Cytochrome $b_{558/566}$ from the Archaon Sulfolobus acidocaldarius
A NOVEL HIGHLY GLYCOSYLATED, MEMBRANE-BOUND B-TYPE HEMOPROTEIN*

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In this study we re-examined the inducible cytochrome $b_{558/566}$ from the archaon Sulfolobus acidocaldarius (DSM 639), formerly thought to be a component of a terminal oxidase (Becker, M., and Schäfer, G. (1991) FEBS Lett. 291, 331–335). An improved purification method increased the yield of the protein and allowed more detailed investigations. Its molecular mass and heme content have been found to be 64,210 Da and 1 mol of heme/mol of protein, respectively. It is only detectable in cells grown at low oxygen tensions. The composition of the growth medium also exerts significant influence on the cytochrome $b_{558/566}$ content of S. acidocaldarius membranes. The cytochrome exhibits an extremely high redox potential of +400 mV and shows no CO reactivity; a ligation other than a His/His-coordination of axial ligands appears likely. It turned out to be highly glycosylated (more than 20% of its molecular mass are sugar residues) and is probably exposed to the outer surface of the plasma membrane. The sugar moiety consists of several O-glycosidically linked mannoses and at least one N-glycosidically linked hexasaccharide comprising two glucoses, two mannoses, and two N-acetyl-glucosamines. The gene of the cytochrome (cbsA) has been sequenced, revealing an interesting predicted secondary structure with two putative α-helical membrane anchors flanking the majority of a mainly β-pleated sheet structure containing unusually high amounts of serine and threonine. A second gene (cbsB) was found to be cotranscribed. The latter displays extreme hydrophobicity and is thought to form a functional unit with cytochrome $b_{558/566}$ in vivo, although it did not copurify with the latter. Sequence comparisons show no similarity to any entry in data banks indicating that this cytochrome is indeed a novel kind of b-type hemoprotein. A cytochrome c analogous function in the pseudoperiplasmic space of S. acidocaldarius is discussed.

Sulfolobus acidocaldarius has been described as an obligate aerobic, facultatively chemolithoautotrophic, hyperthermophilic archaean growing at 75–80 °C and pH 2–2.5 (1). However, it grows much faster under heterotrophic conditions. Its respiratory chain consists of at least one succinate dehydrogenase (2, 3), two Rieske proteins (4), and two terminal oxidases (5, 6). In S. acidocaldarius membranes, three different a-type and two different b-type but no c-type cytochromes can be detected. Most cytochromes can be attributed to either one of the two oxidase complexes. Cytochrome $a_{587}$ (SoxC) and cytochrome $a_{562}$ (SoxB) are part of the SoxABCD quinol oxidase (7, 8), whereas a second cytochrome $a_{557}$ (SoxG) and a cytochrome $b_{562}$ (SoxM) are constituents of the SoxM terminal oxidase complex (9). However, a second b-type cytochrome cannot be attributed to any of these complexes. Previously (10), cytochrome $b_{558/566}$, named after its absorption maxima in the redox difference spectrum, was thought to participate in terminal electron transport due to its positive redox potential of >300 mV, its putative heme and copper content, and the CO difference spectrum typical for an o-type cytochrome obtained with partially purified membrane solubilisates. Because the function of this membrane-residing cytochrome remained obscure and could not be attributed to redox systems of respiratory electron transport, a genetic approach toward its further characterization was taken.

In this report, we present an improved purification method providing high yields of this unusual cytochrome for a fundamentally revised spectroscopic and protein-chemical characterization. Cytochrome $b_{558/566}$ is shown to be highly glycosylated, and the first results of its glycosylation pattern are reported. In addition, an operon has been identified and sequenced encoding this interesting and novel hemoprotein; the transcription pattern is described. From the primary sequence, we concluded that in contrast to previous speculations, this cytochrome has no function as part of a terminal oxidase. A function as an ectoenzyme participating in periplasmic metabolism is discussed on the basis of its significant up- and down-regulation by modifying the growth conditions of the Sulfolobus cells.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions

S. acidocaldarius cells (DSM 639) were grown heterotrophically in a mineral salt medium at 78 °C and at pH 2.5 in a 50-liter fermenter as described previously (11). To establish oxygen-limiting conditions throughout cell growth, aeration was started with a 12 liter/h airflow and was increased in a step-wise manner up to 35 liters/h in parallel to the increasing cell density.

Escherichia coli XL2-blue (Stratagene) was grown at 37 °C in LB medium (12) supplemented with 50 μg of ampicillin/ml. The plasmid pBlue-script II SK* was purchased from Stratagene (Heidelberg, Germany), and pSP64 was purchased from Serva (Heidelberg, Germany).

