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Identification and Characterization of a Novel 9.2-kDa Membrane Sector-associated Protein of Vacuolar Proton-ATPase from Chromaffin Granules*

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Vacuolar proton-translocating ATPase (holoATPase and free membrane sector) was isolated from bovine chromaffin granules by blue native polyacrylamide gel electrophoresis. A 5-fold excess of membrane sector over holoenzyme was determined in isolated chromaffin granule membranes. M9.2, a novel extremely hydrophobic 9.2-kDa protein comprising 80 amino acids, was detected in the membrane sector. It shows sequence and structural similarity to Vma21p, a yeast protein required for assembly of vacuolar ATPase. A second membrane sector-associated protein (M8-9) was identified and characterized by amino-terminal protein sequencing.

Proton-translocating adenosine triphosphatases have fundamental roles in energy conservation, secondary active transport, the acidification of intracellular compartments, and cellular pH homeostasis. They fall into three broad classes, called F, P, and V (1), of which the vacuolar type (V-ATPases)[1] is both the most recently recognized and the least well characterized. ATPases of this class occur in endomembranes bounding the acidic compartments of animal, plant, and fungal cells (2) and also in the plasma membranes of some specialized cell types. They have been purified from several mammalian sources, including adrenal secretory vesicles (3, 4), brain clathrin-coated vesicles, (5, 6), and kidney medulla microsomes (7), as well as from the vacuoles of fungi and higher plants. Most V-ATPases contain some 6–10 different subunits (2), but subunit composition depends on the source of the enzyme, and tissue-specific isoforms exist (8). The V-type ATPases are structurally similar to those of the F-type, having a transmembrane proton-conducting sector and an extramembrane catalytic sector. By analogy with the two sectors of F-ATPases (9–12), these are termed V_0 and V_1, respectively. For a recent review, see Ref. 13.

In this work, the recently developed technique of blue native polyacrylamide gel electrophoresis (BN-PAGE; Refs. 14–17) was employed to purify vacuolar ATPase holoenzyme (V_1V_0) and free membrane sector (V_0) simultaneously from adrenal secretory vesicle membranes. Combined with high resolution Tricine-SDS-PAGE in the second dimension, the subunit composition, particularly with respect to small polypeptides, was determined. Two novel proteins, 8–9 and 9.2 kDa in size, were found in the membrane sector. Here we report the detailed analysis of the larger of these two polypeptides.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and T4-DNA ligase were obtained from New England Biolabs. *Taq* DNA polymerase was from Stratagene, and TA Cloning Kit® was from Invitrogen. Sequenase version 2.0 sequencing kit, [α-^{35}S]dATP and Hybond N$^+$ membranes were obtained from Amersham Pharmacia Biotech. ABI Prism™ dye terminator cycle sequencing kit was purchased from Perkin Elmer. The cDNA library from bovine adrenal medulla was a kind gift from Leonora Ciufo (University of Edinburgh, Edinburgh, Scotland, United Kingdom). Human EST clone ID 143553 (GenBank™accession number R75754) was obtained from the IMAGE Consortium (18). Bovine tissues were frozen in liquid nitrogen several minutes after the death of the animal. The probe against human glyceraldehyde-3-phosphate dehydrogenase was a kind gift from J. Altschmied (Physiologische Chemie I, Universität, Würzburg). Anti-subunit G_1 antibody was kindly provided by Bill P. Crider (University of Texas Southwestern Medical Center, Dallas).

Isolation of V_0 and V_1V_0 ATPase from Chromaffin Granule Membranes by BN-PAGE—Chromaffin granule membranes were prepared according to Apps *et al.* (19). The membranes (11 mg of protein in 1.5 ml of 10 mM Hepes/NaOH, pH 7.4) were solubilized at 4 °C by addition of 1 ml of 1.75 M 6-aminohexanoic acid, 50 mM BisTris-Cl, pH 7.0, and 500 μl of 10% dodecyl maltoside. After 30 min centrifugation at 100,000 × *g*, 200 μl of 5% Serva Blue G in 500 mM 6-aminohexanoic acid was added to the supernatant. One ml of supernatant was loaded onto each of three 3-mm-thick preparative 5–13% acrylamide gradient gels for BN-PAGE (14). After BN-PAGE, the blue bands were excised and the native complexes electroeluted. About 300 μg of V_1V_0 complex and 900 μg of V_0 membrane sector were recovered from 11 mg of membrane protein.

