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A NOVEL SUBUNIT OF VACUOLAR H^+-ATPase RELATED TO THE *b* SUBUNIT OF F-ATPases

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Summary

The subunit structure of the vacuolar H^+-ATPase (V-ATPase) membrane sector is not entirely known. The proteolipid is the only subunit that has been implicated in the mechanism of energy transfer in the enzyme. We have identified a protein (M16) that co-purifies with the V-ATPase complex from bovine chromaffin granules. Information obtained from the amino acid sequence of a proteolytic fragment of M16 was used to clone a bovine adrenal cDNA encoding this protein. The cDNA encodes a hydrophilic protein of 118 amino acid residues with a calculated molecular mass of 13 682 Da. Amino acid sequence analysis revealed that M16 exhibits a significant homology to subunit *b* of F-ATPases. M16 is smaller than subunit *b* and contains no apparent transmembrane segment in its N terminus. The remainder of subunit *b* is related to M16 not only by its amino acid sequence but also in its predicted structure of helix–turn–helix. The structural and evolutionary implications of these findings are discussed.

Key words: V-ATPase, vacuolar system, membrane, subunit structure, proton pumps.

Introduction

Eukaryotic cells contain numerous acidic compartments composed of elements of the vacuolar system. V-ATPase is responsible for acidifying the interior of these organelles and providing the energy for numerous transport processes across their membranes. V-ATPase is related in its structure and mechanism of action to F-ATPase, which is present in chloroplasts, mitochondria and eubacteria. These enzymes not only share a common structure, and presumably mechanism of action, but also have a common evolutionary ancestry. In eukaryotic cells, F-ATPases are confined to the semiautonomous organelles, chloroplasts and mitochondria, all of which contain their own genes that encode some of the F-ATPase subunits (Nelson, 1989, 1992*a*,*b*).

V-ATPase is a complex enzyme containing several subunits that are divided into catalytic and membrane sectors. The catalytic sector (V_1) contains six different polypeptides denoted as A, B, C, D, E and F (Nelson, 1992*b*; Nelson *et al.* 1994, 1995; Gräf *et al.* 1994; Graham *et al.* 1994). The stoichiometry of these subunits excluding F was determined to be 3:3:1:1:1, respectively (Arai *et al.* 1988; Supek *et al.* 1994*a*). Subunit A (69 kDa) of V-ATPases contains the catalytic ATP-binding site of the enzyme (Moriyama and Nelson, 1987; Bowman *et al.* 1988*b*; Zimniak *et al.* 1988). The amino acid sequence of this subunit contains a 'glycine-rich motif' that is common to ATP-binding proteins (Walker *et al.* 1982*a*,*b*; Saraste *et al.* 1990). This motif contains two cysteine residues which, when modified, result in inactivation of the enzyme (Feng and Forgac, 1992). Moreover, modification of a single cysteine residue on subunit A prevents dissociation of the catalytic sector from the membrane by cold treatment (Moriyama and Nelson, 1987; Feng and Forgac, 1992; Taiz *et al.* 1994). These and other observations leave little doubt that subunit A functions in the ATPase activity of V-ATPases by providing the catalytic ATP-binding site.

It is not clear whether subunit B contains an ATP-binding site (Moriyama and Nelson, 1987). Recently it was shown that subunit B could be labeled by 2-azido-[^{32}P]ATP in isolated coated-vesicle V-ATPase and by ^{32}P-labeled ATP or ADP by ultraviolet irradiation in the isolated recombinant subunit B (Zhang *et al.* 1995; Peng, 1995). Sequence analysis revealed an extensive homology to the α subunit of F-ATPases (Bowman *et al.* 1988*a*; Manolson *et al.* 1988). However, subunit B contains no glycine-rich sequence, which is an indication of the existence of a nucleotide-binding site. These and other observations suggest that subunit B may function in regulating the activity of V-ATPases, but may do so with or

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without the direct involvement of bound nucleotides. The remaining subunits in the catalytic sector of V-ATPases have no homology to F-ATPase subunits. There is no assigned function for subunits C, D, E and F of V-ATPases. We have recently cloned a bovine cDNA and a yeast gene, *VMA8*, encoding subunits D of the respective V-ATPases (Nelson *et al.* 1995). Although no significant sequence homology was found between subunit D and the γ subunit of F-ATPases, structural analysis indicated that similar motifs are present in the two proteins.

The function of the membrane sector is to conduct protons across the membrane and to couple this vectorial action with the scalar process of ATP formation or hydrolysis. While the membrane sector of *Escherichia coli* F-ATPase consists of three different subunits, that of mammalian mitochondria may contain up to 10 different polypeptides (Schneider and Altendorf, 1987; Futai *et al.* 1989; Senior, 1990; Walker *et al.* 1991). The operon structure of archaebacterial V-ATPase suggests that its membrane sector may be composed only of the proteolipid (Denda *et al.* 1990). In contrast, the mammalian membrane sector contains multiple subunits (Zhang *et al.* 1992). The proteolipid is the principal subunit of the membrane sectors of both F- and V-ATPases. The proteolipid of V-ATPase is a highly hydrophobic protein that binds dicyclohexylcarbodiimide (DCCD) and contains about 160 amino acid residues (16 kDa) (Sutton and Apps, 1981; Mandel *et al.* 1988; Nelson and Nelson, 1989; Nelson, 1992*c*; Noumi *et al.* 1991; Supek *et al.* 1994*b*). DCCD binding inactivates the proton-pumping and ATPase activities of the enzyme (Arai *et al.* 1987; Sze *et al.* 1992*a*,*b*). The proteolipid is likely to be involved in the process of proton translocation across the membrane. Except for the proteolipid, there is no evidence that other proteins are involved in proton conductance. In contrast, in F-ATPases it has been clearly demonstrated that all three subunits (*a*, *b* and proteolipid) are required for proton conductance and/or proper assembly of the membrane sector (Schneider and Altendorf, 1987). While subunit *a* was implicated in the catalysis of proton conduction across the membrane, subunit *b* was suggested as the provider of the binding site for the catalytic sector. It may also act in the mechanochemical coupling between the ATPase and proton transport sites.

