A Gene Encoding the Proteolipid Subunit of Sulfolobus acidocaldarius ATPase Complex*

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An analysis of genes for the major two subunits of the membrane-associated ATPase from an acidothermophilic archaebacterium, Sulfolobus acidocaldarius, suggested that it belongs to a different ATPase family from the Fo-ATPase (Denda, K., Konishi, J., Oshima, T., Date, T., and Yoshida, M. (1988) J. Biol. Chem. 263, 17251-17254). In the same operon of the above two genes we found a gene encoding a very hydrophobic protein of 101 amino acids (M_t = 10,362). A proteolipid was purified from the membranes of this bacterium in which partial amino acid sequences matched with the sequence deduced from the gene. Significant amino acid sequence homology and a similar hydrophathy profile appeared when the sequence was compared with the 8-kDa proteolipid subunit of FoF1-ATPases. It is about 30 amino acids larger than the 8-kDa proteolipid and has a small (11-amino acid) repeat sequence. However, it is distinct from the 16-kDa proteolipid subunit of an eukaryotic vacuolar H+-ATPase (Mandel, M., Moriyama, Y., Hulmes, J. D., Pan, Y.-E., Nelson, H., and Nelson, N. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5521-5524).

FoF1-ATPases catalyze ATP synthesis with coupled proton flow across membranes and are composed of two parts (1-3). Fo-ATPase, the water-soluble catalytic part, has a subunit structure epsilon epsilon gamma epsilon (4). Fo, the membrane-bound part, can mediate passive proton flow across membranes (4-6). It consists of at least three kinds of subunits whose roles on proton translocation have been extensively studied (9, 10). One of the Fo subunits is a proteolipid of about 8 kDa (that is specifically labeled by N,N'-dicyclohexylcarbodiimide (DCCD) (11). Recent elucidation of the FoF1-ATPase genes from various organisms has revealed that amino acid sequences of the FoF1-ATPase subunits, especially those of the alpha and beta subunits, have been well conserved during evolution. In addition, the structural features of the proteolipid subunit are also definitely conserved (12, 13).

On the other hand, an acidothermophilic archaebacterium, Sulfolobus acidocaldarius, seems to lack typical FoF1-ATPase (14-16). The soluble ATPase purified from its membranes (S. acidocaldarius ATPase) differs from usual Fo-ATPase with respect to subunit properties, enzymatic kinetics, sensitivity toward inhibitors, and pH optima, though it has almost the same molecular shape and size as Fo-ATPase. In fact, the molecular cloning of the major two subunits, alpha and beta, of S. acidocaldarius ATPase and a comparison of their amino acid sequences with those of FoF1-ATPases clearly showed that S. acidocaldarius ATPase does not belong to the FoF1-ATPase family even though they have both probably evolved from a common ancestor (17, 18).

Very recently, three research groups have reported the cDNA nucleotide sequences that code the major two subunits, 70 and 60 kDa, of eukaryotic vacuolar H+-ATPases which belong to another group of H+-ATPase, that is the V-ATPase family (19-22). Members of the V-ATPase are found in eukaryotic endomembrane vesicles such as vacuoles, lysosomes, chromaffin granules, and Golgi apparatus (23-26). The amino acid sequences of the vacuolar H+-ATPase 70- and 60-kDa subunits showed remarkable homology to the alpha and beta subunits of S. acidocaldarius ATPase, respectively (2). This finding raised a possibility that S. acidocaldarius ATPase and eukaryotic V-ATPase have evolved from a common ancestor.

Similar to FoF1-ATPases, V-ATPases are inhibited by DCCD which binds to the 16-kDa proteolipid subunit (27-31). Mandel et al. (32) reported recently on the cDNA sequence of the proteolipid of bovine chromaffin granule H+-ATPase, suggesting that the proteolipids of V-ATPases evolved in parallel with those of FoF1-ATPases from a common ancestral 8-kDa proteolipid gene through gene duplication. Here we report on the amino acid sequence, deduced from the base sequence of the gene, for a proteolipid subunit of the S. acidocaldarius ATPase complex.

MATERIALS AND METHODS

Isolation, Sequencing, and Analysis of the Proteolipid Gene—The proteolipid gene exists on the same EcoRI DNA fragment which contains the alpha and beta subunit genes. Sequencing and computer analysis of the proteolipid gene were carried out as described in our previous papers (17, 18). The hydropathy plot based on the method of Kyte and Doolittle (33) was performed with Genetyx (Software Development Co.).