Isolation of V_0 and V_1V_0 ATPase from a Triton X-114 Extract—Triton X-114-extraction was used for enrichment of V_0 and V_1V_0-ATPase (20). Chromaffin granule membranes (1 mg protein in 0.17 ml of 10 mM Hepes/NaOH, pH 7.4) were centrifuged for 30 min at 100,000 × *g*. The pellet was resuspended in 0.2 ml 150 mM KCl, 10 mM Tris, pH 7.5, and solubilized by addition of 50 μl 10% (w/v) Triton X-114. V_0 and V_1V_0

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y15285 (bovine M9.2 protein) and Y15286 (human M9.2 protein).

The protein sequence data have been submitted to the SWISS-PROT data base with accession numbers P81103 (bovine M9.2 protein) and P81134 (bovine M8-9 protein).

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[1] The abbreviations used are: V-ATPase, vacuolar type ATPase; BN-PAGE, blue native polyacrylamide gel electrophoresis; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis; EST, expressed sequence tag; V_1V_0, V-ATPase, vacuolar proton pumping ATPase (holoenzyme); F_1F_0, complex V, mitochondrial proton pumping ATPase (holoenzyme); V_1 and V_0, hydrophilic (catalytic) and hydrophobic (transmembrane) sectors of V-ATPase, respectively; proteolipid c, subunit c of V-ATPase; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction; bp, base pair(s); BisTris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Mops, 3-(*N*-morpholino)propanesulfonic acid.

were precipitated by a 15-min incubation on ice. After a 10-min centrifugation at 100,000 × *g*, the pellet was washed with 0.5 ml of 10 mM Na^+/Mops, pH 7.2, and centrifuged as before. The pelleted proteins were solubilized by addition of 90 μl 1 M 6-aminohexanoic acid, 50 mM BisTris/HCl, pH 7.0, and 21 μl of 10% dodecyl maltoside, and centrifuged again for 10 min at 100,000 × *g*. After addition of 10 μl of 5% Serva Blue G in 500 mM 6-aminohexanoic acid, 50 μl were applied to 10-mm gel wells for analytical BN-PAGE. After blue native electrophoresis, individual lanes were cut from the gel and processed in a second dimension by Tricine-SDS-PAGE. Electrophoretic techniques, staining techniques, and densitometric quantification followed the protocols described previously (17, 21).

Partial Protein Sequencing—V_0 and V_1V_0 complexes were electroeluted from blue native gels, resolved by Tricine-SDS-PAGE, and electroblotted onto polyvinylidene difluoride (PVDF) membranes. Individual bands were sequenced directly using a 473A protein sequencer (Applied Biosystems), or after various chemical treatments, *e.g.* cyanogen bromide cleavage, partial acidolysis by 80% formic acid (24 h at 37 °C), incubation in a 1:1 (v/v) mixture of trifluoroacetic acid and methanol (16 h at 37 °C) for partial deacylation (22), cleavage between asparagine and glycine (23) by 3 M hydroxylamine, pH 9.6 (7 h at 37 °C), or cleavage at tryptophan (24) by 0.7% iodosobenzoic acid dissolved in 80% acetic acid (24 h at room temperature). For searching genomic data bases with amino acid query sequences, the TFASTA computer program of the Husar package of the German Cancer Research Center (Heidelberg, Germany) was used. Protein secondary structures were calculated using the ANTHEPROT program (25–28).

