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Cytochrome aa_3 from the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*

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Cytochrome aa_3 serves as a terminal oxidase in the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. A procedure for its isolation is described. The purified preparation consists of only one major polypeptide of 38 kDa apparent molecular mass. The enzyme contains two heme *a* molecules with midpoint potentials of +220 and +370 mV, respectively. The copper content is at least 2 Cu/aa_3. It has only negligible capacity to oxidize cytochrome *c*, but rather serves as an oxidase for reduced caldariella quinone as present in the membrane of *Sulfolobus*.

Archaebacteria; Electron transport; Hemoprotein; Copper; Midpoint potential; Caldariella quinone

1. INTRODUCTION

The archaebacterium *Sulfolobus acidocaldarius* grows aerobically at temperatures of about 85°C and pH values between 2 and 3. It has been shown that this extreme thermoacidophilic organism maintains a large proton gradient across its plasma membrane [1] and most likely uses chemiosmotic proton cycling to drive ATP synthesis via an F_0F_1-analogous ATP-synthase [2,3]. Although spectroscopic studies revealed the presence of *b*- and *a*-type cytochromes as well as the involvement of a cytochrome aa_3 as a terminal oxidase [4–6], no details were known either of the components and the exact pathway of electron transport or of the identity of the respiration-driven pumps generating the proton motive force in *Sulfolobus*. Here we report on the partial purification of cytochrome aa_3 and some of its enzymatic and spectroscopic properties.

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2. MATERIALS AND METHODS

Sulfolobus acidocaldarius DSM 639 cells were grown and harvested, and purified membranes prepared as described [4]. All procedures were performed at 4°C. The purified membranes were first exposed to 1 M KSCN/10 mM Tris-Cl (pH 8) in the presence of 5% DMSO for 1 h. After ultracentrifugation at 150000 × *g* for 1 h, the pellet was suspended in a buffer containing 20 mM Tris-HCl, pH 8.0. This homogenate was extracted by adding an equal volume of a buffer containing 2% *N*-lauroylsarcosine (Sarcosyl), 20 mM Tris-HCl, pH 8.0. After 30 min extraction, the sample was spun at 150000 × *g* for 1 h. The resulting pellet was suspended in a buffer containing 50 mM malonate, 1 mM EDTA, pH 5.5, and extracted by adding an equal volume of a buffer containing 2% Sarcosyl, 1 M NaCl, 10 mM Tris-HCl, pH 8.0. After 30 min, the sample was spun at 150000 × *g* for 1 h; polyethylene glycol 6000 (PEG) was added to the supernatant to a final concentration of 10% and the mixture was stirred for 1 h. After centrifugation at 20000 × *g* for 15 min, the supernatant was decanted and PEG was added to a final concentration of 12.5%. The mixture was stirred for 1 h and centrifuged at 20000 × *g* for 15 min. The dark green, oily layer sitting on top of a solid precipitate was carefully removed and suspended in 20 mM Tris-HCl, pH 8.0. This material was passed through a Spherogel TSK 4000 SWG column in a buffer containing 0.2 M Na_2SO_4, 10 mM Tris-SO_4, 0.05% Sarcosyl, pH 7.5. Alternatively, the separation was performed by gel exclusion chromatography on a Sephadex S 200 HR column. For spectroscopic determinations, HPLC-fractions were concentrated by ultrafiltration with an Amicon PM 30 membrane. Membrane protein was determined by a

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modified Biuret reaction [7], and solubilized membrane protein by the Lowry method in the presence of SDS [8]. Polyacrylamide gel electrophoresis was carried out by the Laemmli procedure [9] on 15% gels with an acrylamide to bisacrylamide ratio of 100:1, and proteins were visualized by Coomassie staining. Spectra were recorded by an HP 8450-A diode-array spectrophotometer equipped with special cells to record redox potentials simultaneously according to [10]. The redox titrations were carried out at room temperature and pH 7–7.5 under an argon atmosphere. TMPD (50 μM) oxidation and cytochrome *c* (50 μM) oxidizing activity were followed at 546 nm in 50 mM K-phosphate buffer, pH 7.0, at 60°C. Copper content was determined by atomic absorption spectroscopy at 324.8 nm using a Hitachi 180/80 polarized Zeeman atomic absorption spectrophotometer. Caldariella quinone was extracted from *Sulfolobus acidocaldarius* membranes according to [11], stabilized in micellar form and reduced by $NaBH_4$ or dithionite for use as a substrate.