Cell Harvest and Membrane Preparation

S. acidocaldarius cells were harvested in the late logarithmic phase at an optical density of 1.4–1.5 by continuous flow separation using a
Westfal separator. The cells were resuspended in 50 mM Mes, 1 mM EDTA, pH 5.5; centrifuged at 7800 × g; resuspended in the same buffer; frozen in liquid nitrogen; and stored at −70 °C. Typical yields were 2.4–3 g of wet cell material per liter of culture medium. Cells were disrupted in a Manton-Gaulin press, and membranes were prepared as described previously (11).

Isolation of Cytochrome b₅₅₈/5₆₆

Hydrophobic Interaction Chromatography—For chromatopreextraction, the membranes were diluted to a protein concentration of 10 mg/ml in 50 mM potassium phosphate, 30 mM sodium pyrophosphate, pH 7.5. After stirring for 1 h at room temperature, the suspension was centrifuged (120,000 × g for 1 h at 4 °C). The membrane pellets were resuspended in 50 mM Tris/HCl, pH 7.3, and AS was added to a final concentration of 500 mM. DM was added to a final concentration of 20 mM. After stirring for 1 h at room temperature, the solution was centrifuged (as above) to remove insoluble components. The following steps were performed at 4 °C. A saturated solution of AS was slowly added to the supernatant to achieve 50% saturation. This solution was applied to a propyl-agarose column (Sigma P-5268; 1.5 cm in diameter, 16 cm in length) equilibrated with 50% saturated AS, 1 mM DM, 25 mM Tris/HCl, pH 7.3, at 0.5 ml/min. Subsequently, the column was washed with 120 ml of the same buffer. Elution was performed by a step-wise decrease in pH 7.3, at 0.5 ml/min. Subsequently, the column was washed with 120 ml of 0.5% SB-12 instead of DM. A minor fraction of the cytochrome b₅₅₈/5₆₆ was eluted at step 1 with large amounts of contaminating proteins. The major part eluted at step 2 in a much purer form. Fractions from step 2 were collected and concentrated by ultrafiltration on a PM-30 membrane (Amicon, Beverly, MA) to 3–5 ml.

Gel Filtration—The concentrated cytochrome fraction was further purified by gel filtration (50 mM Tris/HCl, 0.5 mM DM; flow, 1 ml/min; Highload Superdex 200, Amersham Pharmacia Biotech). Elution was monitored by the absorbance at 280 nm and 430 nm. Fractions containing the cytochrome b₅₅₈/5₆₆ were pooled and concentrated by ultrafiltration (as above). Further contaminating proteins were removed by digestion with trypsin (about 1 mg of trypsin/10 mg of protein) and another gel filtration step (as above). If the cytochrome preparations were to be used for sugar analyses other than the chemical deglycosylation procedure (see below), both gel filtrations were carried out with 0.5% SB-12 instead of DM.

Protein and Heme b Determination

Protein concentrations were determined by the modified Lowry method in the presence of detergents (13) using bovine serum albumin as standard or with the Bio-Rad D₆₅ protein assay (Bio-Rad). Heme b concentrations were determined as pyridine hemochromogen according to the method described by Schaeger and von Jagow (18) using a 18.5% separating gel, a 10% spacer gel, and a 4% stacking gel, all with 3% bisacrylamide as cross-linker. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. Glycoprotein staining was performed as described in Ref. 19.

Deglycosylation and Sugar Analysis

Chemical deglycosylation was performed using a commercially available kit (K-500, Oxford GlycoSystems, Abingdon, United Kingdom) that cleaves both O- and N-linked sugar residues by the use of trifluoromethane sulfonic acid. For total sugar analysis, purified cytochrome b₅₅₈/5₆₆ (1 mg) was hydrolysed in 1 ml of 2 M CF₃COOH (100 °C for 4 h), reduced (NaBH₄) per-O-acetylated, and analyzed by GLC-MS. A third aliquot (1 mg) was used to determine N-linked sugars by heating with hydrazine (1 M, pH 12) for 12 h. After the reagent was evaporated under reduced pressure, sugars were N-acetylated (1 ml of saturated aqueous NaHCO₃) and 15 μl of acetic acid anhydride for 30 min. The N-acetylation was repeated twice. After neutralization with ion exchanger (IR 120, H⁺ form), the crude oligosaccharide preparation was purified using a gel permeation chromatography column (2 × 112 cm, Sephadex G-10) in water monitoring the eluate at 206 nm (Uvicord, Amersham Pharmacia Biotech). Oligosaccharide fractions were combined and lyophilized. One aliquot (5%) of the oligosaccharide was analyzed for its components by GLC-MS analysis described above.

Chemical Cleavage and Amino Acid Sequencing

Purified samples of either intact cytochrome b₅₅₈/5₆₆ or of deglycosylated cytochrome (about 20–50 μg) were cleaved by 0.75 μl cyanogen bromide in 75% formic acid in a final volume of 400 μl. The reaction mixture was incubated for 1 h at 37 °C, diluted by addition of 400 μl of distilled water, frozen at −70 °C, and dried in a Speed Vac concentrator. The dried samples were resuspended in distilled water, and the evaporation was repeated. Finally, samples were resuspended in 20 μl of water.

