Properties of the detergent solubilised cytochrome *c* oxidase (cytochrome *cbb*$_3$) purified from *Pseudomonas stutzeri*

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Abstract Cytochrome *cbb*$_3$ is a cytochrome *c*-oxidising isoenzyme that belongs to the superfamily of respiratory haem/copper oxidases. We have developed a purification method yielding large amounts of pure *cbb*$_3$ complex from the soil bacterium *Pseudomonas stutzeri*. This cytochrome *cbb*$_3$ complex consists of three subunits (ccoNOP) in a 1:1:1 stoichiometry and contains two *b*-type and three *c*-type haems. The protein complex behaves as a monomer with an overall molecular weight of 114.0 ± 8.9 kDa and a $s^0_{20,w}$ value of 8.9 ± 0.3 S as determined by analytical ultracentrifugation. Crystals diffracting to 5.0 Å resolution have been grown by the vapour diffusion sitting drop method to an average size of 0.1×0.1×0.3 mm. This is the first crystallisation report of a (*cbb*$_3$)-type oxidase.

Key words: Cytochrome oxidase; Membrane protein; Crystallography; Analytical ultracentrifugation; Mass spectrometry; *Pseudomonas*

1. Introduction

Cytochrome oxidases form a large enzyme family that is characterised by a common design of the active site. This bimetallic site contains a haem group adjacent to a copper ion. Hence, the family is commonly called haem/copper oxidases [1]. Cytochrome *cbb*$_3$ is a cytochrome *c*-oxidising isoenzyme that belongs to the superfamily. It is peculiar to proteobacteria and it has not been found in other bacteria or Archaea.

Spectroscopic and enzyme activity properties have been previously reported for a number of bacterial cytochrome (*cbb*$_3$)-type oxidases belonging to the α subdivision of proteobacteria [2–6]. In nitrogen-fixing bacteria such as *Bradyrhizobium japonicum*, this enzyme has been shown to be involved in respiration at low oxygen tension [2]. Recently, this type of cytochrome oxidase has also been isolated from *Helicobacter pylori* [7] and the thermohalophilic *Rhodothermus marinus* [8]. All these enzymes are cytochrome *bc* complexes that lack the Cu_A centre characteristic of the mitochondrial cytochrome *c* oxidase (cytochrome *aa*$_3$) [5,6].

This class of enzymes has been predicted to be composed of a complex of four integral membrane proteins ccoNOQP (FixNOQP). However, the presence of the small ccoQ subunit, a single transmembrane helix protein, has not been so far reported.

The major catalytic subunit (ccoN, FixN) is homologous to subunit I of the mitochondria-like oxidase [4] and it has been predicted to be a 12 helix transmembrane protein. The six histidine residues involved in chelating the low, high spin haems and the Cu_B site are strictly conserved [9]. Nevertheless, the catalytic binuclear centre consists of a unique association of cofactors in the cytochrome *c* oxidase family, Cu atom (Cu_B site) and a *b*-type haem. Two smaller membrane-bound cytochromes ccoO (FixO) and ccoP (FixP) are part of the enzyme complex. While ccoO is a monohaem cytochrome *c*, the ccoP subunit presents a peculiar dihaem cytochrome *c* arrangement [10].

The *Pseudomonas stutzeri* strain *ZoBell* is a Gram-negative bacterium belonging to the family of the Pseudomonaceae (γ subdivision). This family includes a number of soil- and water-living species as well as bacteria pathogenic for animals and plants. Despite the amount of biochemical information available on the (*cbb*$_3$)-type oxidases belonging to the α subgroup of the proteobacteria, little research has been focused on this class of enzymes from the γ subdivision. Moreover, structural biology and hydrodynamic studies have not been reported for any cytochrome *cbb*$_3$.

The presence of a (*cbb*$_3$)-type oxidase in *Pseudomonas aeruginosa* and *Pseudomonas putida* has been recently confirmed at the DNA level by the complete sequence annotation of these bacterial genomes. In *P. stutzeri* the presence of a (*cbb*$_3$)-type oxidase in the respiratory chain was suggested at DNA and mRNA level [11,12]. However, this enzyme has not been either purified or characterised so far.

In this work we show that membranes from aerobically grown *P. stutzeri* contain a large amount of cytochrome *cbb*$_3$. We present a novel method for the purification of an active enzyme complex with all the redox-competent subunits. The isolation conditions developed allow the purification of a stable three subunit complex suitable for structural studies. The hydrodynamic properties of the ccoNOP complex in *n*-dodecyl-β-D-maltoside (DM) are explored by a thorough analysis of analytical ultracentrifugation data. These results

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Abbreviations: DM, *n*-dodecyl-β-D-maltoside; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenyldiamine; TMBZ, 3,3′,5,5′-tetramethylbenzidine; TFA, trifluoroacetic acid; 1 S, 1×10^{-13} s/rad^{-2}

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are compared with preliminary X-ray diffraction data on 3D crystals.

2. Material and methods

2.1. Bacterial growth and protein purification

P. stutzeri strain *ZoBell* was cultivated at 32°C under low aeration condition in shaking flasks using the following medium: L-asparagine 2.0 g/l, Na_3citrate·$2H_2O$ 7 g/l, KH_2PO_4 2.0 g/l, $MgSO_4$·$7H_2O$, $CaCl_2$·$2H_2O$ 0.1 g/l, NaCl 10 g/l, $FeCl_3$·$6H_2O$ 25 mg/l, $CuCl_2$·$2H_2O$ 0.17 mg/l, pH 8.0 [13,14].