3. RESULTS

Previously we have shown that crude membrane preparations from *Sulfolobus* as well as detergent extracts can utilize reduced cytochrome *c* as an electron donor in cyanide-sensitive respiration [12]. While the membrane was essentially resistant to dissociation of functional respiratory chain complexes by non-ionic detergents like Triton, a sequential extraction using *N*-lauroylsarcosine as described in section 2 allowed the purification of an aa_3 preparation in a catalytically active form. Membrane-associated proteins were removed by pretreatment with KSCN. In a first extraction step with Sarcosyl at low ionic strength, a substantial part of the *b*-type cytochromes was solubilized, whereas the main amount of cytochrome aa_3 was still retained in the membrane. In the final extraction step in the presence of detergent plus NaCl, cytochrome aa_3 was solubilized. By the following fractionated PEG precipitation and gel chromatography, an almost 40-fold purification of aa_3 based on the specific heme content was achieved. Table 1 gives significant data of the purification protocol. Interestingly the ability of crude membranes to oxidize cytochrome *c* was gradually lost during purification of aa_3. Instead, a significant increase of specific activity with TMPD as an artificial electron donor to the fully cyanide-sensitive oxidase was observed. As shown in fig.1 by preparative HPLC, two protein fractions were obtained, of which the first contained most of the heme, as determined by absorption at 426 nm. Calibration of the column with marker proteins (phosphorylase *b*, 188 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsinogen, 25 kDa) gave an apparent relative molecular mass of this detergent-enclosed protein fraction of about 120 kDa. From pyridine hemochrome spectra (not shown), it became evident that this fraction only contains *a*-type heme [13]. Reduced minus oxidized difference spectra of concentrated peak-I fractions showed only one peak in the α-band region with a maximum at 604 nm. Depending on the individual preparation, variable amounts of spectroscopically detectable degradation products were found as a shoulder at 586 nm (fig.2). In the CO-difference spectrum of the same fractions, typical peaks at 429 and 594 nm as well as a trough at 445 nm were indicative for a CO-binding *a*-type cytochrome (fig.3).

The specific copper content of the same peak

Table 1

Typical purification protocol for cytochrome aa_3

Purification state	Total protein (mg)	Spec. heme-*a* content (nmol/mg)	Purif. factor *n*	Cyt-*c* oxid. (U/mg)	TMPD oxid. (U/mg)	Q^{cal} oxid. (U/mg)
Membranes	189.2	0.46	1	0.3–0.4	4.7	0.73
NaCl/sarcos. extract	15.5	3.1	6.7	–	8.3	5.6
FPLC Peak I						
Total	0.68	12.2	26	<0.05	–	19
Maximum	0.16	17	36	0.05	15.7	–

Heme-*a* content was determined using $\Delta\epsilon_{605}$ (red – ox) 12 $mM^{-1} \cdot cm^{-1}$ [18]. Other details as described in section 2; oxidation of reduced caldariella quinone (Q^{cal}) was followed at 351–341 nm, using $\Delta\epsilon$ (red – ox) 1778 $M^{-1} \cdot cm^{-1}$ as determined in our laboratory