Screening of a Bovine cDNA Library by PCR—The NH_2-terminal amino acid sequences of bovine M9.2 and the sequences of two corresponding human cDNA clones, IMAGE consortium clone 143553 (GenBank™ accession number R75754; Ref. 18) and murine MM85D12 (GenBank™ accession number D21772; Ref. 29) were used to deduce a pair of degenerate primers for PCR with the plasmid DNA of the whole bovine adrenal medulla cDNA library: VATPB9.2c, 5′-AT(C/T) GTG ATG AGC GTG TTC TGG GG-3′; and VATPB9.2n, 5′-GCC AAA AIA GAT AGC AGC AIA C-3′. PCR was performed in 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin, 200 μM of each dNTP, 0.5 μM of each primer, 10 mM Tris/HCl, pH 8.8. The temperature cycle was as follows: 94 °C for 1 min, 42 °C for 1 min, 70 °C for 30 s for 30 cycles and a single step of 72 °C for 10 min. The PCR product was cloned into the pCR II vector (Invitrogen) and sequenced. A third perfect match primer was deduced from this sequence: VATPB9.2a, 5′-GGG GCA TCG TCG GCT TCC TGG TGC-3′. The bovine cDNA library was then screened by PCR using the combination of primers VATPB9.2a and VATPB9.2n and the same temperature profile. Six pools, comprising a total of 1500 colonies, were taken as the template for PCR, and examined for the occurrence of a 106-bp PCR product. The positive pool was divided into subpools, and the procedure was repeated until a single clone (BVATPM9.2) was obtained.

Sequencing of Human and Bovine Clones—The insert from clone BVATPM9.2 was cut out with *Bam*HI and cloned into *pBluescript*™ II SK(−). The new clone pBBM9.2 was subcloned by using the *Bst*EII site at nt 69 and the *Xba*I site at nt 246. The insert of human cDNA clone 143553 was cut out with *Eco*RI and *Hin*dIII and cloned into *pBluescript*™ II SK(−). Clones pBBM9.2 and pBHM9.2 were sequenced in both directions.

RNA Isolation and Northern Blotting—Total RNA was prepared according to the method of Chomczynski and Sacchi (30). RNA was separated by formaldehyde agarose gel electrophoresis using 5 mM sodium acetate and 0.1 mM EDTA in running and loading buffers, capillary-blotted on Hybond N^+ membranes (31), and fixed by UV irradiation. DNA probes were labeled with [α-^{32}P]dCTP by random priming, and QuikHyb® solution from Stratagene was used for hybridization. A 900-bp cDNA was excised from pBBM9.2 with *Bam*HI and used as a probe for M9.2. The probe against bovine V_1V_0-ATPase subunit c (proteolipid c), GenBank™ accession number J03835 (32), was made by PCR using primers BVch 5′-TCA GCC GCC ATG GTC TTC AG -3′ and BVcr 5′-CGG CGA AGA TGA GGA TGA GG-3′. Using the bovine adrenal medulla cDNA library as a template, a 358-bp fragment corresponding to positions 190–547 of V-ATPase proteolipid c was amplified by PCR, tested by restriction analysis, and cloned into pCR 2.1 (Invitrogen).

RESULTS

Characterization of V-ATPase by BN-PAGE and Two-dimensional Electrophoresis—Analytical BN-PAGE (Fig. 1) was used for separation of V_1V_0 holocomplex and free V_0-membrane sector from solubilized chromaffin granule membranes (*lane M*), and from a fraction prepurified by Triton X-114 extraction/precipitation (*lane P*). The oxidative phosphorylation complexes from solubilized bovine heart mitochondria served as molecular mass standards (17, 33, 34). The molecular masses assigned to complex I and complex V are minimal values, inasmuch as the copy number of some subunits is not exactly known (35, 36). The prominent band with an apparent mass around 440 kDa was identified as V_0-membrane sector by the characteristic polypeptide patterns in two-dimensional electrophoresis, and by amino-terminal protein sequencing (see below). A faint protein band with an apparent mass of about 1000 kDa was identified as holo V_1V_0-ATPase. The position of free V_1 sector is also indicated in Fig. 1, although the amounts were too low for detection in BN-PAGE (see below).

