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Purification and spectral characterization of a *b*-type cytochrome from the plasma membrane of the archaebacterium *Sulfolobus acidocaldarius*

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For the first time the purification of a heme-*b* containing cytochrome from the plasma membrane of an extremely thermoacidophilic archaebacterium is described The detergent solubilized 30 kDa polypeptide contains two heme-*b* centers and one copper ion According to its low temperature spectra and CO-binding properties, it is likely to function as a cytochrome-*o* like terminal oxidase in the membrane The purified cytochrome does not retain catalytic activity, however

Cytochrome-*b*, Archaebacteria, *Sulfolobus*, Electron transport, Respiration

1 INTRODUCTION

The extremely thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* grows aerobically and has been shown to carry out respiration-linked proton extrusion [1]. The electrochemical gradient of H^+ across its membrane is likely to be used for chemiosmotically driven ATP synthesis [1,2] It is unknown, however, which components of the respiratory system are acting as effective proton pumps allowing for a pH gradient >3 It has also been shown that differential cyanide sensitivity indicates the action of more than one terminal oxidase The membrane-residing respiratory system besides NADH-dehydrogenase [3] and succinate dehydrogenase [4] contains only *a*- and *b*-type cytochromes while *c*-type cytochromes are absent [5–7]. From these we have purified and characterized a novel cytochrome-aa_3 [8,9], acting as a 'quinol oxidase' and using caldariella quinone as substrate.

The isolation of *b*-type cytochromes revealed to be unusually discouraging due to the instability under the harsh conditions necessary for dissociation of the firmly associated respiratory complexes from the membrane A partial purification has been preliminarily reported [10]. Here we describe the isolation and spectral characterization of a purified *b*-type cytochrome from *Sulfolobus acidocaldarius*, likely to function as a cytochrome-*o* in the intact membrane.

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2 MATERIALS AND METHODS

2 1 *Growth of bacteria*

Sulfolobus acidocaldarius DSM 639 cells were grown aerobically in Brock's medium [11] supplemented with 10 mM K_2SO_4, 0 2% sucrose, 0 1% yeast extract (Gibco), pH 2 5, at 75°C Cells were harvested in the early stationary phase, washed once in a buffer containing 50 mM imidazole, 1 mM malonate, pH 7 0, and sedimented for 15 min at 6500 × *g* at 4°C The pellet was stored at −80°C in the same buffer additionally containing 50% glycerol

2 2 *Membrane preparation*

Membranes were prepared as described [9], with the exception that a buffer containing 50 mM imidazole, 1 mM malonate, pH 7 0 was used and the cells were disrupted in a Manton Gaulin press for 5 min at 5×10^7 Pa

2 3 *Purification procedure*

Membrane extraction and protein purification were performed at 4°C First the membranes were incubated in a buffer containing 50 mM imidazole, 20 mM $K_2P_2O_7$, pH 7 5, for 1 h at a final protein concentration of about 10 mg/ml After sedimentation at 200 000 × *g* for 50 min, the resulting pellet was suspended in 50 mM imidazole (final protein concentration of 20 mg/ml) and extracted for 75 min in the presence of 1% dodecylmaltoside (DM) After 1 h sedimentation at 150 000 × *g*, the supernatant was concentrated (Amicon, PM10 membrane), gently shaken with Bio-Beads SM2 for at least 2 h, and subsequently passed through a hydroxyapatite column (2 6 × 26 cm), equilibrated with 0 75 M KH_2PO_4, 0 05% DM, 0 02% *N*-dodecyl-*N*,*N*-dimethylammonio-3-propane-sulfonate (SB12), pH 7 5, (flow rate 40 ml/h) Protein and heme in eluating fractions were monitored at 280 and 420 nm, respectively Heme-containing peak fractions were analyzed by their dithionite-reduced minus oxidized difference spectra Cytochrome containing fractions were concentrated, the buffer exchanged to 50 mM KH_2PO_4, pH 7 5, and loaded on a second hydroxyapatite column (3 × 14 cm) equilibrated with 50 mM KH_2PO_4, 0 05% DM, 0 02% SB12, pH 7 5 (flow rate 30 ml/h) When 100 ml of the equilibration buffer had passed through, a linear phosphate gradient (50–500 mM KH_2PO_4) in 0 05% DM, 0 02% SB12, pH 7 5, was applied Cytochrome-*b*-enriched fractions were pooled and concentrated In case of a residual cytochrome-*a* contamination the material was rechromatographed on hydroxyapatite under analogous conditions Subsequently the material was loaded on a H/Load/600-