The membrane sector of mammalian V-ATPase may be composed of at least five different subunits (Zhang *et al.* 1992; Nelson, 1992*c*). The genes or cDNAs encoding four of the subunits (M115, M45, M39 and proteolipid) have been cloned and sequenced, and the cloning of the gene encoding the 20 kDa polypeptide (presumably subunit *a*) is in progress (Wang *et al.* 1989; Perin *et al.* 1991; Bauerle *et al.* 1993). All the above subunits were identified in V-ATPases of bovine, yeast and several other sources. A novel subunit (M45) was discovered in V-ATPase from bovine chromaffin granules (Supek *et al.* 1994*a*). This subunit was identified as a broad band on SDS–polyacrylamide gels migrating between subunits B and C. Cloning of the cDNA encoding this polypeptide revealed seven potential glycosylation sites positioned on the luminal side of the membrane and a single transmembrane segment at the C-terminal part of the polypeptide. Immunological studies suggest that this polypeptide is restricted to internal organelles of mammalian cells with a potential function as an anchor for the enzyme in these organelles. We anticipate that accessory subunits with similar structure will be identified in V-ATPases from other sources.

It is apparent that more subunits may function in proton conduction through the membrane and/or in the assembly of the V-ATPase membrane sector. In this communication, we report the cloning of a cDNA encoding the M16 bovine V-ATPase subunit. The amino acid sequence of this subunit is homologous to that of *b* subunits of F-ATPases. The structural, mechanistic and evolutionary implications of this finding are discussed.

Materials and methods

Enzymes were purchased from Boehringer, BRL or New England Biolabs. Peroxidase-conjugated protein A and antibodies were obtained from Sigma. Radioactive chemicals were purchased from Amersham. Amplitaq DNA polymerase for PCR amplification was purchased from Perkin Elmer-Cetus Instruments. Published procedures were used for recombinant DNA methods (Sambrook *et al.* 1989), protein determination (Fanger, 1987) and screening libraries (Sambrook *et al.* 1989). Both DNA strands of the cloned cDNA were sequenced using oligonucleotide primers.

Isolation of peptides for amino acid sequencing

Bovine chromaffin granules were prepared as previously described (Wang *et al.* 1989). The V-ATPase (about 0.5 mg of protein) was purified from chromaffin granules and precipitated by addition of three volumes of cold ethanol (Nelson *et al.* 1995), incubation for 15 min at −20 °C and centrifugation at 20 000 ***g*** for 20 min. The resulting pellet was dissolved in 1 ml of buffer containing 20 mmol l^{-1} Mops–NaOH (pH 7.5), 1 % SDS and 0.2 % mercaptoethanol. Two 0.5 ml samples were applied onto 7 %–35 % sucrose gradients in a buffer containing 10 mmol l^{-1} Mops–NaOH (pH 7.5) and 0.02 % SDS. The gradients were centrifuged at 15 °C in an SW60 rotor at 57 000 revs min^{-1} for 15 h. Twelve fractions were collected from each tube and analyzed for subunit content by electrophoresis on a 12.5 % polyacrylamide gel in the presence of SDS. Staining by Coomassie Blue revealed that subunits C, D, E and M16 were present in fractions 8–10 and subunits A and B were present in fractions 6–8. Fractions 8–10 were pooled, precipitated by the addition of three volumes of cold ethanol as above and the resulting pellet was recovered in 0.5 ml of a solution containing 10 mmol l^{-1} Mops–NaOH (pH 7.5), 1 % SDS and 0.2 % mercaptoethanol. The sucrose gradient centrifugation (one tube) was repeated and the fractions containing M16 were pooled and precipitated by ethanol. The resulting pellet was dissolved in 100 μl of electrophoresis sample buffer containing 0.1 mol l^{-1} Tris-Cl (pH 6.8), 2 % SDS, 2 % mercaptoethanol, 0.05 % Bromophenol Blue and 10 % glycerol.

The dissociated sample was electrophoresed using a 15-well 12.5 % polyacrylamide gel and a Mini-PROTEAN II apparatus (Bio-Rad). The proteins were electroblotted onto an Immobilon filter (Millipore) according to the method of Matsudaira (1987). The stained band at the position of 16 kDa (M16) was excised and subjected to cyanogen bromide treatment. The treatment was performed in 1 ml of 70 % formic acid containing 3 mg of cyanogen bromide. After incubation at room temperature for 15 h, the filters were washed five times with 1 ml portions of distilled water and subjected to amino acid sequence analysis in a gas-phase Applied Biosystems sequenator.