After 30 h, cells were harvested by centrifugation on a Sorvall RC3B plus at 4°C for 30 min at 4500 rpm (5–6 g wet cells/l), resuspended in 20 mM Tris–HCl pH 7.5 and stored frozen at −80°C. Cell disruption was achieved by French press fracture using the following buffer: 20 mM Tris–HCl, 2 mM $MgCl_2$, ~1 mM 4-(2-aminoethyl)-benzol-sulphonyl fluoride (AEBSF, Serva), 0.1 mg/g wet cells deoxyribonuclease I, 500 mM NaCl, pH 7.5. EDTA 5 mM was added to the mixture 5–10 min after cell breakage. The solution obtained was centrifuged for 2.5 h at 100 000 × *g*, 4°C. The prepared membranes were stored at −80°C and the total amount of protein was estimated by the bicinchoninic acid method (BCA, Pierce).

Solubilisation of membrane protein (typically 300–500 mg of total protein) was achieved by incubation, for 5 min on ice, of thawed homogenised (homogeniser, Janke and Kunkel GmbH) membranes with DM in a ratio of 2.5 g of detergent per gramme of protein in a buffer containing: 20 mM Tris–HCl, 0.5 mM EDTA, 10% w/v glycerol, 50 mM NaCl, ~1 mM AEBSF, pH 7.5. The supernatant, obtained after centrifugation at 100 000 × *g* for 1 h at 4°C, was loaded on a 48 ml DEAE Toyopearl 650C (Tosohaas) anionic exchanger column equilibrated with: 20 mM Tris–HCl, 0.05 mM EDTA, 10% w/v glycerol, 50 mM NaCl, 0.02% DM, pH 7.5. Elution of the sample was obtained by a linear gradient of 0–25% in 290 ml of 20 mM Tris–HCl, 10% w/v glycerol, 1 M NaCl, 0.02% DM. Fractions enriched in *c* and *b* haem content eluted between 100 and 160 mM NaCl were immediately loaded on a 40 ml imminodiacetic-immobilised resin (Fast Flow Chelating Sepharose, Pharmacia) previously saturated with $CuSO_4$ and extensively equilibrated in 20 mM Tris–HCl, 10% w/v glycerol, 200 mM NaCl, 0.02% DM. Bound proteins were eluted in a 440 ml linear gradient of 0–8 mM imidazole in equilibration buffer. The elution profile was monitored at 280 and 405 nm and the fractions eluted at the end of the imidazole gradient were collected, immediately diluted and loaded on a second anionic exchanger column. This last 35 ml DEAE Toyopearl 650C resin was equilibrated in 20 mM Tris–HCl, 0.5 mM EDTA, 0.02% DM, pH 7.5; the elution was achieved by a 470 ml gradient, 0–30%, of 20 mM Tris–HCl, 1 M NaCl, 0.5 mM EDTA, 0.02% DM, pH 7.5. The cbb_3 oxidase-containing fractions were concentrated, immediately frozen in liquid nitrogen and stored at −80°C.

2.2. Analytical techniques

Oxygen consumption was followed during the purification with a Clark oxygen electrode in a 3.2 ml glass chamber (Eschweiler system 2000). *N*,*N*,*N*′,*N*′-Tetramethyl-*p*-phenyldiamine (TMPD) 1 mM and ascorbate 10 mM were used as electron donors in the following buffer: 20 mM Tris–HCl, 100 mM NaCl, 0.02% DM, pH 7.5.

Haems were quantified by recording pyridine haemochrome spectra, data were analysed by the inverse matrix method to deconvolute the contribution of haems *a*, *b* and *c* [15]. HPLC haem analysis was performed on a Gilson chromatographer Abimed using a Vydac 218TP54 C18 reverse phase column with a linear gradient of 0–100% (1.43%/min) of water and acetonitrile in the presence of 0.1% trifluoroacetic acid (TFA) monitoring the absorbance at 380 nm. The haem standards were obtained from myoglobin (Sigma), haem *b*, cytochrome oxidase aa_3 from *Paracoccus denitrificans*, haem *a* and *Escherichia coli* quinol oxidase, haems *b* and *o* [16]. Haems were extracted by a mixture of acetone:HCl (19:1) according to the protocol of Lübben et al. [17]. SDS–PAGE haem staining was carried out using the 3,3′,5,5′-tetramethylbenzidine (TMBZ) method described in [18].

Metal analysis was carried out by total reflection X-ray fluorescence spectrometry [19] performed at the Institute of Inorganic Chemistry, University of Frankfurt.

N-terminal sequencing was carried out with automated Edman degradation analysis by the analytical chemistry facility of the EMBL, Heidelberg.

MALDI mass spectrometry data were recorded in the positive ion mode with delayed extraction on a Reflex II time-of-flight instrument equipped with a SCOUT multiprobe inlet and a 337 nm nitrogen laser. Mass spectra were obtained averaging 100–300 individual laser pulses. Calibration of the spectra was performed by a three point quadratic fit using bovine serum albumin (66 431 Da), apo-myoglobin (16 952 Da) and apo-cytochrome *c* (12 361 Da). Sample preparation was achieved using reverse phase C4 Ziptip (Millipore) and the sinapinic acid sandwich technique [20]. Briefly, 0.2 μl of a saturated solution of sinapinic acid (Fluka) in ethanol was applied to the individual spots on the target and dried. The eluted samples (1 μl; 50% acetonitrile, 0.1% TFA) from the C4 Ziptip were mixed with 1.0 μl of a saturated solution of sinapinic acid in 30% acetonitrile, 0.1% TFA, and applied to the target.