Superdex 200 column using a buffer containing 50 mM imidazole, 150 mM NaCl, 0 2% SB12, pH 7 5 (flow rate 1 ml/min) The final step of purification was performed on a MonoQ-5/5 column with a linear gradient from 0 to 0 5 M NaCl in 50 mM imidazole, 0 2% SB12, pH 7 5

2 4 *Spectroscopy*

A Hewlett Packard HP8450A diode array spectrophotometer was used for spectra at room temperature, low temperature spectra were measured with a Sigma ZWSII dual wavelength spectrophotometer equipped with a self-designed low-temperature device Pyridine hemochrome spectra were recorded according to [12] Copper content was determined using a Hitachi 180-80 Zeeman atomic absorption spectrophotometer set at 324 8 nm

2 5 *Other methods*

Hydroxyapatite was prepared according to [13] Membrane protein was determined by the biuret method [14], membrane extracted proteins according to Lowry [15] in the presence of SDS PAGE were performed by the Laemmli procedure [16] on 15% gels, proteins were visualised by silver staining [17]

2 6 *Materials*

H/Load/600-Superdex 200 and Mono-Q-5/5 columns were obtained from Pharmacia All chemicals were purchased from Merck, Sigma, Serva, Biomol or Fluka

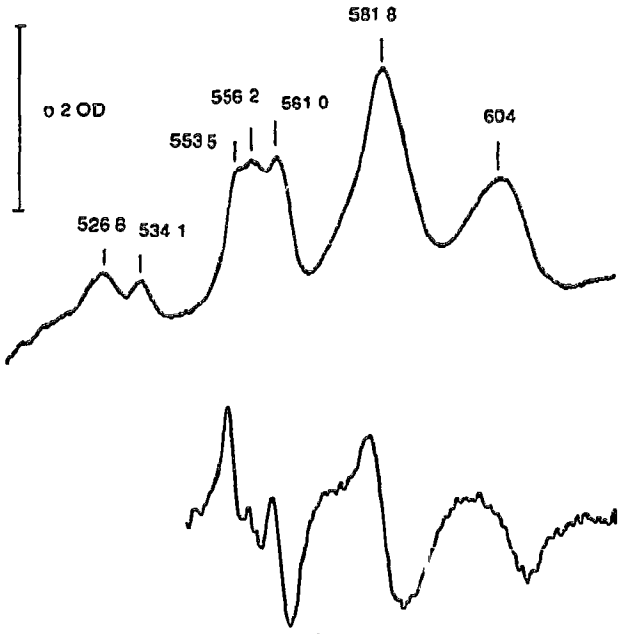

Fig 1 Reduced minus oxidized difference spectrum of a membrane suspension from *Sulfolobus acidocaldarius* taken at liquid nitrogen temperature, 1 5 mm light path, the medium contained 50 mM imidazole pH 7 5 30% glycerol and 2 9 mg protein/ml final concentration The reduced sample was prepared by addition of sodium dithionite The bottom trace shows the 1st derivative, clearly indicating three inflection points in the α-band region of heme-*b*