The fragments were separated by SDS-PAGE, blotted on polyvinylidene difluoride membranes, and stained with Coomassie Brilliant Blue R-250. Bands were cut out, and the amino acid sequence was determined by automated microscale Edman degradation.

DNA Techniques

All general cloning procedures were performed according to standard methods (12). Genomic DNA of S. acidocaldarius was prepared accord-

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1 The abbreviations used are: Mes, 2-(N-morpholino)ethanesulfonic acid; AS, ammonium sulfate; DM, N-dodecyl-β-maltoside; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; SB-12, N-dodecyl-N,N-dimethyl-ammonio-3-propane-sulfonate; GLC, gas liquid chromatography; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s).
Cytochrome b<sub>558/566</sub> from Sulfolobus acidocaldarius

ing to the method of Marmor (22), and plasmid DNA was isolated from E. coli cells on a small scale by lysis with lysosyme and Triton X-100 (23) or on a large scale by anion exchange chromatography (Jetstar, Genomed, Bad Oeynhausen, Germany).

All nonradioactive labeling, blotting, hybridization, and chemiluminescence detection techniques were performed as described in detail in Ref. 24.

**Cloning Procedure**

The longest amino acid sequence obtained (MYEVDTAGIYITPVAVAA) was chosen to derive two oligonucleotide probes (probe 1, 5'-CCWGWCGT-RTCWCAYCTCRTACAT-3'; probe 2, 5'-ATATAAYAAYAACWCTGTC-WGC-3'). The oligonucleotides were 3'-end-labeled with digoxigenin-11-dUTP (24).

*S. acidocaldarius* genomic DNA was digested with various restriction enzymes, separated on agarose gels, and blotted by vacuum onto nylon membrane (Hybond-N, Amersham Pharmacia Biotech). A 2.8-kb PstI/XhoI fragment positive with probe 1 was chosen for insertion into pSP64 and cloning in E. coli XL2-blue (plasmid pCBS1). Transformation into E. coli cells was performed as described in Ref. 25. A 0.9-kb SspI fragment out of the larger 2.8 kb fragment also positive with probe 1 was subcloned into pBluescript II SK × (plasmid pCBS2). Sequencing of the fragments was performed nonradioactively with Thermo Sequenase kit (Amersham Pharmacia Biotech) using a GATC 1500 direct blotting electrophoresis device (GATC) and the DIG system from Boehringer Mannheim as described in Ref. 26.

**Isolation of RNA and Northern Blot Hybridization**

Total RNA was extracted from S. acidocaldarius cells with guanidinium isothiocyanate and phenol using a commercially available kit (Roti-Quick-Kit, Carl Roth, Karlsruhe, Germany). Northern blotting and hybridization was performed as in Ref. 24.

Two RNA probes were produced by in vitro transcription of linearized DNA template (plasmid pCBS2 cut with XhoI or with BglII) with T7 polymerase and the DIG RNA labeling kit (Boehringer Mannheim) as described in Refs. 27 and 24. Hybridization and chemiluminescence detection was also performed as in Ref. 27.

**Influence of Growth Medium on Cytochrome b<sub>558/566</sub>**

To examine the influence of different growth media, S. acidocaldarius cells were grown on a small scale in a 1-liter fermenter under oxygen-limiting conditions. Select yeast extract was purchased from Life Technologies, Inc.; neutralized bacteriological peptone was from Oxoid (Basingstoke, United Kingdom); casein (enzymatic hydrolysate), L-leucine, and L-isoleucine were from Sigma; and 2-oxoglutaric acid was from Fluka (Deisenhofen, Germany).

Cells were harvested by centrifugation at 7800 × g. Cells were disrupted for 5 min by ultrasonification, and membranes were prepared by down-scaling the procedure described previously (11). The cytochrome b<sub>558/566</sub> content of the membranes was calculated based on the ascorbate-reduced minus ferricyanide-oxidized difference spectrum using an extinction coefficient of ε<sub>566–590</sub> = 11,200 M<sup>−1</sup> cm<sup>−1</sup>, determined by pyridine hemochromogen. The protein concentration of the membranes was determined as described above.

**RESULTS**

**Preparation of Cytochrome b<sub>558/566</sub>**—The cytochrome composition of the membranes changes according to the oxygen level at which the cells are grown (7). Under low oxygenation, the expression of cytochrome b<sub>558/566</sub> strongly increases, whereas high oxygen levels (maximum air flow) suppress the production of this cytochrome nearly completely (15).

To isolate the protein, purified membranes were subjected to chaotropic pre-extraction with sodium pyrophosphate, which removed loosely bound proteins from the membrane. DM was used for solubilization as this detergent does not induce spectral changes of α- or b-type cytochromes (7). High ionic strength, achieved by addition of 500 mM AS during the solubilization step, improved the efficiency of the subsequent chromatographic preparation steps.