Analytical gel filtration was performed on a HR10/30 Superdex 200 column (Pharmacia) equilibrated with the following buffer: 20 mM Tris–HCl, 200 mM NaCl, 0.5 mM EDTA, 0.02% DM. Samples of 100–200 μl (1–10 mg/ml) were run at 0.5 ml/min and the elution profile was monitored at 280 nm and 405 nm. Bio-Rad molecular weight gel filtration standards were used to calibrate the column.

2.3. Analytical ultracentrifugation

Experiments were performed on a Beckman XL-A ultracentrifuge using a An60Ti rotor at 4°C. Purified protein samples were investigated at concentrations between 0.02 mg/ml and 4 mg/ml in 20 mM Tris–HCl, 100 mM NaCl and 0.02% DM.

Sedimentation equilibrium data were obtained by centrifuging cbb_3 oxidase samples between 8000 rpm and 14 000 rpm for 20–30 h and recording radial scans at different wavelengths in order to be in the linear response range of the instrument. Data were analysed by ORIGIN-XL-A Beckman, Ultrascan 5.0 [21] (this software was developed by B. Demeler, www.ultrascan.uthscsa.edu) and by Kaleidagraph software using the following exponential model:

$$\mathrm{Abs_r} = \mathrm{Abs_m} e^{\left[\frac{\mathrm{Mp}(1-\Phi\rho)\omega^2(r^2-r^2m)}{2\,RT}\right]} \quad (1)$$

where $\mathrm{Abs_r}$ is the absorbance at any radial position *r*, $\mathrm{Abs_m}$ is the absorbance at the meniscus, Mp is the protein molecular weight, ρ is the density of the solution, ω is the radial velocity (rad/s), *R* and *T* are the gas constant and the absolute temperature. The buoyant term for membrane proteins must be corrected to include the contribution of detergent and lipids bound to the enzyme complex [22]:

$$(1-\Phi\rho) = (1-V_\mathrm{p}\rho) + \delta_\mathrm{d}(1-V_\mathrm{d}\rho) + \delta_\mathrm{l}(1-V_\mathrm{l}\rho) \quad (2)$$

Therefore Φ is dependent on the partial specific volume of the protein V_p (0.7411 cm^3/g), the partial specific volume of detergent V_d (DM, 0.824 cm^3/g [22]), δ_d the amount of detergent bound per gramme of protein (0.65 g/g), the partial specific volume of phospholipids V_l (0.975 cm^3/g [22]) and the amount of phospholipids bound per gramme of protein δ_l (0.15 g/g). The amount of DM bound per gramme of protein was estimated by the [^{14}C]DM method [23] and by a colorimetric method (details will be given elsewhere, Urbani et al., in preparation). The amount of phospholipids bound per gramme of purified enzyme complex was estimated using the molybdate method for the determination of phosphorus [24].

Sedimentation velocity recorded by spinning protein samples at 20 000, 30 000 and 40 000 rpm data were analysed using the computer program Ultrascan 5.0. Stokes radius (Rh) calculation was achieved rearranging the Svedberg equation [25,26]:

$$\mathrm{Rh} = \frac{\mathrm{Mp}(1-\Phi\rho)}{N6\pi\eta_{20,\mathrm{w}}s^0_{20,\mathrm{w}}} \quad (3)$$

where $s^0_{20,\mathrm{w}}$ is the sedimentation coefficient at infinite protein dilution in pure water at 20°C, $\eta_{20,\mathrm{w}}$ is the viscosity of water at 20°C and *N* is the Avogadro number.

2.4. Crystal growth and X-ray analysis

Crystals were grown by vapour diffusion using the sitting drop method in the following conditions: 0.2 M Zn acetate, 0.1 M Na acetate pH 4.5, 19–22% PEG 1000. The final protein concentration was 10 mg/ml after mixing with the reservoir buffer. Crystals were frozen by rapid freezing in liquid nitrogen. Cryo-freezing buffer con-

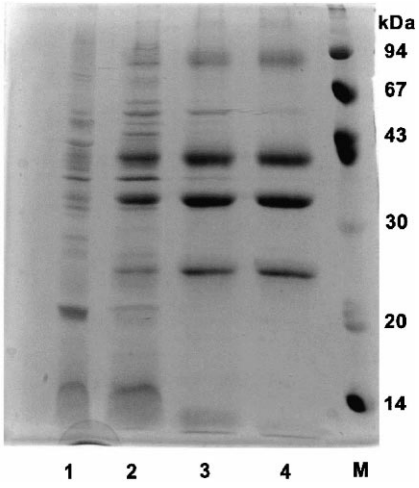

Fig. 1. SDS–PAGE of the three main steps in cytochrome cbb_3 purification. Lane 1: solubilised membrane in DM; lane 2: first anionic exchanger DEAE column; lane 3: Cu(II)-immobilised affinity chromatography; lane 4: final preparation. Protein samples were denatured in a high SDS (16% w/v) concentration loading buffer at 23°C for 5 min before being run on SDS–PAGE at 10°C.

dition was achieved by gradually increasing the concentration of PEG 1000 by exchanging the reservoir buffer with 28–30% PEG 1000, 0.2 M Zn acetate, 0.1 M Na acetate pH 4.5 and incubating the crystals for 6–12 h. Data collection was carried out in the synchrotron facilities of ESRF (Grenoble) beamline ID14-3 and DESY (Hamburg) beamline X11. Data were processed with the DENZO software package [27] and the CCP4 4.1 suite [28].