Preparation of antibody against the M16 protein and western analysis

To generate antibody against M16, the whole coding region of the cDNA was cloned in frame with the carboxy terminus of the maltose-binding protein gene in the pMAL-cRI vector (New England Biolabs). The resulting plasmid was used to transform DH5α *E. coli* cells. Following induction with 0.2 mmol l^{-1} IPTG for 2 h, the fusion protein was purified on a maltose–agarose column according to the manufacturer's instructions. The purified fusion protein was concentrated by acetone precipitation, dissolved in a solution containing 10 mmol l^{-1} Tris-Cl (pH 8.0), 1 mmol l^{-1} EDTA and 0.1 % SDS. Antibody was raised in guinea pigs as previously described (Supek *et al.* 1994*a*). To prepare affinity-purified antibody, about 10 mg of the purified fusion protein were cross-linked to 1 ml of Affi-Gel 10 (BioRad) in 0.2 mol l^{-1} Mops–NaOH (pH 7.5). Solid ammonium sulfate was added to 5 ml of serum to give 50 % saturation. The resulting pellet was dissolved in 5 ml of a phosphate-buffered saline (PBS) solution containing 100 mmol l^{-1} sodium phosphate (pH 7.5) and 100 mmol l^{-1} NaCl, and dialyzed overnight against the same solution. The dialyzed antibody was passed 10 times through the column to which the fusion protein was bound. The column was washed with 50 ml of the PBS solution followed by the second wash with 25 ml of PBS solution containing 0.5 mol l^{-1} NaCl. The antibody was eluted with 0.1 mol l^{-1} glycine–HCl (pH 2.8) and 0.5 ml fractions were collected into tubes containing 0.1 ml of 1 mol l^{-1} Tris-Cl (pH 8.0). Fractions containing the antibody were pooled and dialyzed against PBS solution containing 35 % glycerol and stored at −20 °C.

Western blots were performed according to the protocol of the ECL antibody detection system from Amersham Corp. Samples were denatured in SDS sample buffer and electrophoresed on 12 % polyacrylamide Mini-gels (Bio-Rad). Following electrotransfer at 0.5 A for 15 min, the nitrocellulose filters were blocked for 1 h in the PBS solution containing 0.1 % Tween 20 and 5 % non-fat dry milk. Blocked filters were incubated with an antibody (dilution 1:1000) for 1 h at room temperature in the above solution, which contained dry milk at a concentration of only 1 %. Following four washes with the same solution, peroxidase-conjugated secondary antibody was added to the filters. After incubation for 30 min and four washes with the same solution, the nitrocellulose filters were subjected to the ECL amplification procedure. The filters were exposed to Kodak X-Omat AR film for 5–30 s.

Results

Identification of potential new subunits in chromaffin granule V-ATPase

Although preparations of V-ATPases from mammalian sources exhibited similar patterns on SDS gels, the identity of protein bands as subunits of the enzyme was not certain (Cidon and Nelson, 1986; Percy and Apps, 1986; Xie and Stone, 1986; Arai *et al.* 1988). Therefore, different subunits were postulated to constitute V-ATPase in various laboratories. Fig. 1 shows an SDS gel of purified V-ATPase from bovine chromaffin granule membranes. The catalytic sector subunits are denoted as A–F and the membrane sector subunits are labeled as M followed by a number indicating their apparent molecular mass calculated from their migration on the SDS gel. Three different proteins (M115, M39 and proteolipid) were clearly shown to comprise the V-ATPase membrane sector (Wang *et al.* 1989; Nelson and Nelson, 1989; Perin *et al.* 1991). In Fig. 1, we indicate additional protein bands that co-purified with the enzyme and

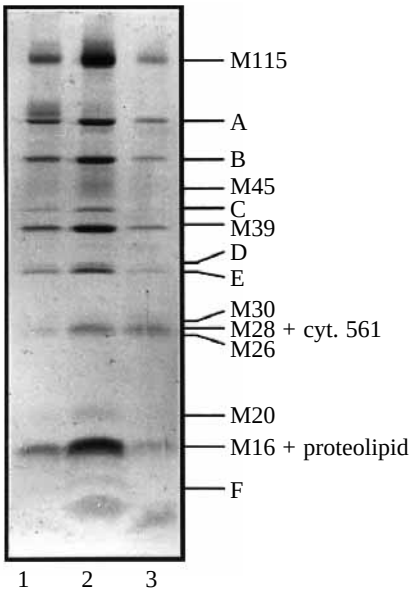

Fig. 1. Polypeptide composition of purified V-ATPase from bovine chromaffin granules. The V-ATPase complex was purified from the chromaffin granules by centrifugation through a glycerol gradient as described elsewhere (Moriyama and Nelson, 1987). The gradient was fractionated and the fractions were analyzed by electrophoresis on an SDS–polyacrylamide gel. The gel was stained with Coomassie Blue. Three fractions (lanes 1–3) containing the peak of ATPase activity are shown. In addition to previously identified subunits (M115, A, B, M45, C, M39, D, E and proteolipid), the preparation contains the new subunit M16 reported in this manuscript as well as two other putative subunits of V-ATPase (M20 and F). The preparation also contains additional polypeptides (M30, M28, M26 and cytochrome 561). While M30, M28 and M26 co-purify with the enzyme, cytochrome 561 does not coincide with the fractionation of the V-ATPase.