The following hydrophobic interaction chromatography and gel filtration (see “Experimental Procedures”) separates the cytochrome b<sub>558/566</sub> from other cytochromes. Fig. 1 documents the process of purification resulting in a single protein band (right lane), susceptible to Coomassie Blue staining but not to silver staining. This unusual behavior led us to suspect that cytochrome b<sub>558/566</sub> might be a glycoprotein. Staining for sugar residues (not shown) confirmed this assumption. It was further corroborated by the observation that alkali treatment caused a considerable decrease of apparent molecular mass (not shown).

This unexpected result also explains the observation that cytochrome b<sub>558/566</sub> is resistant to protease degradation (trypsin or V8; data not shown), a feature that was then included in the purification procedure. A tryptic digestion cleaved remaining contaminating proteins, and the final gel filtration removed these cleaved proteins as well as the added trypsin (Fig. 1, lane 5). The use of SB-12 instead of DM did not induce any spectral changes of cytochrome b<sub>558/566</sub> but led to a minor decrease in long-term stability and a slight increase in susceptibility to proteolytic digestion.

**Spectral Characterization**—In native membranes, cytochrome b<sub>558/566</sub> is the only cytochrome present in significant amounts that is readily reducible by ascorbate (6, 15). Difference spectra (reduced minus oxidized) of the purified cytochrome b<sub>558/566</sub> resembled the spectral features of ascorbate-reduced S. acidocaldarius membranes, exhibiting maxima at 566 and 558 nm and a minimum at 561 nm in the a-band region and lower maxima at 530 and 538 nm in the ε<sub>558/566</sub>-region of the visible spectrum (Fig. 2). The Soret band in the reduced minus oxidized difference spectrum was located at 430 nm. In the reduced spectrum it was found at 430 nm as well, whereas it shifted to 419 nm in the oxidized spectrum. The differential extinction coefficients are ε<sub>566–575</sub> = 10,800 M<sup>−1</sup> cm<sup>−1</sup>, ε<sub>566–590</sub> = 11,200, and ε<sub>410–439</sub> = 65,900 M<sup>−1</sup> cm<sup>−1</sup> based on the heme b content determined as pyridine hemochromogen. At liquid nitrogen temperature, the α-bands clearly resolved into two distinct and narrow peaks at 563 and 553 nm, whereas the β-bands moved to 527 and 534 nm (data not shown).

The pyridine hemochrome spectrum (Fig. 2) with a maximum at 556 nm indicates that the purified compound contained only heme b. In the above spectral region, neither the isolated cytochrome, prepared according to the described procedure, nor native membranes displayed a CO-inducible difference spectrum in the reduced state, thus indicating a hexa-coordinated heme center without exchangeable ligand positions. This is in sharp contrast to former observations, in which the cytochrome had been extracted from membranes immersed in an imidazole buffer during detergent extraction (10). Actually, prolonged exposure of membranes or detergent extracts to imidazole (0.1–
The concentration was 0.67 mg/ml.

Top curve: oxidation with K₃[Fe(CN)₆] and kept in the oxidized state at room temperature. Protein concentration was 0.35 mg/ml. Middle curve: pyridine hemochrome difference spectrum of the respective preparation. Bottom curve: CO difference spectrum (dithionite reduced with CO minus (dithionite reduced)) of cytochrome b₅₅₈/₅₆₆ in imidazole buffer (0.1 M imidazole, pH 7.0, 0.5% SB-12, 30% glycerol). Protein concentration was 0.67 mg/ml.

0.6 M) causes the appearance of a confluent α-peak at 560–562 nm (28) with a high absorption coefficient, a behavior that we were able to reproduce with the purified cytochrome. Under the latter conditions, a reduced minus oxidized difference spectrum resulted, which upon addition of CO clearly indicated the formation of a carbon monoxide compound yielding a sharp trough at 560 nm (556 nm at liquid nitrogen temperature) in the difference spectrum (reduced plus CO, minus reduced) (Fig. 2).

Obviously, imidazole promotes a ligand exchange and conformational distortion of the protein, facilitating the binding of CO to the heme center. This process is reversible, however, as demonstrated in independent experiments. Removal of imidazole, for example, by buffer exchange during chromatography restored the native situation with respect to spectral properties and eliminated any CO reactivity. This lack of CO reactivity and the fact that direct reoxidation of the easily reducible cytochrome by molecular oxygen could not be observed exclude a function as part of a terminal oxidase complex. The cytochrome is most stable when kept in the reduced form. If it is oxidized with K₃[Fe(CN)₆] and kept in the oxidized state at neutral pH for a prolonged time, the cytochrome is damaged irreversibly, as can be seen directly from a shift of the Soret band from 419 to 405 nm (data not shown). To minimize oxidative damage, the samples used for the EPR measurements were frozen shortly after oxidation with cerium(IV) salts.

The most important feature of the EPR spectrum (Fig. 3) is a highly anisotropic low spin heme signal with a g value of 2.09 (tentatively), due to a hexa-coordinated heme iron. The g value is superimposed by another signal, possibly caused by loosely bound copper.

