15 min prior to the activity measurement. TCHQ was made up as a 50 mM stock solution in ethanol. Activity measurements were made by adding 5 mM sodium ascorbate followed by addition of either TMPD or TCHQ and allowing the assay to run for a few minutes to establish a background rate of oxygen consumption prior to addition of enzyme. Horse heart cytochrome *c* was added after a few minutes to test its effect on oxygen uptake.

The subunit composition was determined by electrophoresis in discontinuous polyacrylamide slab gels run in the presence of 1 % SDS. The gel system used was essentially the same as described by Robinson et al. [12]. Samples were incubated overnight in 2 % SDS in Tris/phosphate buffer, pH 6.8, with 10 mM dithiothreitol. The gels were stained with Coomassie Blue R-250 and scanned using a densitometer from E-C Apparatus Corp. Molecular mass markers were from Amersham Canada Ltd., Oakville, ON, Canada.

RESULTS

Table 1 summarizes a typical preparation of the *caa*₃ and *aa*₃ oxidases. The preparation began with 586 nmol of haem A and 359 mg of total membrane protein, giving an initial haem A/protein ratio of 1.57 nmol/mg. The two haem A oxidases are not resolved by the hydroxyapatite chromatography step but are resolved on the S-Sepharose column. The final Q-Sepharose step gave a yield of 60 % of the haem A applied to the column, resulting in an overall yield of 35 % in this preparation. In three replications of this procedure the average yield of haem A was 36 %. The final haem A to protein ratios of the cytochrome *caa*₃ oxidase and the cytochrome *aa*₃ oxidase were 18.4 and 14.8 nmol of haem A/mg of protein respectively, representing about 20-fold purification above the membrane extract for each enzyme.

Table 1 Purification of *caa*₃-type and *aa*₃-type oxidases from *B. subtilis*

Step	Haem A (nmol)	Protein (mg)	Yield (%)		Purification (-fold)
			Haem	Protein	
Membrane extract	586	359	100	100	1.0
Hydroxyapatite	461	143	82	40	2.1
S-Sepharose					
<i>caa</i> ₃	177	13.2	32	3.7	9.1
<i>aa</i> ₃	156	17.2	28	4.8	5.8
Q-Sepharose					
<i>caa</i> ₃	101	5.5	18	1.5	12.5
<i>aa</i> ₃	98.8	6.7	17.5	1.9	9.6

Figure 1 Densitometric scans of purified cytochrome *caa*₃ and *aa*₃ oxidases

Trace a is a scan of the standards, and their molecular masses (kDa) are indicated along the top of the scan. Trace b is a scan of the *caa*₃ oxidase and trace c of the *aa*₃ oxidase. A 12 μ g sample of protein was loaded for each of the oxidase samples. The conditions of electrophoresis are described in the Materials and methods section. The molecular masses of the oxidase subunits were estimated by comparison with the molecular mass standards on a plot of log molecular mass versus relative mobility.

Figure 2 UV-visible difference spectra of purified oxidases from *Bacillus subtilis*

Cytochrome *caa*₃ oxidase (—) is at a concentration of 4.65 μ M, and cytochrome *aa*₃ oxidase (---) is at 2.25 μ M. The reduced state was generated by addition of 5 mM sodium ascorbate plus 200 μ M TMPD to samples made anaerobic by equilibration with N₂. There was no further change upon addition of 10 mM sodium dithionite to the ascorbate/TMPD-reduced samples. The reference in each case is the air-oxidized state.

Densitometric scans of the two purified oxidases are compared with a set of molecular mass standards in Figure 1. Both enzymes contain two major subunits, and the apparent molecular masses are 71.6 and 34.3 kDa for the *caa*₃ oxidase, and 67.6 and 37.2 kDa for the *aa*₃ oxidase. The molecular masses indicate that the two purified enzymes are composed of the two largest subunits specified by their respective operons [3,4].