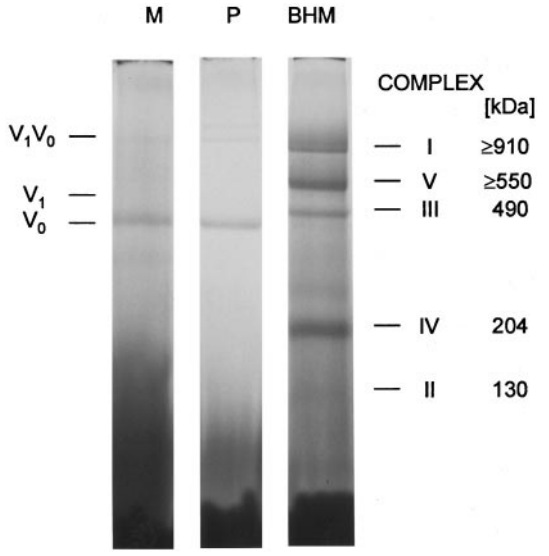

FIG. 1. **Separation of V_1V_0 ATPase and V_0 membrane sector by BN-PAGE.** Solubilized chromaffin granule membranes (180 μg of total protein; *lane M*) and a fraction enriched in V-ATPase, prepared from chromaffin granule membranes (420 μg of total protein; *lane P*), were applied to a 5–13% acrylamide gradient gel and resolved by BN-PAGE. The five oxidative phosphorylation complexes from solubilized bovine heart mitochondria (150 μg of total protein) were used for molecular mass calibration (*lane BHM*). The position of V_1 is indicated, although it was detected only after two-dimensional resolution (Fig. 2).

Second-dimensional SDS-PAGE of *lane M* from BN-PAGE (*cf.* Fig. 1) revealed the characteristic polypeptide patterns of V_1V_0 holoenzyme and V_0 membrane sector. Additionally, minor amounts of free V_1 sector and some contaminating mitochondrial F_1F_0-ATP-synthase could be detected (Fig. 2, *upper panel*). F_1F_0 was removed by Triton X-114 extraction/precipitation (Fig. 2, *lower panel*). Using the migration distances of V_1V_0 (≈1000 kDa), F_1F_0 (≥550 kDa), and V_0 (≈440 kDa) for calibration, the apparent mass of free V_1 sector was estimated at around 500 kDa. Several staining maxima of the 14-kDa proteolipid c in addition to those at the positions of V_1V_0 and the major band of V_0 indicated the positions of minor amounts of V_0 sector in higher oligomeric states.

The molar ratio of V_0/V_1V_0 in isolated chromaffin granule membranes was deduced from Coomassie Blue-stained two-dimensional gels by densitometric quantification of proteolipid c and 115- and 39-kDa proteins (data not shown). A 5-fold molar excess of free V_0 sector over assembled holocomplex was determined (*cf.* "Discussion"). Variation of the detergent/protein ratio for membrane solubilization by ±50% had no effect on the V_0/V_1V_0 ratio, which was 5.1 ± 0.3 (n = 5).

Identification of Protein Subunits of V-ATPase and Membrane Sector—Analysis of polypeptide composition by SDS-PAGE was performed directly from lanes of BN-PAGE (Fig. 2)