3 RESULTS

Previous spectroscopic studies have shown that more than one heme-*b* center is present in the membranes of *Sulfolobus*, as indicated by an absorption maximum at 565 5 nm and the pronounced shoulder at 558 nm in reduced-oxidized difference spectra taken at room temperature [9]. Partial reduction by ascorbate and spectral subtraction from the fully dithionite-reduced state suggested a third compound to be present absorbing at 562 nm. In Fig 1, a low-temperature spectrum of freshly prepared *Sulfolobus* membranes is shown where indeed three individual maxima in the α-region of *b*-type cytochromes can be clearly distinguished At low temperature the absorptions are shifted slightly to lower wavelengths located at 553.5, 556 2 and 561 nm, respectively Also the β-bands are nicely developed In addition, the spectrum shows the typical band of the aa_3-oxidase and the prominent peak at 582 nm which could not be functionally attributed so far though being the most significant spectral band While we have tentatively assigned it as a cytochrome-a_1 [6,7], it was suggested to be a constituent part of a terminal aa_3-type oxidase described for another species of *Sulfolobus* [18] During preparation of *b*-type hemoproteins from solubilized membranes the separation from this latter

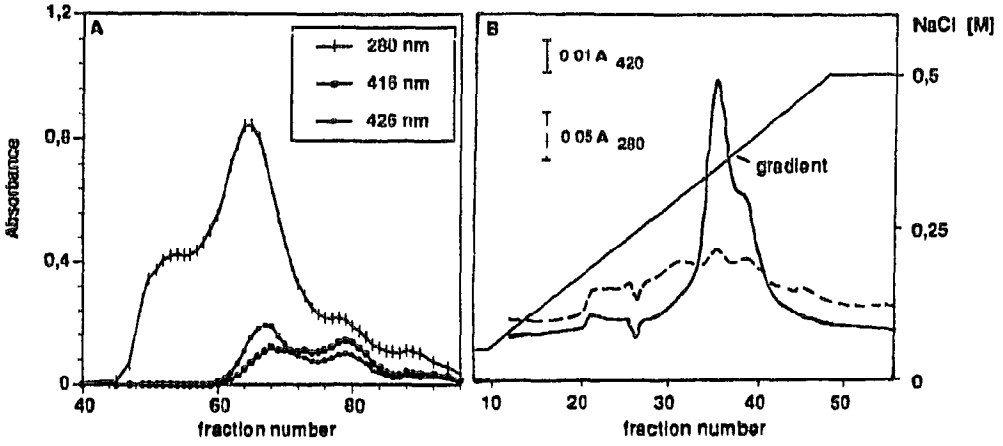

Fig 2 Elution profiles of chromatographic separation steps (A) Superdex-200 column, the fractions were tested individually at 280 nm for protein, at 416 nm for oxidized, and at 426 nm for reduced heme absorption, respectively It was observed that during the course of preparation the hemes can be partially reduced (B) Mono-Q 5/5 column, in this case protein and heme were monitored automatically at 280 nm for protein and 420 nm as an intermediate wavelength for the hemes Other conditions as described in the text