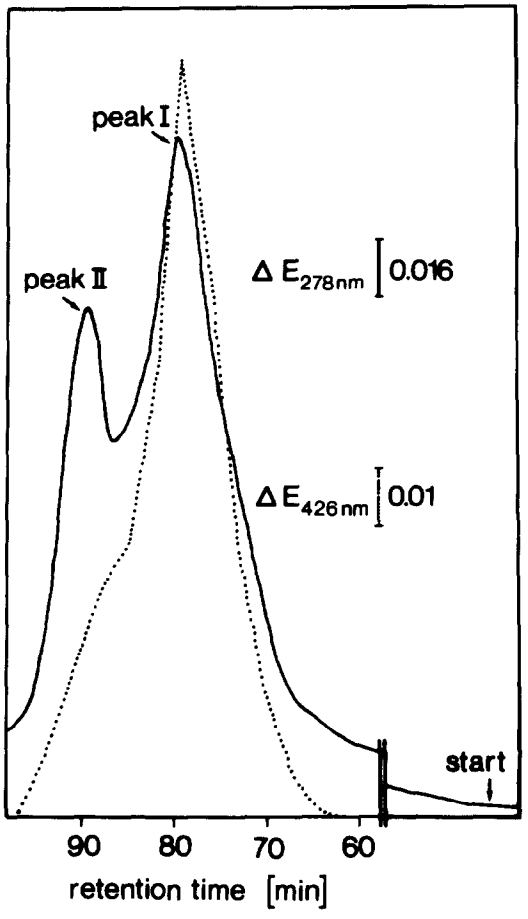

Fig.1. Elution profile of the preparative HPLC column. Continuous line: detection at 278 nm (proteins); dotted line: detection at 426 nm (hemes). Sample volume: 2 ml. The elution buffer contained 0.2 M Na_2SO_4, 10 mM Tris, 0.05% Sarcosyl, pH 7.5. Flow rate: 2 ml/min.

fractions was determined from three independent preparations to be 2 or 3 Cu/aa_3. After dialysis against 1 mM EDTA overnight, only 10% of the copper was removed. However, in neither the crude detergent extract nor the partially purified preparations could an absorption at 830 nm be verified.

SDS-PAGE of the peak-I fractions revealed only one major band with an apparent molecular mass of about 38 kDa (fig.4). Despite the presence of a series of minor contaminant polypeptides, this protein band was the only one which could be directly correlated to the specific heme content in the peak-I elution profile.

As seen from fig.4, the redox titration of the

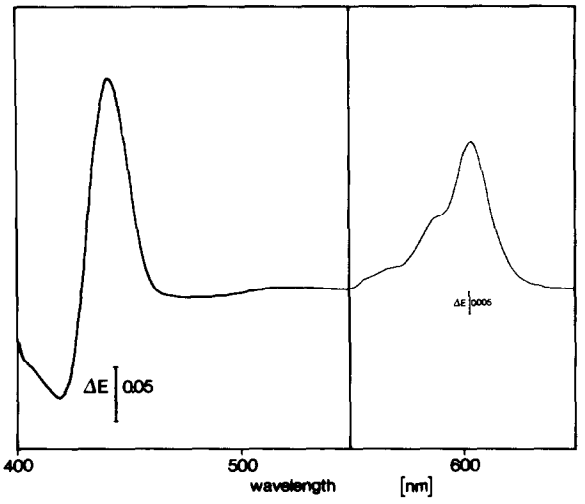

Fig.2. Reduced-oxidized spectrum of a concentrated fraction from the first peak (see fig.1) of the HPLC elution profile; protein concentration: 0.16 mg/ml.

604 nm absorption band in the final preparation was in perfect agreement with the presence of two heme-*a* centers. Deconvolution of the data on the bases of 60% contribution of heme *a* and 40% contribution of heme a_3 [14] to the total absorption resulted in midpoint potentials of about +220 and +370 mV, which coincide nicely with values of known eucaryotic terminal oxidases.

More recently, we discovered that the enrich-

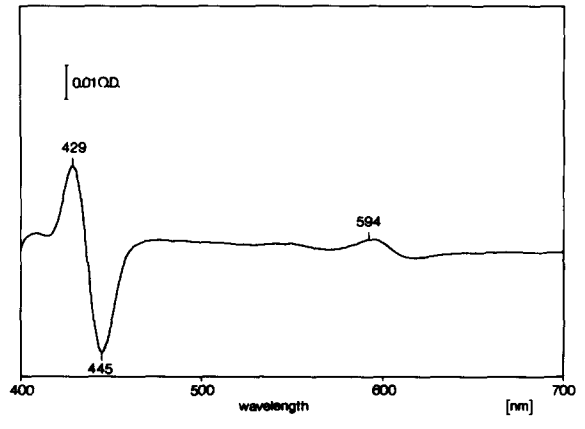

Fig.3. CO-difference spectrum of a concentrated fraction from the first peak (see fig.1) in the HPLC elution profile. The sample was reduced by adding a few grains of sodium dithionite. CO was bubbled for 30 s through the sample; protein concentration: 0.38 mg/ml.