Although the most prominent feature of the spectrum is a sharp high-spin signal at g = 6.05, this is most likely a preparation-induced property. As mentioned above, isolated cytochrome b₅₅₈/₅₆₆ undergoes decay when kept in the oxidized state. Even if only a minor amount of the cytochrome is damaged, it will be enough to cause an apparently strong high spin resonance signal around g = 6. Due to the intense g value of the low spin heme, the high spin signal can be estimated to account for only 10–15% of the amount of the low spin signal.

**Molecular Properties**—When using a pH 2–6 polyacrylamide gel, the PI was found to be in the range of 4–4.5 (data not shown). The apparent molecular mass (SDS-PAGE) was about 66 kDa (Fig. 1). This is in good agreement with the molecular mass determined by mass spectrometry: the positive ion MALDI-time of flight mass spectrum of cytochrome b₅₅₈/₅₆₆ recorded in the linear mode is shown in Fig. 4. The spectrum comprises two prominent mass peaks representing the single- and double-charged quasimolecular ions at m/z = 64,210 and 32,091, respectively.

Chemical deglycosylation led to a single band of about 55 kDa (Fig. 5, lane 5), cytochrome b₅₅₈/₅₆₆, as prepared; lane 6, after deglycosylation, which is in fair agreement with the molecular mass of 50,736 Da according to the gene-derived sequence (see below). The cytochrome b₅₅₈/₅₆₆ precipitated almost completely during the neutralization step of the protocol, so that the soluble fraction of the deglycosylation procedure contains no protein (Fig. 5, lane 7). Bovine serum albumin used as a negative control (Fig. 5, lane 8) showed no decrease in molecular mass (lane 9), whereas the deglycosylation of the positive control α₁-glycoprotein (lane 2) led to the expected decrease in molecular mass (lanes 3 and 4). The deglycosylation of the cytochrome worked best with samples prepared in buffer containing DM as detergent. Preparations with SB-12 as detergent did not show a decrease in molecular mass when the same procedure was applied (data not shown). On the other hand, cytochrome preparations containing SDS (samples cut out and eluted from an SDS-polyacrylamide gel) could be deglycosylated, but only at the cost of great losses of protein (data not shown).

Total sugars analysis of purified cytochrome b₅₅₈/₅₆₆ resulted in mannose, glucose, and 2-deoxy-2-N-acetylamino-β-glucose (GlcNAc) in relative proportions of approximately 7:2:2. Upon analysis of O-linked sugars, only mannose was detected. N-linked sugars were analyzed after hydrazinolysis and found to be exclusively present in a hexasaccharide fragment obtained after gel permeation chromatography. This oligosaccharide consisted of Man, Glc, and GlcNAc in a 1:1:1 ratio. Further analysis of this oligosaccharide is presently under way in our laboratory.

The heme content was found to be in the range of 0.6–0.8 mol of heme/mol of protein. This is in good agreement with the iron content of 0.8 mol/mol of protein. Although variable copper contents of 0.2 to 0.5 mol of copper/mol of protein could be found in the cytochrome preparations, this amount was totally removable by dialysis and is considered as unspecifically bound metal ion.

Redox titrations of S. acidocaldarius membranes had already suggested a b-type cytochrome with a potential >300 mV. By multiple titrations of the purified cytochrome b₅₅₈/₅₆₆, an exact value of +400 ± 5 mV at pH 6.5 could be established; in accord with a single-heme cytochrome, the slope of the Nernst plots was 60 mV. Also, in these experiments, an increasing instability during prolonged exposure to strong oxidants (ferricyanide) was observed.

**Isolation and Sequencing of the Gene**—Direct N-terminal amino acid sequence analysis of purified cytochrome b₅₅₈/₅₆₆ after SDS-PAGE and electroblotting onto polyvinylidene difluoride membrane failed, irrespective whether or not the sample was deglycosylated. Only CNBr cleavage of a deglycosylated sample of cytochrome b₅₅₈/₅₆₆ produced a peptide fragment sufficient for synthesis of oligonucleotide probes, suitable for
Southern blot hybridization. Restriction enzyme-digested chromosomal DNA from *S. acidocaldarius* gave positive signals only with probe 1. Probe 2 did not give any signal due to two protein sequencing errors, detected after the gene had been sequenced. The gene, including its flanking regions, was cloned and sequenced from a 2.8-kb *Pst*I/*Xba*I-fragment and from a 0.9-kb *Ssp*I/*Ssp*I-fragment subclone as described under “Experimental Procedures.”