3. Results

3.1. Purification and subunit composition

The membrane fraction of aerobically grown *P. stutzeri* was used to prepare the active enzyme complex. Solubilised membranes did not show any presence of haem *a* as estimated by the pyridine haemochrome method (data not shown). The solubilised membranes were loaded on a first anion exchange resin (DEAE Toyopearl) and eluted in a gradient of NaCl. The mild nature of the Toyopearl exchanger matrix was necessary to prevent complex dissociation. Proteins containing bound haem were eluted in a broad peak around 100–200 mM NaCl. A second chromatographic step, Cu(II) chelate affinity, cleaned the preparation from most of the contaminating proteins (Fig. 1). At this stage, particular care was taken to immediately remove the imidazole used for the elution of the IMAC column with a second anionic exchanger. The specific activity is increased during all the purification steps (Table 1), suggesting that none of the chromatographic separations was deleterious for enzyme activity. This purified enzyme quickly turns over oxygen to water at a rate of 180 ± 50 s^{-1} when ascorbate and TMPD are used as electron donors and this activity is inhibited by the presence of KCN (IC_{50} value below 1 μM). Storage at −80°C of the purified material did not result in any significant loss of enzyme activity. The purification procedure is generally carried out in a single day. We did not observe any appreciable difference in purity or in enzyme activity when the preparation was carried out at 4°C or in a N_2-saturated buffer system.

SDS–PAGE analysis of the purified material, using the protocol described above, is presented in Fig. 1. Three distinctive polypeptide bands are shown by Coomassie blue staining with apparent molecular weights of 41, 35 and 26 kDa.

The two smaller subunits, 35 and 26 kDa, show peroxidase activity when assayed with the TMBZ, hydrogen peroxide method, pointing to the presence of covalently bound *c* haems (data not shown). An additional band around 90 kDa is also present, its intensity changing from preparation to preparation and being much dependent on the SDS–PAGE running temperature. This might be due to some impurity or more probably to an aggregate of denatured subunits. Polypeptide molecular weights were more accurately estimated by MALDI-TOF-MS of the purified material after washing the sample with 10% acetonitrile, 0.1% TFA in water on a reverse phase C4 material in order to reduce the contribution of detergent and lipids. The following masses with their respective S.D. ($n = 3$) were measured: $23\,470 \pm 28$ Da, $34\,840 \pm 50$ Da and $58\,360 \pm 60$ Da. A large bandwidth distribution (> 1000 Da) was observed for the presumed ccoN subunit signal (58 360 Da). This broadening might be due to the presence of strongly bound lipids or detergent molecules which are not removed by the C4 treatment.

In order to achieve a unique identification of the three polypeptide chains, N-terminal sequence analysis was performed on each of the three bands after transfer on a polyvinylidene fluoride membrane. Multiple sequence alignment shows a significant homology between the N-termini of the *P. stutzeri* preparation and the subunits ccoO and ccoP from other bacteria (Table 2a,b). Sequence data from the apparent 41 kDa polypeptide chain (ATSTAYLY) were more difficult to compare in a multiple sequence alignment, most probably due to the presence of proteolytic degradation of signal peptidases. A tentative alignment of the obtained sequences with other species ccoN N-termini is presented in Table 2c.

Pyridine haemochrome analysis on the purified protein showed a ratio of two *b* to three *c* haems as expected for this class of oxidases (Table 1). The non-covalently bound haems of the purified enzyme have been additionally identified by HPLC analysis of the acetone/HCl-extracted fraction. These showed the same behaviour of the *b* haems extracted

Table 1
Cytochrome cbb_3 purification table

	Specific activity (μM/s mg)	Total protein (mg)	Ratio *c*/*b* haems	Yield (%)
Membranes	102.6	514		100
S.M.P.	170.8	356.5	2.10 ± 0.07	
DEAE	184.7	227	2.42 ± 0.10	79
Cu(II) IMAC	950.7	37.7	1.40 ± 0.07	67
Final preparation	1869.6	18.4	1.50 ± 0.03	65

S.M.P.: solubilised membrane protein in DM. DEAE: first anionic exchanger column. Cu(II) IMAC: Cu(II)-immobilised affinity column. Yields in percentage are based on the recovery of activity according to the formula: $100 \times$(specific activity $\times$ total protein)/(specific activity $\times$ total protein)$_{membranes}$.

Table 2
N-terminal sequence alignment of (cbb_3)-type oxidase subunits

a

N-term ccoO																		
Ps_st						M	K	S	H	E	K	L	E	K	N	V	G	L	L
Ps_ae						M	K	N	H	E	I	L	E	K	N	V	G	L	L
Pa_de	M	A	I	L	E	K		H	K	V	L	E	K	N	A	T	L	L
Br_ja	M	S	F	W	T	R		H	Q	V	F	E	K	N	S	I	I	L
Rh_sp	M	G	I	L	A	K		H	K	I	L	E	T	N	A	T	L	L

b

N-term ccoP								
Ps_st	M	T	X	F	W	S	X	Y
Ps_ae	M	T	T	F	W	S	L	Y
Pa_de	M	A	D	T	D	D	E	H
Br_ja	M	T	D	H	S	E	F	D
Rh_sp	M	S	V	K	P	T	K	Q

c

N-term ccoN															
Ps_st	.	.	.	.	.	.	.	A	T	S	T	A	Y	L	Y
Ps_ae				M	S	T	A	I	S	Q	T	A	Y	N	Y
Pa_de	.	.	.	A	L	E	P	H	P	E	T	Q	Y	M	D
Br_ja	.	.	.	P	A	E	I	N	G	R	P	N	Y	N	M
Rh_sp	.	.	.	P	E	P	A	L		Q	T	E	Y	M	D

The letter x has been used in the amino acid positions were sequence uncertainty was found. Ps_st: *P. stutzeri* (this study); Ps_ae: *P. aeruginosa* (EMBL: AE004583); Pa_de: *P. denitrificans* (EMBL: PD34353); Br_ja: *B. japonicum* (EMBL: BJAJ5001); Rh_sp: *Rhodobacter sphaeroides* (EMBL: RS58092).

from myoglobin and *E. coli bo* oxidase (data not shown). These data are in agreement with the metal analysis, which indicates the presence of 5.3 Fe atoms per enzyme complex. Moreover, metal analysis returned a ratio of 0.96 mol Cu per mol of protein (ccoNOP), which might be indicative of the presence of the sole Cu_B site.