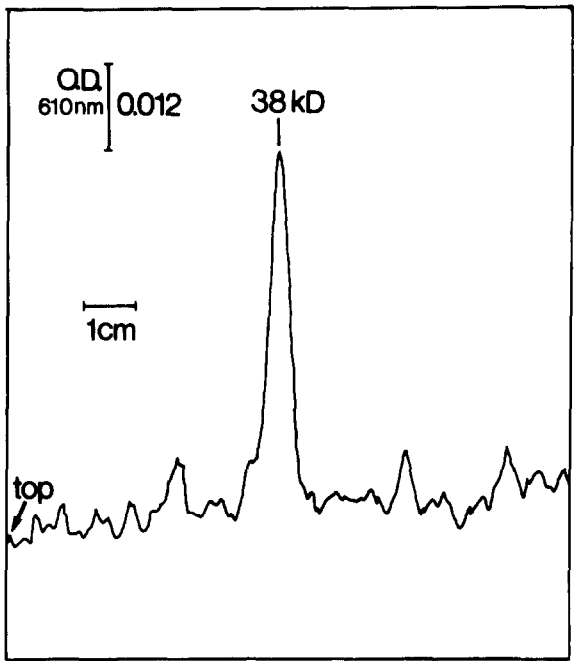

Fig.4. SDS-PAGE of the partially purified cytochrome aa_3. 21 μg cytochrome aa_3 was analyzed on 15% SDS-PAGE, stained with Coomassic blue and scanned by a densitometer.

ment of the specific heme aa_3 content occurred in parallel with the specific activity of this preparation as a completely cyanide-sensitive oxidase of reduced caldariella quinone (see also table 1). The

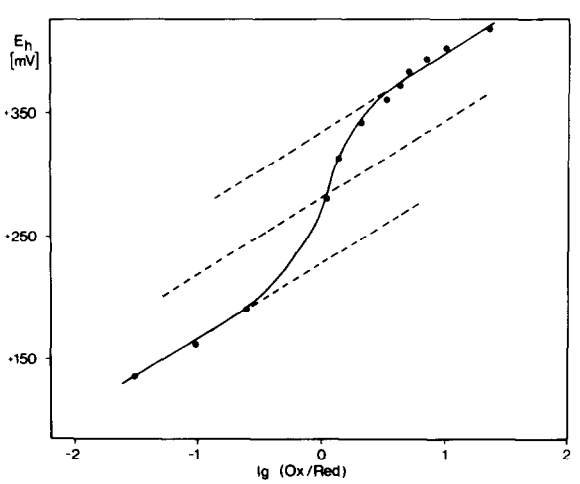

Fig.5. Redox titration of hemes *a* at (604–630) nm; protein concentration: 0.3 mg/ml. Redox mediator concentrations: phenazine methosulfate (40 μM), phenazine ethosulfate (40 μM) and diaminodurol (20 μM). For further details, see section 2 and [10].

latter was extracted from *Sulfolobus* membranes; details of its properties and interaction will be communication elsewhere [15].

4. DISCUSSION

Here for the first time the essential properties of an aa_3-type cytochrome extracted from an extremely thermoacidophilic archaebacterium could be studied in a largely purified preparation. The spectroscopic properties fit well with those from known cytochrome aa_3 as do the redox potentials of the heme-*a* centers which support its function as a terminal oxidase. Although a stoichiometry of at least 2 Cu/aa_3 could be shown, the structural and functional assignment of the two copper atoms needs further investigation. Since the electrophoretic pattern indicates only one major subunit, the latter should host both hemes and both copper atoms; based on the observed molecular mass, the functional complex in its native form might be composed of a dimer as proposed also for the aerobic photoheterotroph *Erythrobacter longus* [16]. *Sulfolobus acidocaldarius* does not contain any *c*-type cytochrome and is lacking a bc_1 complex [4,6]. Consequently, the native electron donor for this terminal oxidase must be different, and it is not surprising that increasing purification results in a gradual loss of cytochrome-*c* oxidase activity, which anyway appeared too small to account for respiratory rates of *Sulfolobus* as observed in vivo. Instead, other donors capable of single electron transfer proved to be valuable substrates, such as TMPD as an artificial donor or reduced caldariella quinone as a natural redox system present in the plasma membrane of *Sulfolobus*. Thus, a direct pathway between the latter and the oxidase may be postulated, similarly to the electron transport system of *E. coli* [17]. Of course, many details are subject to further investigation of this newly described aa_3-type terminal oxidase as, for instance, the influence of the replacement of tetraether lipids in the membrane by detergent molecules, the reconstitutive properties of the complex and the question whether or not it acts as a redox-driven proton pump.

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