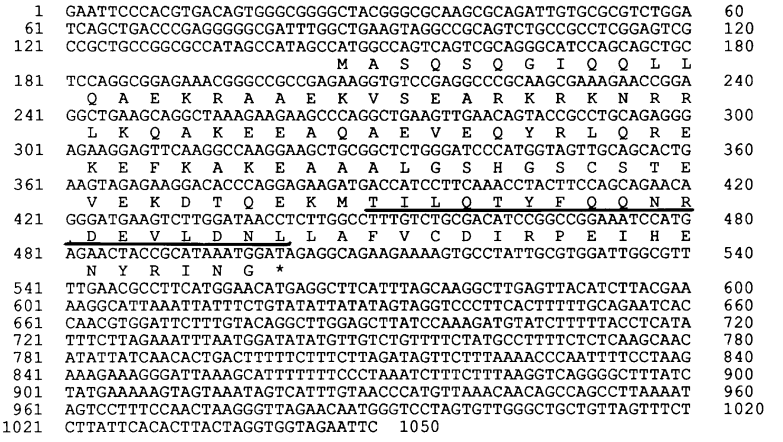

Fig. 2. Nucleotide and derived amino acid sequences of the cDNA coding for the bovine M16. The sequence of the cDNA insert in the YPN1 vector is shown. The sequence includes *Eco*RI sites at the beginning and end of the sequence added to the cDNA by linker cloning. The amino acid sequence obtained by sequencing of CNBr-treated protein is underlined.

therefore may be potential subunits of V-ATPase. One of those protein bands (M16) was transferred onto an Immobilon filter, cleaved by cyanogen bromide and the resulting proteolytic fragments were subjected to amino acid sequencing analysis (see Materials and methods). One of the peptides yielded the amino acid sequence ILQTYFQQNXDEVLDNL. A search in the GenBank database revealed significant homology with partial human cDNA sequences (Z20553 and Z21399) encoding unknown proteins. In addition, the amino acid sequence exhibited a significant identity with yeast M16 (Vma10p) which is a novel V-ATPase subunit recently discovered in our laboratory (Supekova *et al.* 1995).

Screening of bovine adrenal medulla cDNA library

Four oligonucleotide probes were designed on the basis of the above amino acid sequence and the human cDNA: (1) GCC ATC CTG CAG ACC TAC TTC CAG CAG AA(CT) GT; (2) CAT ACT TCC GGC AGA ACA GGG ATG AAG TCT; (3) GGA CAA CCT CTT GGC TTT TGT CTG TGA CAT; and (4) CGG CCA GAA ATC CAT GAA AAC TAC CGC AT. The first probe was synthesized according to Lathe's prediction (Lathe, 1985) and the other three according to the partial human cDNA. The screening of the bovine adrenal medulla library was performed as previously described (Supek *et al.* 1994*a*). Several positive colonies were analyzed by dot-blot and Southern hybridization. Among them, five showed a very strong hybridization signal with the oligonucleotide probes and had a size of about 1 kb. The cDNA fragments were sequenced in both directions using oligonucleotide-directed sequencing. Fig. 2 shows the nucleotide and predicted amino acid sequences of the bovine cDNA encoding M16. The sequence predicts a protein of 118 amino acid residues with a molecular mass of 13 682 Da and a calculated isoelectric point of pH 7.96. Except for the initiator methionine residue, which is probably blocked, there is a single methionine residue positioned in the middle of the protein. This arrangement resulted in our obtaining a single amino acid sequence following *in situ* cyanogen bromide treatment on the Immobilon filters.

Identification of related sequences in GenBank

The search in the GenBank database was quite rewarding. First, it picked up human partial cDNA clones of about 0.3 kb (GenBank Z20553, Z21399, Z15027, Z21353 and T25065). These human cDNAs showed more than 80 % identity in their nucleotide sequences over their whole length to the bovine cDNA of M16, and the translated amino acid sequences were almost identical to that of M16. Therefore, all of these human cDNAs appear to encode the human M16. The search also revealed significant homology with a part of chromosome VIII that was recently identified by us to constitute the *VMA10* gene encoding subunit M16 of *Saccharomyces cerevisiae* V-ATPase (Supekova *et al.* 1995). Among sequences derived from prokaryotes, the search revealed potential homology between M16 and part of *Vibrio alginolyticus* unc operon (X16050) encoding F-ATPase subunits (Krumholz *et al.* 1989; McCormick *et al.* 1993).

M16 is a membrane sector subunit of V-ATPase

M16 contains the peptide sequence that is present in the highly purified V-ATPase preparation from the chromaffin granules. It is also homologous to Vma10p, which has recently been demonstrated to be a part of the membrane sector of the V-ATPase from *S. cerevisiae* (Supekova *et al.* 1995). This suggests that bovine M16, like the yeast protein, is a part of V-ATPase complex. To verify this assumption, a specific antibody against M16 was raised and affinity-purified on a column with the bound antigen. Fig. 3 shows the distribution of V-ATPase subunits in a glycerol gradient after solubilization of chromaffin granule membranes with the detergent $C_{12}E_9$. The experiment shows that M16 co-purified with other subunits of V-ATPase. A cold-inactivation experiment, depicted in Fig. 4, demonstrated that, like its yeast counterpart, the bovine M16 protein is a genuine membrane sector subunit of V-ATPase.