3.2. Complex properties

The overall complex composed of the ccoNOP subunits is eluted as a single symmetric peak on the analytical gel filtration column (data not shown) with an apparent molecular weight of 190 ± 30 kDa. This estimation reflects the sum of three diverse contributions: the molecular weight of the protein complex itself, the lipid fraction still bound and the detergent DM in complex.

A more precise estimation of the purified enzyme complex molecular weight and hydrodynamic properties was achieved by analytical ultracentrifugation. The contribution of detergent and phospholipids to the overall molecular weight of the complex is excluded from the calculation by taking into account the amount of detergent and phospholipids bound per cbb_3 complex and their partial specific volumes (Eq. 2). Sedimentation equilibrium data (Fig. 2) were collected at different protein concentrations and centrifugation speeds. All these data fitted to a single non-interacting model (Eq. 1), the average ($n = 16$) of the calculated molecular weights is 114 090 ± 8930 Da. This value is in agreement with the presence of a monomer of three (ccoNOP) subunits according to the mass spectrometry data, 116 600 Da.

The conformational and molecular mass homogeneity of the sample was evaluated by analyzing sedimentation velocity data by the method of van Holde and Weischet [29] (Fig. 3). The $s_{20,w}$ values remain constant across the boundary, indicating that the macromolecular sample has a homogeneous mass and shape [30]. Once the homogeneity was assessed, sedimentation velocity data could be analysed by a more rigorous treatment to obtain best-fit values for $s_{20,w}$ and $D_{20,w}$. To this aim, data were fitted to the Lamm equation using finite element analysis. Fits were obtained by this analysis method with almost random residual (Fig. 4A,B). A number of sedimentation velocity data sets were analysed by this procedure yielding the average best fit values ($n = 6$) of 8.6 ± 0.3 S and $5.04 \pm 0.50 \times 10^{-7}$ cm^2/s for the calculated sedimentation and diffusion coefficient in water at 20°C, respectively. These coefficients together with the partial specific volume accurately calculated by Eq. 2 gave a protein molecular mass of 111 220 ± 6470. Sedimentation velocity data were also analysed by the time derivative method of Stafford [31] and yielded similar results. Moreover, knowing the protein hydrodynamic coefficients and molecular weight it is possible to estimate the expected Stokes radius of the cbb_3 complex. Using a modified form of the Svedberg equation [25,26] (Eq. 3) we obtained a value of 40–45 Å for the detergent/lipid/protein complex.

The dependence of the $s_{20,w}$ over the protein sample concentration loaded per cell was explored to better evaluate complex stability and its oligomerisation state. A $s^0_{20,w}$ value

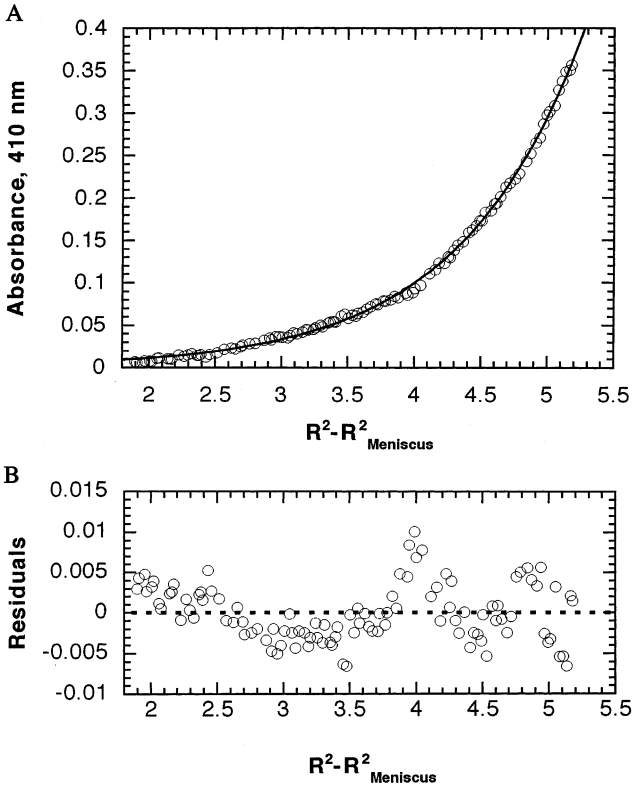

Fig. 2. Sedimentation equilibrium data of cytochrome cbb_3. The experiment reported was performed at 4°C on a protein sample of 0.2 mg/ml. Data were collected at 410 nm after centrifugation for 24 h at 11 000 rpm. A: Experimental data (open circle) and fitting of a single non-interactive component model (Eq. 1) (solid line). B: Residual distribution.