Isolation of Proteolipid—Membranes of S. acidocaldarius (690 mg of protein), prepared as described previously (14), were suspended in 60 ml of 50 mM Hepes/NaOH (pH 7.2) and were washed with the same buffer twice by centrifugation. The precipitated membranes were suspended thoroughly in 60 ml of diethyl ether and were precipitated by centrifugation. This ether wash was repeated twice. The pellet was then suspended with 50 ml of chloroform/methanol (2:1, v/v) containing 5 mM diithiothreitol and stirred vigorously for 10 h at 4°C. The solution was centrifuged, and the upper aqueous phase and the interface were removed. The lower organic solvent phase was evaporated to dryness.

1 The abbreviations used are: DCCD, N,N'-dicyclohexylcarbodiimide; S. acidocaldarius ATPase, a water-soluble ATPase solubilized from membranes of Sulfolobus acidocaldarius; Heps, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

2 A detailed comparison of the primary structure of vacuolar ATPase and S. acidocaldarius ATPase and its implication in cellular evolution will be published elsewhere as a collaborative work of the laboratories of B. J. and E. J. Bowman, R. J. Poole, L. Taiz, and M. Yoshida.
concentrated to 3 ml, mixed with 12 ml of diethyl ether at −20 °C, left at −20 °C for 1 h, and centrifuged at −20 °C. The resulting pellet was washed with ice-cold acetone and dried under vacuum. The dried material was suspended with chloroform/methanol (2:1, v/v) and subjected to a 

Cm-cellulose column (1.5 × 20 cm) equilibrated with chloroform/methanol/H2O (5:2:1). The nonadsorbed fractions were collected, dried down with diethyl ether, dissolved in 1 ml of chloroform/methanol (2:1, v/v), and stored at −20 °C. Before use, an aliquot was taken out and dried down.

Peptide Sequencing—To determine the NH2-terminal sequence of the proteolipid, dried pellet material was dissolved either in 75% trifluoroacetic acid, or 1% sodium dodecyl sulfate, and applied directly on a gas phase peptide sequenator (Applied Biosystems 470A). To determine the internal peptide sequence, the proteolipid was cleaved by CNBr treatment. Dried pellet material containing about 10 µg of protein was dissolved in 200 µl of 75% trifluoroacetic acid, and 5 µg of CNBr was added. The mixture was incubated overnight at room temperature in darkness and was subjected directly to a gas phase peptide sequenator. Since the possibility was considered that some of the CNBr peptides were so hydrophobic that they might be lost during Edman degradation procedures, solid phase techniques fixing the CNBr peptides to an aminopropyl glass filter were also employed. Details of this method were based on Ref. 34.

RESULTS AND DISCUSSION

As described previously (18), a gene for the β subunit (atpB) of S. acidocaldarius ATPase follows a gene for its α subunit (atpA). About 830 bases downstream of the atpB, we found an open reading frame that encodes a very hydrophobic protein of 101 amino acid residues (Fig. 1). Between this gene (atpP) and atpB, no obvious promoter or terminator sequences are found, and there are two open reading frames with a short interacistronic noncoding region or even overlapping translation stop/start codons. They probably code minor subunits of S. acidocaldarius ATPase, and work is currently going on to confirm this from the peptide sequences. Therefore, it is most likely that the atpA, atpB, and atpP genes are in the same operon and the product of the atpP gene is one of the membranous subunits of the S. acidocaldarius ATPase complex.

The above assumption was supported by the finding that the atpP gene product has significant amino acid sequence homology and similar hydropathy profiles to the proteolipid subunits of F0F1-ATPases (Figs. 2A and 3). It probably penetrates the membrane twice and contains the typical hydrophobic stretch in the NH2-terminal side hydrophobic segment. The arginine in the middle hydrophilic segment is also conserved. Although the ATPase activity of S. acidocaldarius membranes is relatively resistant to DCCD inactivation (14), it has a glutamic acid residue at position 83, in the middle of the second hydrophobic segment, that is equivalent to the glutamic acid (or the aspartic acid residue in the case of Escherichia coli) of proteolipid subunits of F0F1-ATPases, which is specifically labeled by DCCD when the enzymes are inactivated by this reagent (35, 36). In addition to the data shown in Fig. 2A, several proteolipid sequences (11) from other sources were compared, and it was found that the sequence of S. acidocaldarius proteolipid is generally more similar to those from chloroplasts than to those from mitochondria. The implication of this fact is yet not known.