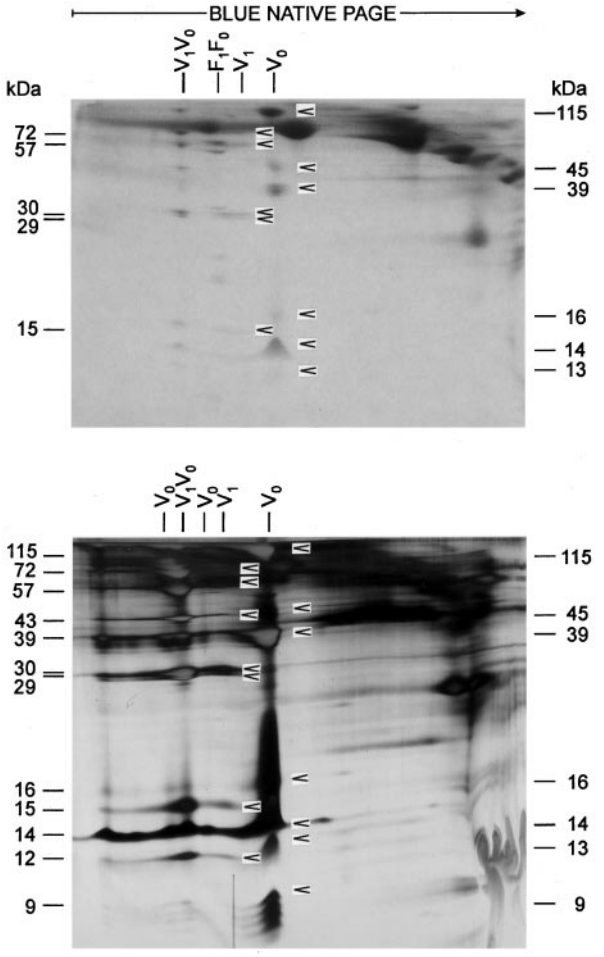

FIG. 2. **Two-dimensional separation of multiprotein complexes from chromaffin granule membranes.** *Lane M* from Fig. 1 comprising separated multiprotein complexes from chromaffin granule membranes was resolved by Tricine-SDS-PAGE, using a 16.5% acrylamide gel. Coomassie Blue G 250 stain was used for detection (*upper panel*). *Lane P* from Fig. 1 starting from a fraction enriched in V-ATPase was resolved and silver-stained (*lower panel*). Apparent molecular masses were assigned to subunits of the V_0 membrane sector (*right side*), to V_1 sector subunits (*upper panel, left side*) and to all detectable subunits of the holoenzyme (*lower panel, left side*). In addition to a major form of the V_0 membrane sector, the positions of minor forms with higher oligomeric states are indicated.

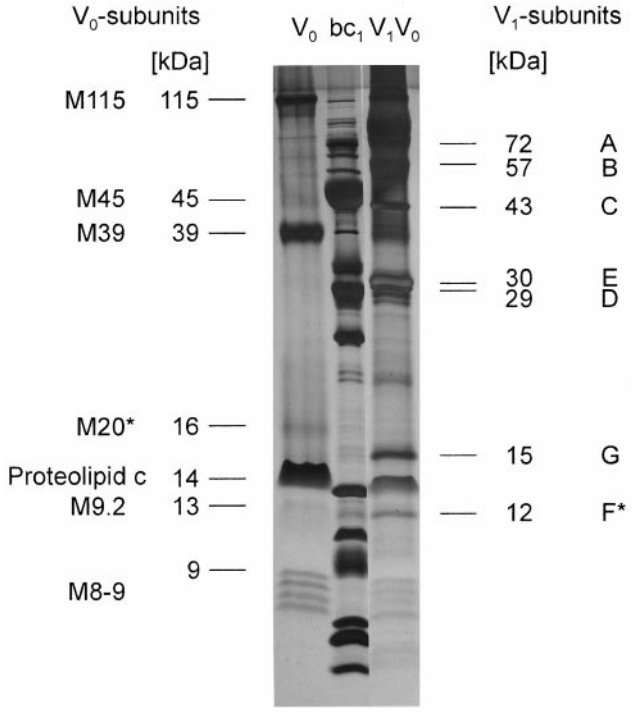

FIG. 3. **Subunit composition of V_1V_0 ATPase and V_0 membrane sector.** The multiprotein complexes recovered by electroelution from preparative blue native gels were resolved by Tricine-SDS-PAGE using a 16.5% acrylamide gel. Bovine heart bc_1 complex subunits (33) were used as molecular mass standards. *Asterisks* (*) mark subunits which were not verified by amino acid sequencing or Western blotting (*cf.* "Results").

and after electroelution of the complexes from blue native gels (Fig. 3).