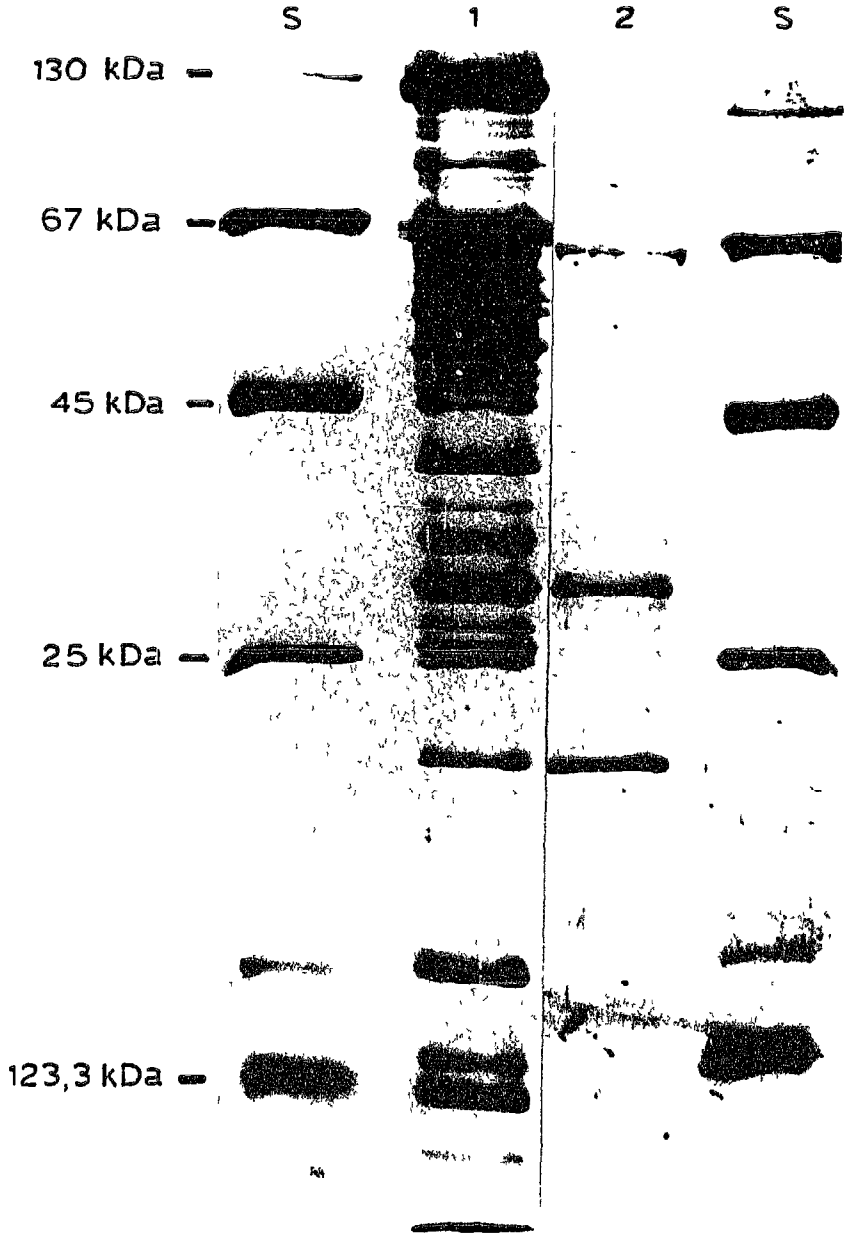

Fig 3 SDS-gel electrophoresis of membrane extract (lane 1, 10 μg/ml) and purified cytochrome-*b* (lane 2, 3 μg/ml) from *Sulfolobus acidocaldarius* Protein bands were visualized by silver staining, S = molecular weight markers (β-galactosidase 130 kDa, bovine serum albumin 67 kDa, ovalbumin 45 kDa, chymotrypsin 25 kDa, and cytochrome-*c* 12 3 kDa)

compound was the most critical challenge No detergent was found which preferentially solubilized *b*-type cytochromes. In contrast, most detergents failed to preserve the native state of hemoproteins as concluded from the disappearance of spectral resolution resulting in only one coalescent heme-*b* absorption maximum at 560 nm (not shown) This was not the case when membranes were solubilized by 1% dodecylmaltoside after preextraction with 20 mM pyrophosphate which removed 7–15% of loosely membrane associated proteins (for details see Methods) On a first hydroxyapatite column, a change of detergent was achieved together with partial retention of *a*-type cytochromes. The second hydroxyapatite column removed a large portion of contaminating protein from a slowly migrating broad heme-containing band Actually, one of the major obstacles is the obviously strong association of the various respiratory carriers resulting in a smear of components over a wide range of fractions, thus, an inverse gradual distribution of *a*- and *b*-containing hemoproteins over a broad peak could be achieved, rather than a clear separation A subsequent gel chromatographic step on a Superdex-200 column had the purpose to change the detergent again to a higher concentration of SB-12 only, yielding two peaks, one of which indicated a high specific enrichment of heme-*b* Anion ex-