Figure 2 shows the UV/visible, reduced-minus-oxidized difference spectra for the two purified enzymes. The *caa*₃ oxidase

Figure 3 Comparison of visible and near-IR spectral regions for *aa*₃-quinol oxidase, *caa*₃ oxidase and mitochondrial cytochrome *c* oxidase

(a) Spectral region from 400 to 700 nm; (b) spectral region from 600 to 900 nm. The traces are designated as: —, *aa*₃-quinol oxidase at 9.5 μ M; ---, cytochrome *caa*₃ at 18 μ M; ····, mitochondrial cytochrome *c* oxidase at 9.5 μ M.

has peaks in the visible region at 550 and 603 nm and in the Soret region at 416 and 443 nm, due to cytochrome *c* and cytochrome *aa*₃ respectively. In our preparations the ratio of cytochrome *c* to *aa*₃ varied from 0.9 to 1.1. The quinol oxidase shows absorbance at 600 nm and 442 nm due to the haem of cytochrome *aa*₃. The spectra of both oxidases show that the preparations are free from other haem contamination.

A closer look at the absorbance difference spectra in the visible region for the purified oxidases from *B. subtilis* is given in Figure 3(a), where they are compared with cytochrome *c* oxidase from beef heart mitochondria. The position of the α -band maximum is shifted to 600 nm for the *aa*₃-quinol oxidase, whereas the *caa*₃ oxidase is at 604 nm, close to the maximum of mitochondrial cytochrome *c* oxidase. The beef heart oxidase has a broad absorbance centred at 830 nm that disappears upon reduction and hence appears as a trough in this representation (Figure 3). In the *aa*₃-quinol oxidase this band is absent, whereas in the *caa*₃ oxidase there is a transition in this region centred at 790 nm. In addition, both *Bacillus* enzymes show an absorbance trough

centred at 650 nm which is equal in intensity to that seen for the mitochondrial oxidase.

Both of the enzymes from *B. subtilis* are reducible with the artificial substrate ascorbate plus TMPD (see Figure 3). However, the cytochrome *aa*₃ oxidase shows only feeble turnover with TMPD, whereas the cytochrome *caa*₃ oxidase reaches a maximal turnover of greater than 220 s⁻¹ with a *K*_m for TMPD of 2 mM. The activity of the *caa*₃ oxidase was not stimulated by addition of horse heart cytochrome *c*. The *aa*₃ oxidase exhibited a maximal turnover of 45 s⁻¹ with a *K*_m of 1 mM for the quinol analogue TCHQ as substrate.

DISCUSSION

This paper describes a conventional purification protocol for preparing high yields of two haem A-containing oxidases of *Bacillus subtilis*. We think that this procedure will be useful for studying the biophysical and biochemical properties of these two members of the cytochrome oxidase family, because such studies often require large amounts of enzyme. It should be possible to isolate 25 mg of each of these enzymes from just 16 l of liquid culture using the procedure we have described. The procedure reported previously [6] relied largely on ion-exchange chromatography on Mono-Q beads using an FPLC system for purification. At low ionic strength the *aa*₃ oxidase binds to Mono-Q, whereas the *caa*₃ oxidase does not bind [6]. However, we have obtained very poor yields of protein from the Mono-Q matrix (i.e. < 5% of haem A from the original extract), making it an impractical step for efficient purification to obtain the amounts of protein required for biophysical studies.

Both the *aa*₃-quinol and *caa*₃ oxidases are purified in our procedure as two-subunit proteins. However, the gene structure shows four open reading frames for each of these enzymes [3,4] and so our purification procedure may result in the loss of genuine subunits from the membrane complex. Such a result has been seen previously for other members of the cytochrome oxidase family. For example, the *aa*₃-type cytochrome *c* oxidase from *Paracoccus denitrificans* [13,14] and the cytochrome *bo*-oxidase from *E. coli* [15,16] have been purified in forms with differing subunit contents. We are currently exploring the use of different detergents during our purification procedure to see if we can purify these complexes with more than two subunits, but preliminary studies with lauryl maltoside yielded enzymes with the same two-subunit structure we have seen when using Triton X-100 (B. C. Hill and J. Albanese, unpublished work).