Direct application of the two-dimensional technique had the disadvantage that the protein amounts were limited by the maximum load applicable to the first-dimension native gel. The presence of the novel M9.2 and M8-9 proteins (apparent mass in SDS-PAGE of 13 and 8–9 kDa, respectively) in V_1V_0 holoenzyme could therefore be detected only with prolonged silver staining (data not shown). However, because subunits of V_1V_0 and V_0 complexes appeared as clearly recognizable columns of bands in the two-dimensional gels, a smearing 75-kDa band could be easily identified as the only major contaminant of both complexes (Fig. 2). This contaminant was identified as dopamine-β-monooxygenase (GenBank™ accession no. P15101) by amino-terminal sequencing (data not shown).

V_1V_0 and V_0 complexes which were electroeluted from preparative blue native gels and resolved by SDS-PAGE (Fig. 3), revealed essentially the same polypeptide patterns as observed with the direct two-dimensional technique. However, at the higher protein loading, the low molecular mass bands in the holoenzyme now were easier to detect, and apparent molecular masses could be assigned using bovine bc_1 complex subunits for calibration (33).

The polypeptide number and masses in the 29–115-kDa range (37–47) matched those for other V-ATPase preparations (3–6). Smaller protein subunits in the range between 29 kDa and proteolipid c (32) have already been described, *e.g.* subunit G (48), which has also been termed M16 (49), and M20 (50) in the membrane sector. Anti-subunit G_1 antibody reacted with the 15-kDa band of V_1V_0, but did not detect any protein of the V_0 sector (*cf.* "Discussion"). The unidentified 16-kDa band of the V_0 sector therefore was tentatively assigned to M20. Only one protein component running below proteolipid c in SDS gels, namely subunit F, has been identified so far (51). We could detect more components in this low molecular mass range; M9.2 protein with an apparent mass of 13 kDa, and M8-9 protein, represented by a stack of four bands in the 8–9-kDa range, were identified in the membrane sector and in the holoenzyme. According to the electrophoretic mobility, the unidentified 12-kDa protein was tentatively assigned to subunit F.

Comparison of 10%, 13%, and 16.5% acrylamide gels led to the identification of anomalous migration behavior of some subunits, which is often observed with hydrophobic membrane proteins. The M9.2 membrane sector-associated protein had an apparent mass around 10 kDa in 10% gels, which shifted to 13 kDa in 16.5% gels. In 16.5% acrylamide gels (Fig. 3), the M9.2 protein appeared as a diffuse background to the sharp 12-kDa band, but the 15-kDa subunit G and the 16-kDa band were resolved. Subunit G and the 16-kDa band comigrated in 13% acrylamide gels, but the M9.2 and 12-kDa proteins were separated (data not shown).

13% acrylamide gels were preferentially used for the densitometric quantification of Coomassie stain intensities of V_0 and V_1V_0 subunits, and for determination of their staining ratios relative to the M39 subunit (Table I). The stain intensities of

TABLE I
Ratios of Coomassie stain intensities of V_0 and V_1V_0 subunits

The data were obtained by densitometric quantification of different V_0 and V_1V_0 preparations resolved by 13% acrylamide Tricine-SDS-gels, and supplemented by data (in parentheses) from 16.5% acrylamide gels. Stain intensities (arbitrary units) were divided by the molecular masses of the individual subunits, and normalized to M39. Asterisks (*) mark subunits that were not verified by amino acid sequencing or Western blotting (*cf.* "Results").

Subunit	kDa	Staining ratios of subunits of V_0 (n = 5)	Staining ratios of subunits of V_1V_0 (n = 5)
M115	96.3	0.72 ± 0.14	0.40 ± 0.09
A	68.5		1.95 ± 0.13
B	56.6		2.69 ± 0.07
M45	25.4	0.94 ± 0.11	1.17 ± 0.28
C	44		0.88 ± 0.04
M39	39	1.00	1.00
D + E	26.1 + 28.3		1.68 ± 0.08
M20*	16	1.42 ± 0.10	(0.97 ± 0.19)
G	13.7		(3.67 ± 0.67)
Proteolipid c	15.7	3.48 ± 0.52	(3.02 ± 0.55)
F*	12.5		1.10 ± 0.14
M9.2	9.2	0.41 ± 0.05	0.48 ± 0.07
M8–9	9	0.67 ± 0.10	0.62 ± 0.10