M16 is related to subunit b *of the F-ATPase membrane sector*

Very recently we demonstrated that yeast *VMA10* is an intron-containing gene encoding the membrane-sector subunit M16 of V-ATPase (Supekova *et al.* 1995). The integrity of Vma10p (M16) as a V-ATPase subunit was checked by all the available techniques. Disruption of the *VMA10* gene yielded a

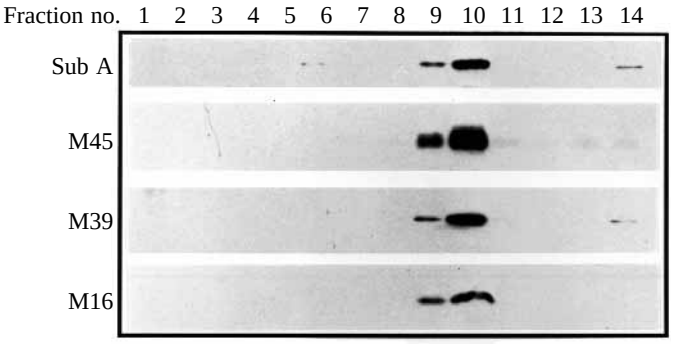

Fig. 3. The M16 protein co-purifies with V-ATPase on a glycerol density gradient. Chromaffin granule membranes (15 ml; protein concentration 5 mg ml^{-1}) were solubilized in a buffer (10 mmol l^{-1} Mes, 10 mmol l^{-1} Tricine, pH 7.0, 5 mmol l^{-1} thioglycerol) containing 1 % $C_{12}E_9$, and V-ATPase was purified by hydroxylapatite column chromatography and ammonium sulfate precipitation as described previously (Moriyama and Nelson, 1987). The resulting partially purified V-ATPase preparation was overlaid on a 10 %–30 % glycerol gradient in the solubilization buffer and centrifuged at 56 000 revs min^{-1} in an SW-60 rotor for 4 h. Fourteen fractions of 0.8 ml were collected from the bottom of the tube. Samples of the fractions were dissociated in SDS-containing buffer and 5 μl samples were electrophoresed on four separate 12 % polyacrylamide gels. Following electrotransfer onto nitrocellulose filters, each filter was incubated with the antibody indicated on the left. The filters were then reacted with secondary peroxidase-conjugated antibody and subjected to ECL amplification as described in Materials and methods. Sub A, subunit A.

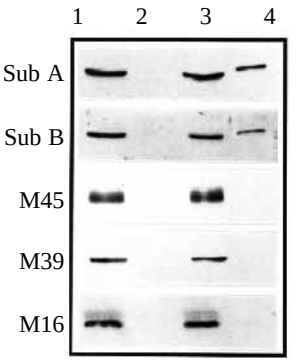

Fig. 4. M16 associates with the membrane sector of V-ATPase. Chromaffin granule membranes were prepared as described previously (Moriyama and Nelson, 1987). Membranes (0.5 mg) were diluted into 2 ml of buffer containing 20 mmol l^{-1} Mops–Tris (pH 7.0), 250 mmol l^{-1} NaCl and 1 mmol l^{-1} dithiothreitol, and then divided into two parts. MgATP was added to one of them to give a final concentration of 5 mmol l^{-1}. After 2 h of incubation on ice, the samples were centrifuged at 150 000 ***g*** for 25 min. The supernatants were concentrated by trichloroacetic acid precipitation and dissolved in 0.1 ml of SDS dissociation buffer. Similarly, the pellets obtained from high-speed centrifugation were dissolved in 0.1 ml of SDS dissociation buffer and 10 μl of each sample was electrophoresed on a 12 % polyacrylamide gel. After transfer to nitrocellulose, the membranes were incubated with antibody against the subunit indicated on the left. Lane 1, pellet after cold treatment without MgATP. Lane 2, supernatant after cold treatment without MgATP. Lane 3, pellet after cold treatment with MgATP. Lane 4, supernatant after cold treatment with MgATP. Sub A, subunit A; Sub B, subunit B.

mutant with a phenotype identical to those of all the other null mutations in V-ATPase subunits (Nelson and Nelson, 1990; Umemoto *et al.* 1990; Noumi *et al.* 1991). This phenotype could be complemented by transformation with a plasmid carrying the *VMA10* gene (Supekova *et al.* 1995). Other criteria indicated that M16 was a genuine subunit of the membrane sector. It co-purified with V-ATPase and it was present in stoichiometric amounts with other subunits of the enzyme. Fig. 5A depicts the amino acid sequence alignment between the bovine and yeast M16 proteins. The two sequences showed 37 % identity and 57 % similarity over 110 overlapping residues. This high degree of identity together with several conservative replacements suggested that the two proteins are related. Amino acid sequence alignment between M16 and subunit *b* of *Vibrio alginolyticus* F-ATPase showed 31 % identity and 52 % similarity (Fig. 5B). This observation prompted us to investigate the relationship between V-ATPase M16 and subunits *b* of F-ATPase. Sequence homologies among bovine and yeast M16, as well as the corresponding F-ATPase subunits from bacteria or chloroplasts, are summarized in Fig. 6. The common evolutionary origin of bacterial subunit *b* and the chloroplast F-ATPase subunits CF_o I and CF_o II has already been established (Bird *et al.* 1985; Westhoff *et al.* 1985; Herrmann *et al.* 1993). Sequence identity among these subunits varies from 17 to 26 %, indicating that the amino acid sequence of F-ATPase *b* subunits is poorly conserved. The mitochondrial homolog of subunit *b* (OSCP) is even less conserved than its bacterial and chloroplast counterparts (Walker *et al.* 1982*a*,*b*). It was observed with several V-ATPase subunits that their amino acid sequences are more conserved than the corresponding subunits in F-ATPases (Nelson, 1992*a*; Nelson *et al.* 1995). As shown in Figs 5 and 6, the amino acid sequences of bovine and yeast M16 are relatively more conserved than the various *b* subunits of F-ATPases. Moreover, alignment of bovine M16 with *Vibrio alginolyticus* subunit *b* exhibited a higher degree of homology when compared with that of *Vibrio versus* other *b* subunits or related proteins such as CF_o I, CF_o II or OSCP (not shown). These observations suggest that M16 subunits of V-ATPases are related to subunits *b* of F-ATPases and that all of these proteins may have evolved from a common ancestral gene.