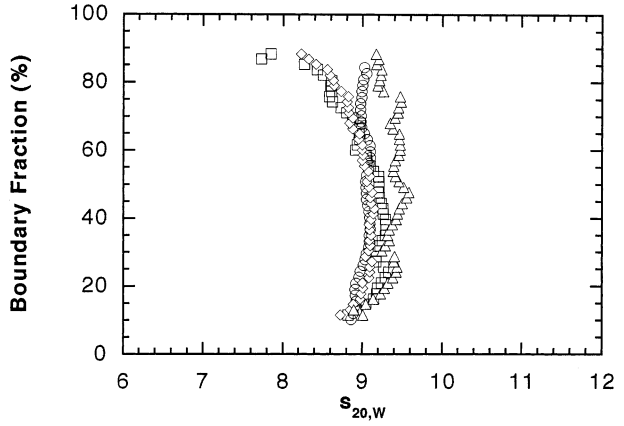

Fig. 3. Sedimentation velocity analysis of cytochrome cbb_3 as analysed by the van Holde–Weischet method. The symbols represent the distribution of sedimentation coefficients across the sedimenting boundary. The cbb_3 oxidase in 0.02% DM, 20 mM Tris–HCl and 100 mM NaCl, pH 7.5 was run at concentrations of 2 mg/ml (open circle), 0.4 mg/ml (open diamond), 0.24 mg/ml (open square) and 0.08 mg/ml (open triangle).

of 8.9 ± 0.3 S was calculated from the intercept on the y-axis of the plot (Fig. 4C). The sedimentation coefficient does not vary significantly over a wide range of protein concentrations, 0.02–2 mg/ml (Fig. 4C). This profile indicates no evidence of subunit dissociation or dimerisation of the complex, highlighting the monodisperse properties of this preparation and its suitability for protein crystallisation.

3.3. Crystallisation and preliminary X-ray diffraction

Hexagonal red crystals (Fig. 5) of the cbb_3 oxidase appeared after 3 days at 20°C and they reached a final average size of $0.1 \times 0.1 \times 0.3$ mm after 10–12 days. All three subunits (ccoNOP) were visible by SDS–PAGE (data not shown) in washed and dissolved crystals, the high molecular weight band (~90 kDa) was not observed in these samples. Anisotropic diffraction patterns were observed using synchrotron radiation sources. The crystal features are a hexagonal lattice with a particularly long C axis (717 Å) with resolution limits of about 5.0 Å on the long axis and around 7.0 Å on the others. This long axis partially impaired data acquisition due to the overlaps of neighbouring reflections on detector area. In order to circumvent this problem the detector distance was increased to a resolution edge of 9.0 Å and the crystals were aligned so as to have the long C axis almost lying on the spindle axis of the goniometric head. We obtained a complete (94% completeness) data set at 9.5 Å resolution (R-merge 0.069) assuming a space group $P6_{5/1}22$ and a cell dimension of $101 \times 101 \times 717$ Å. With these parameters it is reasonable to assume the presence of one cbb_3 molecular complex (~114 kDa) per asymmetric unit with a solvent content of 74% (Matthews coefficient 4.9). The presence of one single molecule per asymmetric unit is also consistent with results of self-rotation function [28] assuming a spherical model with a radius of 40 Å as estimated by hydrodynamic coefficients. The self-rotation function routine did not provide evidence for non-crystallographic symmetries in the generated polar plots (data not shown). Moreover, assuming a second molecule per asymmetric unit would give a solvent content of ~45%, which is considered not probable due to the diffraction properties of these crystals.

4. Discussion

The (cbb_3)-type cytochrome oxidase from *P. stutzeri* has been purified, characterised and crystallised. The homogeneous enzyme preparation is composed of the three subunits ccoNOP, analytical ultracentrifugation data suggest an equi-