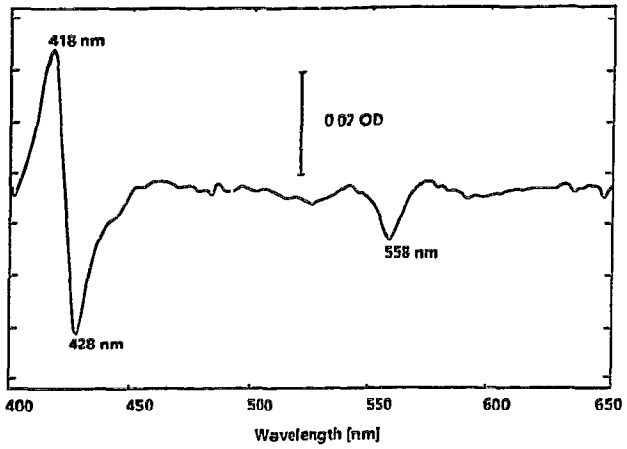

Fig 4 CO/reduced minus reduced spectrum of purified cytochrome-*b* from *Sulfolobus acidocaldarius* Protein concentration was 0 045 mg/ml in a buffer containing 50 mM imidazole, 0 2% SB12, pH 7 5, *d* = 1 cm

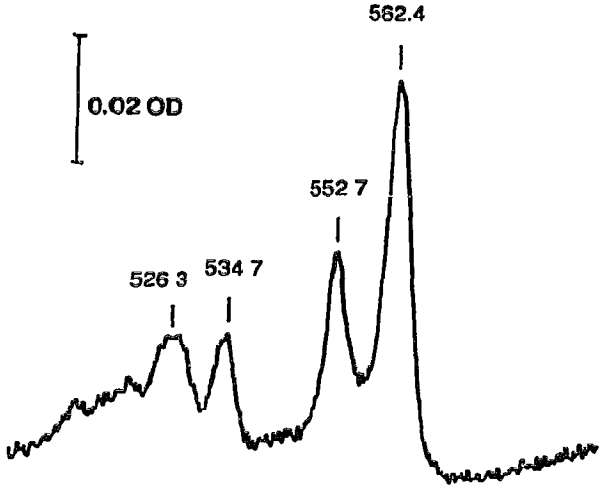

Fig 5 Low-temperature reduced minus oxidized difference spectrum of purified cytochrome-*b* The sample was oxidized by ferricyanide (0 6 mM), after the spectrum was run and stored as a reference, the sample was reduced by a small grain of dithionite and the spectrum scanned again at liquid nitrogen temperature, light path 1 mm, 0 8 mg protein/ ml in a buffer containing 0 2 M potassium phosphate pH 6 5 30% glycerol

change chromatography on a Mono- Q column finally gave a fraction of *b*-type cytochrome, free of any contaminating heme-*a* as verified by difference spectroscopy and pyridine hemochrome analysis Elution profiles of the last two separation steps are given in Fig. 2 From its elution volume on a calibrated S-200 gel chromatography column a molecular mass of 48 kDa was calculated for the detergent-embedded cytochrome Fig 3 shows a silver stained SDS-gel of the preparation compared to the membrane extract. The band of 30–31 kDa was identified to carry the heme-*b* by heme-staining of gels run in the absence of mercaptoethanol, allowing to prevent a complete loss of the prosthetic iron porphyrin during SDS gel electrophoresis A smaller peptide of about 18–20 kDa was frequently observed as well as a tendency to form aggregates of about 67 kDa apparent molecular mass. It is unclear whether or not this polypeptide is an additional constituent part of the cytochrome-*b* complex.

Table I gives a typical purification protocol Though a high degree of enrichment was achieved, it also illustrates the poor yield of purified cytochrome Obviously also some loss of heme-*b* occurred, especially during the 2nd and 3rd column steps. The final product shows a typical cytochrome-*b* reduced minus oxidized difference spectrum at room temperature, with maxima at 427 nm, 558 nm and a pronounced shoulder at 566 nm. It binds carbon monoxide, yeilding a CO/reduced minus reduced difference spectrum typical for terminal oxidases as given in Fig 4 The characteristic maximum at 418 nm and troughs at 428 and 558 nm are clearly developed, resembling the typical spectrum of CO-binding heme-*b* containing cytochromes. In Fig. 5 a low-temperature difference-spectrum (dithionite-reduced minus oxidized) is shown, indicating the presence of two heme-*b* centers It parallels nicely spectra of well-characterized *o*-type cytochromes [19], displaying at 554 and 561 nm the same maxima in the α-band region as found in intact *Sulfolobus* membranes (Fig. 1). The purified cytochrome also contained tightly bound copper withstanding extensive dialysis against 10 mM EDTA From several preparations an average copper content of 0.4– 0.6 mol/mol heme-*b* was determined, suggesting that the comparatively small 30 kDa-polypeptide hosts two