Structural features of M16 and b *subunits*

Comparison of the hydropathy profiles of different *b* subunits from various sources indicated that they have similar structural features. The structure presumably contains a hydrophobic N-terminal domain and the remaining protein is hydrophilic. In *E. coli*, this hydrophobic domain has been interpreted as an anchor of subunit *b* to the membrane in association with subunits *a* and *c*, and the remaining protein was proposed to lie outside the lipid bilayer and to interact with the F_1 subunits (Walker *et al.* 1982*b*). This model for the *b* subunit has been supported subsequently by experimental

evidence and thorough computer analysis (Hoppe *et al.* 1983; Vik and Dao, 1992). Fig. 7 depicts hydropathy profiles of various *b* subunits and for bovine and yeast M16. It is apparent from the hydropathy profiles that some of the *b* subunits, for example those of *Rhododospirillum rubrum* and *Synechocystis* sp. PCC 6803 as well as the chloroplast CF_o II, exhibit a significant hydrophobic domain in the N terminus that can be interpreted as a transmembrane helix. In contrast, the hydropathy profiles of subunits *b* from *E. coli*, *Vibrio* and the chloroplast-encoded CF_o I show no apparent N-terminal hydrophobic domain. However, the available experimental data support the proposed structure of subunit *b* of F-ATPases as being anchored into the membrane by their N termini. The subunit M16 of V-ATPase, however, shows no hydrophobic segment in its N terminus and it is likely to be lacking this part of the protein. Nevertheless, it is not released by cold-inactivation of the yeast and bovine V-ATPases and therefore it can be considered to be a subunit of the membrane sector of the enzyme (Supekova *et al.* 1995; L. Supekova and N. Nelson, unpublished observations).

A

Yeast M16
Bovine M16

```
  2 SQKNGIATLLQAEKEAHEIVSKARKYRQDKLKQAKTDAAKEIDSYKIQKD 51
    ||..||. |||||| | | ||.||| :. :|||||.:|. |:: |::|::
  3 SQSQGIQQLLQAEKRAAEKVSEARKRKNRRLKQAKEEAQAEVEQYRLQRE 52

 52 KELKEFEQKNAGGVGELEKKAEAGVQGELAEIKKIAEKKKDDVVKILIET 101
    ||:|. |. . |: |. ....| :.|:.:. :... :..:|:|:. |:.
 53 KEFKAKEAAALGSHGSCSTEVEKDTQEKMTILQTYFQQNRDEVLDNLLAF 102

102 VIKPSAEVHIN 112
    |.. .:|:| |
103 VCDIRPEIHEN 113
```