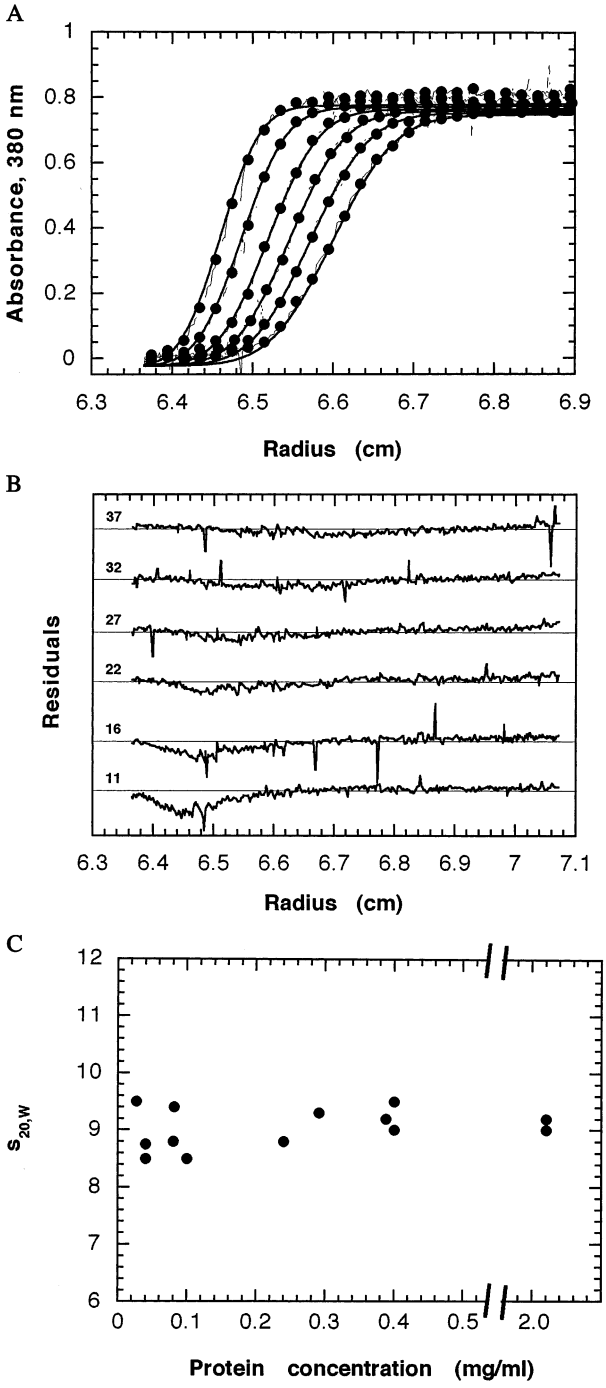

Fig. 4. Sedimentation velocity data of cytochrome cbb_3 fitted by finite element analysis. Sample (0.2 mg/ml protein) was centrifuged at 30 000 rpm at 4°C, scans were taken every 2 min (not all the scans are shown for picture clarity). A: Randomly selected experimental data points (filled circle) from radial scans 11, 16, 22, 27, 32, 37; computed fitting (solid line). B: Distribution of residuals. C: Concentration dependence of $s_{20,w}$ for cytochrome cbb_3 in DM. $s_{20,w}$ values were calculated by finite element and time derivative analysis.

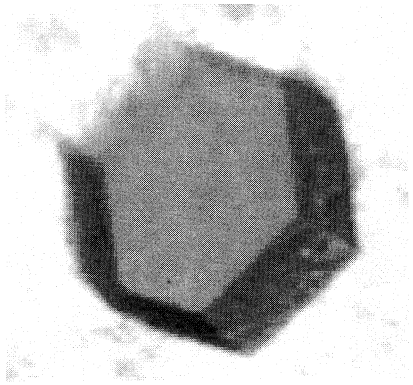

Fig. 5. A crystal of cytochrome cbb_3 oxidase.

molar composition of these three proteins in the complex. Preparations of (cbb_3)-type oxidase from other bacteria indicate a similar SDS–PAGE profile [3,5,6,32,33] with the two smaller subunits stainable by the TMBZ peroxidase method. N-terminal sequence analysis unequivocally identified the 41, 35 and 26 kDa bands with the ccoN (FixN), ccoP (FixP) and ccoO (FixO) proteins. The specific activity in the presence of ascorbate and TMPD, 180 O_2 s^{-1}, is comparable with the oxygen consumption rates reported so far for other (cbb_3)-type oxidases [5,6]. Interestingly, this enzyme preparation shows nitric oxide reductase activity (Forte et al., in preparation).

The hydrodynamic properties of the purified cytochrome cbb_3 were thoroughly explored by analytical ultracentrifugation studies. Iterative fitting of sedimentation velocity data to the Lamm equation yielded a molecular weight value (111 220 ± 6470 Da) close to the ones estimated by equilibrium velocity runs (114 090 ± 8930 Da) and mass spectrometry data (116 600 Da). In solution the DM-solubilised complex behaves as a homogeneous molecular species of 114 090 ± 8930 Da suggesting that the enzyme is active as a monomer of three subunits (ccoNOP). The sedimentation coefficient $s^0_{20,w}$ of 8.9 ± 0.3 S is in agreement with the values reported for other oxidases solubilised in DM [34,35]. The calculated hydrodynamic radius of 40–45 Å is close to the one reported for the monomeric form of mitochondrial cytochrome oxidase (49–51 Å) solubilised in DM [34]. Both values are in agreement with the overall dimensions observed in the high-resolution structures of the cytochrome *c* oxidase from bovine heart [36], of the aa_3 oxidase from *P. denitrificans* [37] and of the ba_3 oxidase from *Thermus thermophilus* [38].

The van Holde–Weischet analysis of sedimentation velocity data pointed out the conformational homogeneity and stability of the protein preparation in DM. These conditions are important factors in obtaining type II crystals suitable for X-ray diffraction studies [39]. The cytochrome cbb_3 enzyme gives hexagonal crystals that show an anisotropic diffraction pattern, with a limit of diffraction between 5 and 7 Å. The size of the unit cell (100 × 100 × 717 Å) is compatible with the analytical ultracentrifugation data. In fact, assuming a hydrodynamic radius of 40–45 Å, arising from the sedimentation data, one could suggest that the protein complex is lying on the edge of a 100 Å hexagon. The screw axis arrangement of the asymmetric units would then propagate to fill the 717 Å edge of the hexagonal parallelepiped. Data analysis of the X-ray diffraction patterns would indicate that cbb_3 oxidase is a monomeric complex in the crystallographic lattice as well as in solution.

These crystals are not yet suitable for high-resolution structure determination due to poor diffraction properties, high solvent content and the long C axis. Moreover, most of the crystals showed multiple lattices and could not be used; this drastically reduced the number of suitable material for this study. Further optimisation of this crystal form might open up the possibility of obtaining the 3D structure of this integral membrane protein.

The high yield of protein purification and the stability of this cytochrome cbb_3 preparation make this enzyme complex particularly suitable for spectroscopic and structural characterisation. Moreover, the study of this oxidase from *P. stutzeri* might provide a useful model to investigate the function and structure of cytochrome (cbb_3)-type oxidase from the bacterial pathogen species of the γ subdivision.