**Gene Organization and Transcription Analysis**—Fig. 6 demonstrates the organization of the cytochrome *b*$_{558/566}$ operon. Three open reading frames could be found within the sequenced DNA region: an open reading frame from nucleotide 179 to 1564 encoding *cbsA* (cytochrome *b*$_{558/566}$ from *Sulfolobus*), a second open reading frame from nucleotide 1564 to 2490 encoding *cbsB*, and a third open reading frame (orf1) starting at nucleotide 2568 and extending beyond the end of the sequenced clone at nucleotide 2769. Fig. 7 shows both the gene and the protein sequences of cytochrome *b*$_{558/566}$ and of an extremely hydrophobic protein (CbsB) not identified by protein chemistry so far. Some characteristic features are emphasized, such as, for example, a putative Box A and a possible terminating structure following the *cbsB* gene; possible transmembrane sections are underlined, and the only three histidines that are candidates for heme *b* coordination are circled.

Northern blot analysis using a homologous probe derived by

*in vitro* transcription of a region comprising the *cbsA* and *cbsB* sequences revealed a single mRNA transcript of 2.5 kb (data not shown). A second probe specific only for *cbsB* hybridized to a single mRNA transcript of the same size (data not shown; location of probes is shown in Fig. 6). The length of this transcript corresponds well with the full length of the *cbsAB* genes (2312 bp). It might also include orf1, leading to a transcript of at least 2591 bases. However, that would imply that the stop codon of orf1 is located immediately after the end of the sequenced clone. In addition, the above-mentioned terminator suggests that orf1 is transcribed independently.

**Amino Acid Sequence and Composition of Cytochrome b$_{558/566}$**—The *cbsA* gene product contains 462 amino acids and has a predicted molecular mass of 50,736 Da. However, sequence comparisons with protein data bases revealed no significant similarity to any other cytochrome or known hemoprotein.

In accordance with the high degree of glycosylation of the protein (>20%), the amino acid composition displays a high content of serine and threonine (>9% each, Table 1 and Fig. 6). In addition, 16 amino acid triplet sequences of NXS or NXT denote possible N-glycosylation sites.
CbsA contains three histidines (residues 82, 190, and 307) and nine methionines in addition to the starting methionine. One of the former serves as ligand for the heme group. As discussed below, the other ligand of the heme might be either His or Met.

A hydrophobicity plot (Fig. 8) of CbsA reveals a hydrophobic stretch at the N terminus and a second one at the C terminus; both of these may serve as membrane anchors, with the hydrophilic bulk of the hemoprotein reaching out into the aqueous phase.

The cbsB gene product contains 310 amino acids and has a predicted molecular mass of 35,155 Da. The hydrophobicity profile of CbsB displays an extreme hydrophobicity, suggesting at least 9 transmembrane helices (Fig. 8), with only short intervening sequences. Remarkable is its enormous content of leucine and other apolar amino acids (~53%); however, other typical sequence signatures could not be detected. Although it was cotranscribed with the cbsA gene, the respective protein band was not detected in any of the cytochrome b558/566 preparations.

Influence of Growth Medium on Cytochrome b558/566 Content of Membranes—Previous experiments showed that even under oxygen-limiting conditions, S. acidocaldarius membranes contain only barely detectable amounts of cytochrome b558/566 when the cells had been grown in a medium largely deprived of nitrogen sources, i.e. yeast extract and l-glutamate were omitted and ammonium sulfate was reduced from 10 to 1.5 mM.2 This was the basis for experiments to grow S. acidocaldarius in media containing different carbon and nitrogen sources. The standard medium contained sucrose (0.2%), ammonium sulfate (10 mM), glutamate (5.8 mM), and 0.1% yeast extract. The most significant effects were perceived by replacement of the yeast extract. Under oxygen-limiting conditions, when the yeast extract was substituted for by peptone (data not shown), the content of cytochrome b558/566 doubled; when it was substituted for by casein hydrolysate, the content more than tripled (Fig. 9, column 4). Because the main component of peptone and casein are enzymatically digested proteins, a few selected amino acids were tested as to whether they could produce the same effect when they replaced the yeast extract. A mixture of L-leucine and L-isoleucine (each 1.25 mM in the growth medium) also caused a doubling in cytochrome b558/566 content (data not shown), whereas L-isoleucine alone (2.5 mM) caused more than a tripling of the content (Fig. 9, column 5). The content of the other cytochromes did not change significantly under any of these conditions. When the yeast extract was replaced by l-tryptophan, no growth occurred.

2 Christian L. Schmidt, unpublished results.
The purification method by hydrophobic interaction chromatography previously described in Ref. 29 and further improved here led to a significant increase in the yield of cytochrome \( \text{b}_{558/566} \) preparations, unexpectedly revealing its character as a membrane-residing glycoprotein. Most likely due to its high glycosylation, the protein does not stain with silver, a property that in previous studies (10) caused the erroneous attribution of the spectrum to a well-staining 30-kDa band that in reality represented a copurifying contaminant polypeptide. The correct assignment to a 66-kDa Coomassie-staining band was now confirmed by mass spectrometry, which gave a single signal at 64.2 kDa for the molecular ion, thus simultaneously illustrating the homogeneity of the preparation. Consequently, the revised estimate of its heme content amounts to 1 heme per protein monomer, as determined by reversed-phase HPLC. No tightly bound copper was associated with these preparations.