Table I

Typical purification protocol for cytochrome-*b* from *Sulfolobus acidocaldarius*

Purification state	Total protein (mg)	Specific heme-*b* content (nmol/mg)	Purification factor *n*
Membranes	715	0 29	1
Preextract	55	–	–
DM extract	163	0 63	2 2
(1) Hydroxyapatite column	66	1 39	4.8
(2) Hydroxyapatite column	11	1 74	6
Gel filtration	1 22	4 65	18
Anion exchange	0 033	39 2	134

es and one copper associated with one of the hemes xpected for an *o*-type terminal oxidase. Despite e similarities no significant catalytic activity as a xidase or a TMPD-oxidase could be verified Al- igh an almost negligible activity with caldariella ione could be detected, the preparation is likely to iactive due to the loss of either additional subunits, f specific lipids essential for catalytic activity Addi- of soybean asolectins or a crude *Sulfolobus* lipid act did not improve catalytic activity It should be tioned, however, that in membrane extracts by ox titrations the presence of high- and low-potential pe cytochromes could be established which would ie values for a functional *o*-type cytochrome [6,20]

DISCUSSION

he presence of *b*-type cytochromes in aerobically vn archaebacteria has been shown for *Halobacte- cutirubrum* [21] and *H halobium* [22,23]. None of e has been purified so far and, moreover, their redox ntials differed significantly when determined either olution or in membranes, respectively Nevertheless, ast one of those might represent a terminal oxidase oncluded from its CO-binding properties Here for first time isolation and spectral characterization of ghly purified *b*-type cytochrome from a thermoaci- hilic archaebacterium, *Sulfolobus acidocaldarius*, d be demonstrated Though its heme-*b* content, per content, and CO-binding properties resemble e of Q-oxidizing *o*-type cytochromes, a corres- ding catalytic activity could not be preserved. An- r difference to known *o*-type cytochromes [24] is the apparent molecular mass of the isolated poly- ide carrying the heme centers In this regard it falls er to *b*-type cytochromes acting as intermediary iron carriers However, since more than only one inal oxidase is present in this archaebacterial genus , together with the fact that a low molecular weight quinol-oxidase was identified [9], the assumption of a rather simple *o*-type terminal oxidase may be fied. Actually, the partially cyanide-insensitive iration at low inhibitor concentration, sufficient to k cytochrome-aa_3 completely, would be in line with concept. Besides that, it was shown in previous ies [6] that a high-potential *b*-type cytochrome (E'_0 300 mV) could be detected by redox-titration in ibrane extracts from *Sulfolobus*, supporting the ence of an alternative terminal oxidase apart from chrome-aa_3 In addition, qualitative observations indicating that the ratio of *a*-type versus *b*-type cy- romes in *Sulfolobus* varies with oxygen availability published results) Caldariella quinone serves as an rmediary pool for reducing equivalents in the membrane of *Sulfolobus* and may provide the specific reductant for both terminal oxidases presumably differ- ing in oxygen affinity. Regrettably, it was impossible to follow the catalytic activity during purification due to competition with the highly active aa_3-oxidase. The failure to isolate the inferred *o*-type cytochrome in ac- tive form may result from the loss of specific lipids or of other constituent polypeptides for an active complex The latter is not unlikely, taking into account an obvious tendency of *Sulfolobus* membrane proteins to desintegrate under conditions necessary to resolve the extraordinary rigid membrane structure. Sequencing of the *b*-polypeptide is in progress and is expected to allow a definite functional assignment by comparison to known heme-*b* containing cytochromes

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