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References

[1] Ferguson-Miller, S. and Babcock, G.T. (1996) Chem. Rev. 96, 2889–2907.
[2] Preisig, O., Anthamatten, D. and Hennecke, H. (1993) Proc. Natl. Acad. Sci. USA 90, 3309–3313.
[3] Preisig, O., Zufferey, R., Thony-Meyer, L., Appleby, C.A. and Hennecke, H. (1996) J. Bacteriol. 178, 1532–1538.
[4] de Gier, J.W., Schepper, M., Reijnders, W.N., van Dyck, S.J., Slotboom, D.J., Warne, A., Saraste, M., Krab, K., Finel, M., Stouthamer, A.H., van Spanning, R.J. and van der Oost, J. (1996) Mol. Microbiol. 6, 1247–1260.
[5] Garcia-Horsman, J.A., Berry, E., Shapleigh, J.P., Alben, J.O. and Gennis, R.B. (1994) Biochemistry 33, 3113–3119.
[6] Gray, K.A., Grooms, M., Myllykallio, H., Moomaw, C., Slaughter, C. and Daldal, F. (1994) Biochemistry 33, 3120–3127.
[7] Tsukita, S., Koyanagi, S., Nagata, K., Koizuka, H., Akashi, H., Shimoyama, T., Tamura, T. and Sone, N. (1999) J. Biochem. (Tokyo) 125, 194–201.
[8] Pereira, M.M., Carita, J.N., Anglin, R., Saraste, M. and Teixeira, M. (2000) J. Bioenerg. Biomembr. 32, 143–152.
[9] Toledo-Cuevas, M., Barquera, B., Gennis, R.B., Wikstrom, M. and Garcia-Horsman, J.A. (1998) Biochim. Biophys. Acta 1365, 421–434.
[10] Arslan, E., Schulz, H., Zufferey, R., Kunzler, P. and Thony-Meyer, L. (1998) Biochem. Biophys. Res. Commun. 251, 744–747.
[11] Vollack, K.U., Xie, J., Hartig, E., Romling, U. and Zumft, W.G. (1998) Microbiology 144, 441–448.
[12] Vollack, K.U., Hartig, E., Korner, H. and Zumft, W.G. (1999) Mol. Microbiol. 31, 1681–1694.
[13] Matsubara, T., Frunzke, K. and Zumft, W.G. (1982) J. Bacteriol. 149, 816–823.
[14] Coyle, L., Zumft, W.G., Kroneck, P.M.H., Koerner, H. and Jakob, W. (1985) Eur. J. Biochem. 153, 459–467.
[15] Berry, E.A. and Trumpower, B.L. (1987) Anal. Biochem. 161, 1–15.
[16] Warne, A., Wang, D.N. and Saraste, M. (1995) Eur. J. Biochem. 234, 443–451.
[17] Lübben, M. and Morand, K. (1994) J. Biol. Chem. 269, 21473–21479.
[18] Thomas, P.E., Ryan, D. and Levin, W. (1976) Anal. Biochem. 75, 168–176.

[19] Wittershagen, A., Rostam-Khani, P., Klimmek, O., Grosse, R., Zickerman, V., Zickerman, I., Gemeinhardt, S., Kroeger, A., Ludwig, B. and Kolbesen, B.O. (1997) Spectrochim. Acta B 52, 1033–1038.
[20] Onnerfjord, P., Ekstrom, S., Bergquist, J., Nilsson, J., Laurell, T. and Marko-Varga, G. (1999) Rapid Commun. Mass Spectrom. 13, 315–322.
[21] Musatov, A. and Robinson, N.C. (1994) Biochemistry 33, 13005–13012.
[22] Le Maire, M., Kwee, S., Andersen, J.P. and Moller, J.V. (1983) Eur. J. Biochem. 129, 525–532.
[23] Møller, J.V. and le Maire, M. (1993) J. Biol. Chem. 268, 18659–18672.
[24] Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496.
[25] Taylor, A.M., Boutler, J., Harding, S.E., Coelfen, H. and Watts, A. (1999) Biophys. J. 76, 2043–2055.
[26] Le Maire, M., Aggerbeck, L.P., Monteilhet, C., Andersen, J.P. and Møller, J.V. (1986) Anal. Biochem. 154, 525–535.
[27] Otwinowski, Z. and Minor, W. (1997) Methods Enzymol. 276, 307–326.
[28] Collaborative Computational Project, number 4 (1994) Acta Crystallogr. D 50, 760–763.
[29] Van Holde, K.H. and Weischet, W.O. (1978) Biopolymers 17, 1387–1403.
[30] Hansen, J.C., Lebowitz, J. and Demeler, B. (1994) Biochemistry 33, 13155–13163.
[31] Stafford III, W.F. (1992) Anal. Biochem. 203, 295–301.
[32] Tamegai, H. and Fukumori, Y. (1994) FEBS Lett. 347, 22–26.
[33] Arslan, E., Kannt, A., Thony-Meyer, L. and Hennecke, H. (2000) FEBS Lett. 470, 7–10.
[34] Musatov, A., Ortega-Lopez, J. and Robinson, N.C. (2000) Biochemistry 39, 12996–13004.
[35] Hendriks, J., Warne, A., Gohlke, U., Haltia, T., Ludovici, C., Lubben, M. and Saraste, M. (1998) Biochemistry 37, 13102–13109.
[36] Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1995) Science 269, 1069–1074.
[37] Iwata, S., Ostermeier, C., Ludwig, B. and Michel, H. (1995) Nature 376, 660–669.
[38] Soulimane, T., Buse, G., Bourenkov, G.P., Bartunik, H.D., Huber, R. and Than, M.E. (2000) EMBO J. 19, 1766–1776.
[39] Ostermeier, C. and Michel, H. (1997) Curr. Opin. Struct. Biol. 7, 697–701.