The S. acidocaldarius proteolipid sequence was also compared with that of bovine chromaffin granule H*-ATPase. As expected, the former shows homology to both the NH2-terminal half and COOH-terminal half of the latter (Fig. 2B). The hydropathy profile of the former resembles that of the COOH-terminal half of the latter (Fig. 3). The molecular size of the chromaffin granule proteolipid (16 kDa) is about double that of the F0F1-ATPase proteolipid (8 kDa), and Mandel et al. (32) suggested that this has resulted from gene duplication of the ancestral 8-kDa proteolipid. Since the protein encoded by the atpP gene of S. acidocaldarius has an intermediate size between the chromaffin granule and F0F1-ATPase proteolipids (10 kDa), the possibility of the occurrence of a S. acidocaldarius proteolipid by gene duplication was checked carefully. However, no reminiscence of gene duplication of

**Fig. 2.** Aligned sequences of a proteolipid subunit of the S. acidocaldarius ATPase complex with (A) proteolipids of F0F1-ATPases from spinach chloroplast, E. coli, and bovine mitochondria, and (B) a proteolipid of bovine chromaffin granule H*-ATPase. Boxes (A) or asterisks (B) have been placed around identical or conservative residues. The following amino acid residues are considered as conservative replacements: E and D, G, and A, and L. Sequence data are based on Refs. 11 and 32.

**Fig. 3.** Comparison of the hydropathy profiles of proteolipid subunits of ATPases from chromaffin granule, S. acidocaldarius, spinach chloroplasts, E. coli, and bovine mitochondria (11, 32). They were calculated with the computer program HYDROPLPOT (33). The locations of the residues that are labeled by DCCD are circled.

RNA gene for the peptide coding subunit of the S. acidocaldarius ATPase complex. The sequences confirmed from peptide sequences are underlined. Amino acids and nucleotides beginning with the initiaer methionine are numbered on the right and on the left, respectively.
been differentiated from ancestral archaebacterial ATPase. The cation occurred in a proteolipid after ancestral V-ATPase had been purified directly from membranes as described under "Materials and Methods." The purified proteolipid, dissolved in 75% trifluoroacetic acid, chloroform/methanol (2:1), or 1% sodium dodecyl sulfate, was applied to sequencing to a glass filter treated in order to have amine-reactive groups blocked. The sequences of peptide mixtures produced by CNBr cleavage were then analyzed. The sequence corresponding to a mixture of the second (AAAAGIGV..) and the fourth peptides (LFGKF) appeared. To avoid peptide loss during organic solvent wash in Edman degradation, we also tried to immobilize covalently the CNBr peptides prior to sequencing to a glass filter treated in order to have amine-reactive groups blocked. This procedure yielded the sequence corresponding to a mixture of the second and the third peptides (FGLTLL...). However, the sequence corresponding to the first NH2-terminal CNBr peptide (KKTWLPLF...) was never obtained, though it should appear after CNBr treatment, even in the case when it is blocked by formylation. Therefore, we tentatively conclude that the translational product of the atpP gene is subjected to processing, producing a mature proteolipid polypeptide which has a stable blocked NH2 terminus such as pyrrolidone glutamic acid. Detailed characteristics of the purified proteolipid are currently being studied and will be published elsewhere.

Thus, in contrast to a proteolipid subunit of bovine chromaffin granule H+ATPase, a proteolipid subunit of the S. acidocaldarius ATPase complex has not undergone gene duplication. In this respect, it is more similar to proteolipid subunits of F0F1-ATPases. On the other hand, the two major subunits of ATPase of this archaeabacterium show a much stronger homology to those of eukaryotic V-ATPases than to those of F0F1-ATPases. We proposed in another paper that eukaryotic V-ATPase and S. acidocaldarius ATPase are derived from a common ancestral H+ATPase. In this context, the simplest interpretation to account for the chimeric nature of the S. acidocaldarius ATPase complex is that gene duplication occurred in a proteolipid after ancestral V-ATPase had been differentiated from ancestral archaeabacterial ATPase. Accumulation of the sequence data of proteolipids from other archaeabacteria and endomembranes will clarify the relationship between them.

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Proteolipid Gene of S. acidocaldarius ATPase Complex

Amino acids
37-47 KKKWLPF
46-58 IGAVYAVSMA

Nucleotides
109-141 ATACCTGATATGGCCTTGTACTCTCT
48-58 LCGAVGMVGM

FIG. 4. Alignment of internal repetitive sequences in a proteolipid subunit of the S. acidocaldarius ATPase complex. Identical residues are indicated by an asterisk.