Spectroscopic features of the two haem A oxidases which were observed in membrane extracts of *B. subtilis* [7] are retained in the two-subunit products of this isolation protocol. For example, the position of the α -band maximum at 600 nm in the quinol oxidase and the Soret/visible absorption ratios for both enzymes are features that are also seen in the membrane extract. This indicates that the local environment around the prosthetic groups is retained in the two-subunit oxidases as isolated from *B. subtilis* and that these preparations are useful for structural and functional studies.

The most striking spectral feature of the *aa*₃-quinol oxidase is the lack of an absorption band in the near-IR region of the spectrum. This result is expected because the gene sequence shows that the putative ligands to Cu_A are not present in this enzyme [4], and is consistent with the earlier assignment of this band to the Cu_A chromophore in mitochondrial oxidase ([17], but cf. [18]). In addition, the lack of any cytochrome *c* oxidizing capability for the *aa*₃ oxidase from *B. subtilis* supports the view that Cu_A is the initial electron input centre in cytochrome *c* oxidases [19].

We do observe a trough at 650 nm in both *B. subtilis* oxidases which is very similar in appearance to the band found in the mitochondrial oxidase. This transition has been assigned to arise from the high-spin cytochrome *a*₃ centre interacting with the nearby Cu_B, and thus is a signature of the binuclear centre [20]. However, we do not see any evidence of the near-IR transition recently reported for cytochrome *a*₃ in mitochondrial cytochrome *c* oxidase [21], but further examination of this region at higher concentrations of the *B. subtilis* oxidases and with different cytochrome *a*₃ adducts is warranted.

The cytochrome *caa*₃ enzyme does possess the ligands to Cu_A in subunit II [3] and a Cu²⁺ electron paramagnetic resonance spectrum [6]. In this paper we have measured the near-IR spectrum of our preparation, and found a band centred at 790 nm with a $\Delta\epsilon$ (reduced-minus-oxidized) of 2 mM⁻¹·cm⁻¹ which is assigned to the Cu_A centre. The band position and intensity are different from the Cu_A band found in mitochondrial cytochrome *c* oxidase. This indicates a slightly different ligand field strength for the Cu_A centre in the *caa*₃ oxidase from *B. subtilis* as compared with the mitochondrial oxidase, despite the similarities between the two subunit II protein sequences.

Our steady-state kinetic studies confirm the clear differences in substrate preference reported previously by Lauraeus et al. [6]. The cytochrome *caa*₃ oxidase uses TMPD very efficiently as an artificial electron donor and all the flux in this assay is carried by the endogenous cytochrome *c*, as indicated by the lack of any stimulation by exogenous cytochrome *c*. However, when the *caa*₃ enzyme is assayed spectrophotometrically it can be shown to oxidize ferrocyclochrome *c*, whereas the *aa*₃ oxidase cannot (B. C. Hill and J. Albanese, unpublished work). Therefore we conclude that the cytochrome *caa*₃ oxidase of *B. subtilis* is a cytochrome *c* oxidase whereas the *aa*₃ oxidase is not. Our *aa*₃-quinol oxidase preparation shows similar activity with TCHQ as found by Lauraeus et al. [6], but much less than the value reported by Lemma et al. [22] using a water-soluble menaquinol, dimethylnaphthoquinol. These authors found that the higher activity of their *aa*₃ preparation was positively correlated with a stronger reducing potential of the substrate. Dimethylnaphthoquinol has a mid-point potential of -80 mV, close to the mid-point potential of the natural, menaquinol, substrate.

This work was supported by a Natural Sciences and Engineering Research Council of Canada research grant to B.C.H.

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Received 21 September 1994/13 February 1995; accepted 23 February 1995