**Discussion**

The two putative transmembrane helices (tmh) of CbsA are marked. Because all above growth media contained \( \text{L}-\)glutamic acid as a second amino acid source, the effect of omitting both yeast extract and \( \text{L}-\)glutamic acid from the standard growth medium was checked. In cells grown on sucrose as sole carbon source and ammonium sulfate as sole nitrogen source (minimal medium) the cytochrome \( \text{b}_{558/566} \) content decreased to about 10% of the standard growth medium (Fig. 9, column 1), whereas the other cytochromes remained in the normal range. When casein hydrolysate was added to this minimal medium, the cytochrome \( \text{b}_{558/566} \) content more than doubled compared with the standard medium (Fig. 9, column 6), whereas it dropped to a level of about 60% when replaced by \( \text{L}-\)isoleucine (column 7). However, it should be noted that in absence of \( \text{L}-\)glutamate, it was possible to adapt the cells only to 1.5 mM \( \text{L}-\)isoleucine but not to 2.5 mM, as in its presence. Thus, it is a synergistic effect of \( \text{L}-\)glutamic acid and casein hydrolysate or of \( \text{L}-\)glutamic acid and \( \text{L}-\)isoleucine that causes the tremendous increase in the cytochrome \( \text{b}_{558/566} \) content. Experiments to examine whether the carbon or the nitrogen moiety of glutamic acid is responsible for its positive effect on the cytochrome content failed; it was not possible to grow cells in a medium containing 2-oxoglutaric acid instead of glutamic acid.

**Table I**

| Amino acid | No. of residues | Percentage | Amino acid | No. of residues | Percentage |
|------------|----------------|------------|------------|----------------|------------|
| Ser        | 44             | 9.52       | Leu        | 49             | 15.81      |
| Thr        | 43             | 9.31       | Ile        | 46             | 14.84      |
| Gly        | 39             | 8.44       | Ser        | 31             | 10.00      |
| Leu        | 39             | 8.44       | Phe        | 23             | 7.42       |
| Ile        | 35             | 7.58       | Tyr        | 21             | 6.77       |
| Val        | 35             | 7.58       | Gly        | 19             | 6.13       |
| Ala        | 31             | 6.71       | Val        | 19             | 6.13       |
| Asn        | 29             | 6.28       | Met        | 14             | 4.52       |
| Pro        | 29             | 6.28       | Pro        | 13             | 4.19       |
| Tyr        | 27             | 5.84       | Thr        | 13             | 4.19       |
| Phe        | 19             | 4.11       | Asn        | 12             | 3.87       |
| Glu        | 17             | 3.68       | Lys        | 11             | 3.55       |
| Trp        | 17             | 3.68       | Ala        | 10             | 3.23       |
| Asp        | 13             | 2.81       | Glu        | 10             | 3.23       |
| Lys        | 13             | 2.81       | Arg        | 7              | 2.26       |
| Gln        | 10             | 2.16       | Asp        | 5              | 1.61       |
| Met        | 10             | 2.16       | His        | 3              | 0.97       |
| Arg        | 9              | 1.95       | Gln        | 2              | 0.65       |
| His        | 3              | 0.65       | Trp        | 2              | 0.65       |
| Cys        | 0              | 0.00       | Cys        | 0              | 0.00       |

\( ^a \) Length, 462 amino acids; molecular weight, 50,736; statistical pI, 4.79.

\( ^b \) Length, 310 amino acids; molecular weight, 35,155; statistical pI, 9.10.
knowledge, the composition and the structure of the sugar moiety has not been elucidated in these examples. Hence, this study is the first time that the sugar part of a glycoprotein from the genus *Sulfolobus* has undergone close examination.

Sequencing of the *cbs* operon provides interesting insights into the primary structure of this novel hemoprotein. The two hydrophobic stretches at the N and C termini (Fig. 8) are thought to serve as membrane anchors, whereas the hydrophilic major part of the protein is highly glycosylated and therefore is supposed to be exposed to the outer surface of the plasma membrane. Notably, secondary structure predictions for this middle part suggest mainly loop regions and β-strands, with less than 10% α-helix contribution. Possible glycosylation sites are mainly found in the predicted loop regions. High glycosylation of this middle part probably serves as a stabilizing element within acidic environments and, more important, as a protection against proteolytic attack. In fact, only after deglycosylation was the cytochrome accessible to proteolytic digestion *in vitro*.

Because the N terminus of cytochrome *b* 558/566 was not accessible to chemical amino acid sequencing, the actual N terminus in the native protein is still matter of debate. Because the sequence after the second methionine (Met 74) in the DNA-derived protein sequence could be obtained from a CNBr-cleaved fragment, the real N terminus must be located somewhere between Met 1 and Met 74. It appears possible that part of the first amino acids might form a leader sequence targeting the cytochrome to the membrane and/or causing its glycosylation. If a leader sequence is cleaved from the protein, the relative sugar content of the glycoprotein would even increase in order to account for the molecular mass determined by mass spectrometry. In addition, only the C-terminal α-helix would then function as a membrane anchor in the mature protein.