B

Bovine M16
Vibrio subunit *b*

```
  3 SQSQGIQQLLQAEKRAAEKVSEARKRKNRRLKQAKEEAQAEVEQYRLQRE 52
    .|..: :|| :|.: |.| :.:|.|||.. |.:|:|||||| :..  | |
 31 AQANASDQLKEAKRTATEIIEQANKRKSQILDEAREEAQAERQKILAQAE 80

 53 KEFKAKEAAALGSHGSCSTEVEKDTQEKMTILQTYFQQN.RDEVLDNLLA 101
     |:.|. . | :.  .  ..:. ...||  ||:  ::.: ..::|||: |
 81 AELEAERNRARDDLRKQVATLAVAGAEK..ILERSIDKDAQKDILDNITA 128

102 FV 103
    :
129 KL 130
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Fig. 5. Protein sequence alignment between bovine M16 and the corresponding yeast and *Vibrio* proteins. Protein sequences are given in single-letter code. Sequences were aligned using the BESTFIT program, and identity is indicated by a solid line between residues. Two dots indicate conservative amino acid replacement. Single dots above the upper of the aligned sequences indicate multiples of ten. (A) Alignment of yeast Vma10p and bovine M16. (B) Alignment of bovine M16 and the *b* subunit of F_1F_o-ATPase from *Vibrio alginolyticus*.

Discussion

The F- and V-ATPases are protein complexes related both in their subunit structure and in their evolutionary origin (Nelson, 1992*a*,*b*,*c*). Moreover, it is assumed that their mechanism of action is similar. Both enzymes contain distinct catalytic and membrane sectors functioning in ATP hydrolysis and proton conductance without an apparent phosphoenzyme intermediate. Sequence analysis has revealed significant homology between subunit A of V-ATPase and the β subunit of F-ATPase, and between subunit B of V-ATPase and the α subunit of F-ATPase (Bowman *et al.* 1988*a*,*b*). It is assumed that these subunits have the same function within their respective enzyme complexes. The membrane sector of V-ATPases contains several subunits. Analysis of the amino acid sequences of proteolipid subunits from V- and F-ATPases revealed that they evolved from a common ancestral gene which underwent a duplication event to give rise to the V-ATPase type of eukaryotes (Mandel *et al.* 1988; Nelson and Nelson, 1989; Nelson, 1992*b*). To date, the only membrane

1 Bovine M16	100									
2 Yeast M16	37 (57)	100								
3 *Vibrio b*	31 (52)	24 (39)	100							
4 *E. coli b*	17 (32)	24 (41)	71 (80)	100						
5 *Rho b*	20 (42)	23 (47)	21 (43)	25 (56)	100					
6 *Rho b′*	26 (46)	19 (42)	27 (42)	23 (39)	25 (49)	100				
7 *Syn b*	18 (41)	22 (42)	28 (51)	27 (48)	25 (49)	23 (47)	100			
8 *Syn b′*	23 (41)	23 (48)	30 (48)	27 (48)	25 (47)	23 (45)	24 (50)	100		
9 CF_o I	17 (39)	15 (40)	20 (43)	20 (48)	20 (40)	17 (40)	23 (49)	22 (44)	100	
10 CF_o II	20 (44)	18 (43)	24 (42)	24 (45)	18 (42)	17 (42)	22 (43)	25 (53)	25 (52)	100
	1	2	3	4	5	6	7	8	9	10

Fig. 6. Comparison of bovine M16 with corresponding proteins from V- and F-ATPases. The diagram shows the percentage identity and similarity (the similarity is indicated by the number in parentheses) between *b* subunits from different organisms. The values were obtained from pairwise comparisons of the indicated proteins using the BESTFIT program. Rho, *Rhodospirillum rubrum*; Syn, *Synechocystis* sp. PCC 6803; CF_o, membrane sector of chloroplast F-ATPase. The F-ATPases from *R. rubrum*, cyanobacteria and chloroplasts comprise two different variants of subunit *b*, which are marked as *b*, *b′* and CF_o I, CF_o II, respectively. Sequences were taken from references mentioned in the text and from Lill and Nelson (1991).

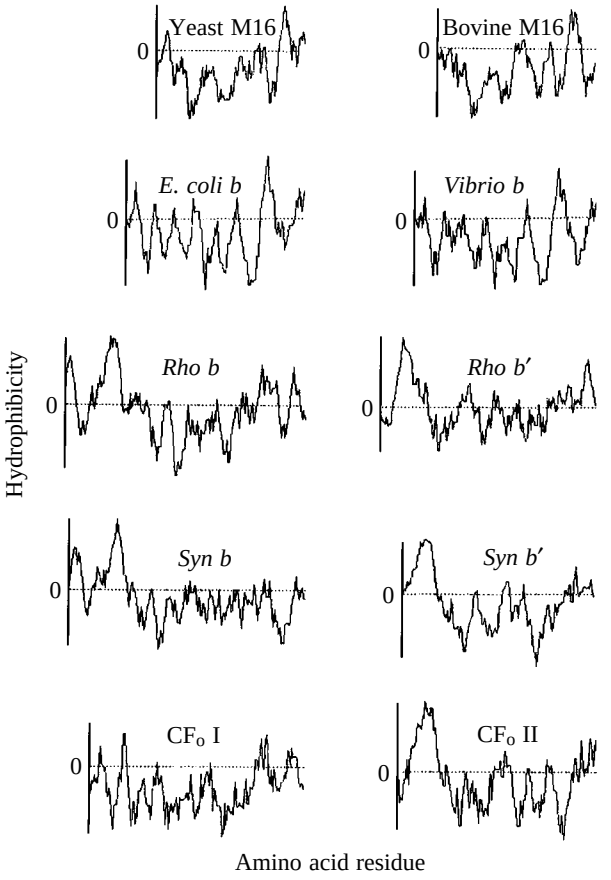

Fig. 7. Hydropathy plot analysis of selected *b* subunits. The indicated proteins were analyzed by the DNAstar package using the Kyte and Doolittle program with a window size of nine amino acids. The yeast and the bovine proteins are shorter than their counterparts from F-ATPases and are apparently missing the amino-terminal transmembrane segment. The identity of the proteins is indicated in Fig. 6. CF_o II is missing the cleaved N-terminal signal sequence.

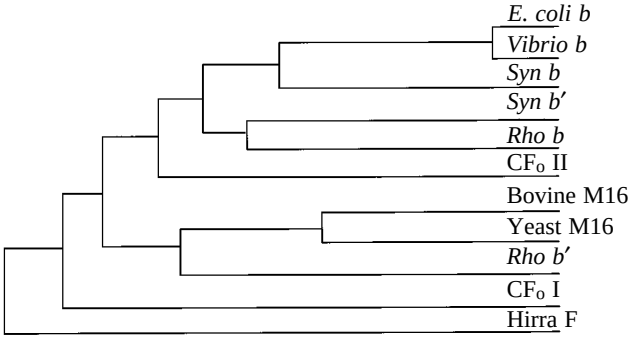

Fig. 8. Phylogenetic tree of *b* subunits of V-type and F-type H^+-ATPases. The tree was calculated from the pairwise comparison of protein sequences of *b* subunit from the indicated organisms. The comparison was performed using the program BESTFIT. In addition to proteins shown in Figs 6 and 7, the depicted phylogenetic tree also includes NtpG of the Na^+-ATPase from *Enterococcus hirae* (Hirra F).

sector subunit that has shown significant homology among various V- and F-ATPases is the proteolipid. In this work, we report on a second membrane sector subunit (M16) from V-ATPases that is homologous to the corresponding subunit in the membrane sector of F-ATPases. The evolutionary tree depicted in Fig. 8 indicates that M16 of V-ATPases and subunit *b* of F-ATPases evolved from a common ancestral gene. Analysis using the BESTFIT program also revealed potential homology to the NtpG gene product of the operon encoding the Na^+ pump in *Enterococcus hirae* (Takase *et al.* 1994). However, as shown in Fig. 8, this is not as significant as the relationship between M16 and subunit *b* of F-ATPases. The H^+-ATPase of archaebacteria has clearly been shown to be related to V-ATPases (Denda *et al.