The extremely hydrophobic protein encoded by *cbsB* probably enhances the linkage of the cytochrome to the membrane. It could also establish a link to an additional protein complex of yet unknown function. *CbsB* could not be identified on the protein level so far. Nevertheless, the common transcript of *cbsA* and *cbsB* strongly suggests that both proteins form a functional unit. Due to the great difference in hydrophobicity, the two proteins are probably dissociated during the purification procedure.

Properties and Function—An important alteration in the purification procedure was to avoid imidazole in any buffer. Imidazole presumably is able to alter the ligation of the heme-iron, replacing a weaker ligand provided by the amino acid side chains of the protein. As such, 9 out of 10 methionines located in the central hydrophilic core of the protein may be envisaged. Thus, imidazole treatment appears likely to pave the way for a further ligand exchange against CO. Cytochrome *b* 558/566 prepared according to the described protocol does not form a CO compound, but it was shown to reversibly assume this property after exposure to 0.1 M imidazole. Whether a his-His or a His/Met ligation is present in the cytochrome cannot be decided from the EPR spectra, because the only unambiguously detected g value falls into the overlap region for both types of ligands. In analogy to c-type cytochromes with His/Met ligation, the novel cytochrome *b* 558/566 exhibits a reduction potential >+350 mV, whereas species with his-His ligation have reduction potentials in the range of 0 to −400 mV (33). Furthermore, c-type cytochromes with His/Met ligation distorted by, for example, imidazole treatment also become capable of binding CO, whereas integral c-type cytochromes are not (34). In this context, the observed CO binding behavior of cytochrome *b* 558/566 can be sufficiently explained. But also, even more unusual types of ligation cannot definitely be excluded.

For example, cytochrome *f* of the chloroplast cytochrome *b* f complex from *Brassica rapa* binds heme with the N terminus of the polypeptide chain as secondary ligand (35). However, the g 1 value of 3.13 in the EPR spectrum of cytochrome *b* 558/566 is not in accord with this type of ligation. Regardless how the heme group is coordinated, it is certainly not embedded within the membrane-spanning regions of the protein, because all three histidines providing at least the primary axial ligand to the heme iron are also located in the large hydrophilic core of the protein.

Although data bank searches did not disclose any sequence similarities to known hemoproteins, the properties of the cytochrome *b* 558/566 may give a hint to a possible physiological function. Due to the likely pseudoperiplasmic location and the above-mentioned characteristics of the heme center, we propose a cytochrome c-like function analogous to the situation in *Paracoccus denitrificans* (36) linking pseudoperiplasmic redox metabolism to membrane-residing electron transport systems. Nothing is known in detail about pseudoperiplasmic metabolism of *Sulfolobus* as yet; however, this hypothesis fits with the observation that cytochrome *b* 558/566 is significantly up-regulated under certain organotrophic growth conditions (Fig. 9) in combination with low oxygen tension. Because these essentially involve the supply of specific amino acids or protein hydrolysates, future experiments have to prove whether or not such pseudoperiplasmic redox reactions occur in their metabolism. The prominent effect of L-isoleucine on the abundance of cytochrome *b* 558/566 may additionally be due to the fact that CbsB contains an unusual large number of isoleucine residues. If its synthesis is limited by the availability of this amino acid, then that of CbsA, which is cotranscribed, would also be affected. Whether or not the synthesis of cytochrome *b* 558/566 (CbsA) is regulated solely on the level of transcription remains to be investigated.

The unusual properties of cytochrome *b* 558/566 could possibly define a novel class of hemoproteins. First, the molecular mass is unexpectedly high for a monoheme cytochrome. Second, the visible spectrum comprising two distinct α-band peaks (563 and 553 nm at liquid nitrogen temperature) is surprisingly complicated for a hexa-coordinated, monoheme cytochrome. Third, cytochrome *b* 558/566 is glycosylated. Very few glycosylated and membrane-bound cytochromes are known. Probably the best examined is flavocytochrome *b* 558, formerly named cytochrome *b* 245, being part of a NADPH oxidase that generates superoxide in phagocytic cells (respiratory burst oxidase) (37, 38). Although it is tempting to assume a similarity to cytochrome *b* 558/566, the difference in redox potentials (+400 compared with −245 mV) clearly shows that these two cytochromes share no common function. Another example is the ferrieductase system from *Saccharomyces cerevisiae* (39), which exhibits striking similarities to the same NADPH oxidase. Further examples include a member of the P-450 system (40) and porcine thyroid peroxidase (41). All of these examples have been found in the domain of euarya. In the domain of archaea, cytochrome *b* 558/566 is the first protein displaying this combination of properties. This provides strong evidence that this protein does not belong to any known cytochrome family but is indeed a novel kind of prokaryotic hemoprotein, the physiological function of which has to be elucidated.

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