* 1990). This is especially evident in the gene encoding the catalytic sector of the enzyme. However, even though a clear relationship has been shown between subunits A and B of V-ATPases from archaebacteria and eukaryotes, the other subunits show little or no homology to their corresponding subunits (Nelson, 1992*b*). Remarkably, the archaebacterial operon encoding V-ATPase contains only a single hydrophobic protein that encodes a short version of the proteolipid (Denda *et al.* 1990). This was fully expected in the light of the function of this proton pump (Nelson, 1989, 1992*a*,*b*,*c*). It was pointed out that the short version of the proteolipid (about 80 amino acid residues in length) correlates with those H^+-ATPases that can function in ATP formation (Nelson, 1989). Accordingly, the archaebacterial ATPase contains the short version of the proteolipid that spans the membrane only twice. If the archaebacterial operon encodes all the necessary ATPase subunits, it is inferred that the proteolipid is the only membrane sector subunit of this enzyme. In view of the probable common mechanism of action of F- and V-ATPases, it is unlikely that the entire function of the membrane sector can be fulfilled solely by the proteolipid. Our discovery that M16 is homologous to *b* subunits of F-ATPase strongly suggests the existence of a second operon in archaebacteria encoding additional subunits of V-ATPase. We anticipate that the sequence of this second operon will unveil not only a gene homologous with M16 but also a gene equivalent to subunit *a* of F-ATPases. The discovery of M16 has somewhat altered the perceived evolutionary pattern of F- and V-ATPases. It is assumed that both of these ATPases evolved from a single long ancestral gene that encoded both the membrane and the catalytic sector (Nelson, 1992*b*). Its gene product probably functioned as a hexamer to fulfill the symmetry that is maintained in both V- and F-ATPases. The finding described in this paper suggests not only that the proteolipid and the catalytic sector were separated very early during the evolution of the ancestral gene but also that the *b* subunits of the membrane sector underwent an early separation. Only later did the V- and F-ATPases diverge to form distinct subfamilies.

The hydrophilic part of subunit *b* of F_o has been identified as part of the stalk structure of the enzyme. A substantial body of evidence suggests that the *b* subunit participates in the interaction between F_1 and F_o (see Senior, 1990). Therefore, the *b* subunit is likely to play a direct role in the transmission of energy derived from ATP hydrolysis to proton translocation

across the membrane. Recently, using recombinant DNA methods, the hydrophilic part of the *b* subunit was expressed in *E. coli* (Dunn, 1992). The polypeptide formed a dimer with a sedimentation characteristic indicating an elongated shape and displayed a circular dichroism spectrum suggesting that it is probably in the α helix conformation. This evidence is consistent with the structural predictions from several computational methods (Vik and Dao, 1992). Consequently, the *b* subunit is viewed as having two long α helices broken by an apparent turn. Structural analysis of the yeast M16 V-ATPase subunit indicates an almost identical structure to this subunit (not shown). It has a very high tendency to form an α helix and, like the subunit *b* of F-ATPase, is broken in the middle by an apparent turn. These structural similarities between M16 of V-ATPase and subunit *b* of F-ATPases suggest that they may play a similar role in the mechanism of action of the respective enzymes. However, in contrast to *b* subunits of F-ATPases, M16 most probably contains no transmembrane helix in its N terminus and it is essentially a truncated form of a subunit-*b*-like protein.

Recently, the structure of the catalytic sector F_1 of beef heart mitochondria was resolved at 0.28 nm (Abrahams *et al.* 1994). Among the striking features that were revealed was a rod-shaped structure that extends through the entire length of the enzyme, protruding as a stalk. It was suggested that this structure is the γ subunit of the enzyme. There is evidence indicating that the function of the γ subunit is in coupling the conformational changes necessary for energy transfer from ATP hydrolysis in F_1 to H^+ conductance in the membrane sector F_o (Futai *et al.* 1989; Senior, 1990; Shapiro *et al.* 1991). We have proposed that subunit D of V-ATPase is analogous to the γ subunit of F-ATPase and that they are likely to have a similar role (Nelson *et al.* 1995). The structural analysis of the γ subunit of the catalytic sector and the *b* subunit of the membrane sector suggested that both of them form the stalk which connects the two sectors of the enzyme. The structure of V-ATPase may be very similar to that of F-ATPase (Bowman *et al.* 1989; Taiz and Taiz, 1991). Therefore, it is likely that subunit D of the catalytic sector and M16 of the membrane sector form the stalk of V-ATPases. Further studies are required to determine the specific role of M16 and subunit D in the mechanism of action of V-ATPases.

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