M9.2 and M8-9 relative to M39 were almost identical in the V_0 membrane sector and in the V_1V_0 holoenzyme which indicates almost identical stoichiometries in V_0 and V_1V_0. However, it was not possible to decide whether these proteins are present in stoichiometric or substoichiometric amounts, because Coomassie Blue staining intensities are not reliable indices of copy number. Subunit G and proteolipid c in the holoenzyme had comparable stain intensities, which might also indicate a higher copy number for subunit G.

Amino-terminal Protein Sequences—The complexes resolved by preparative BN-PAGE were electroeluted, and the protein subunits resolved by Tricine-SDS-PAGE and electroblotted onto PVDF membranes for direct amino-terminal protein sequencing (Fig. 4). Only a few of the proteins had free amino termini accessible to Edman degradation. Among these proteins were the major bovine brain subunit B, identified by the sequence MRGIVNGAAPELPV (39, 40); M45, also called glycoprotein IV or Ac45 protein (41, 42); and proteolipid c (32). However, more than 90% of proteolipid c appeared to be amino-terminally blocked, because the signal intensities of phenylthiohydantoin amino acids from cyanogen bromide fragments were up to 10-fold higher than after direct sequencing. The novel M9.2 and M8-9 proteins were also directly accessible to Edman degradation (*cf.* Table II). The amino-terminal sequences obtained from the four bands of the M8-9 protein (Fig. 3) suggested that M8-9 might be present in a "full length" form (largest band 1) and three amino-terminally shortened forms (smaller bands 2–4).

Subunit A (38) was identified after cleavage at tryptophan by iodosobenzoic acid, subunit C (46) after hydroxylamine cleavage between asparagine and glycine, subunits D (47), E (45), proteolipid c (32), and M45 (41, 42) after cyanogen bromide cleavage, M115 (37) after partial acidic hydrolysis, and M39 (43, 44) after use of deacylating conditions. The sequences obtained from V_0 subunits are summarized in Table II. We could not obtain internal protein sequences from the protein with an apparent mass of 16 kDa, because this protein tends to aggregate during electrophoresis, and is hardly transferred to PVDF membranes by electroblotting. These properties seem to indicate a hydrophobic membrane protein. We assume that this protein represents subunit M20 (50).

Primary Structure and Properties of the M9.2 Protein—TFASTA computer searching using the amino-terminal protein sequence of the bovine M9.2 protein revealed a high degree of homology to several human as well as one murine cDNA clone. The function of these proteins was not known. The M9.2 cDNA from one of the human cDNA clones, IMAGE Consortium Clone 143553 (GenBank accession no. R75754), was sequenced (Fig. 5). The sequence around the initiator codon matches exactly the optimal sequence for initiation by eukaryotic ribosomes ACCATGG as described by Kozak (52). The sequenced M9.2 cDNA clone from a bovine adrenal medulla cDNA library was incomplete (Fig. 5). However, the full bovine M9.2 protein sequence, except the amino acids at positions 4, 14, and 17, was obtained by Edman degradation (Fig. 4*A*), which also showed that the amino-terminal methionine residue was processed in the mature protein. The almost perfect conservation between human and murine proteins, which differed only at position 22, strongly suggests that the three unidentified residues may be conserved in the bovine protein as well. In this case, the bovine protein would be completely identical to the human protein.

The human M9.2 protein has a calculated molecular mass of 9.243 kDa if processing of the amino-terminal methionine is assumed, as in the bovine protein. It is an extremely hydrophobic membrane protein, with a polarity index of 22.5%, according to Capaldi and Vanderkooi (53), comprising 15 aromatic amino acids from a total of 80 amino acids. Helical profiles according to Mohana Rao and Argos (25) predict two membrane-spanning helices from positions 1 to 25 and from positions 36 to 59 (*cf.* Figs. 4*A* and 6*B*, *upper panel*). There are two basic amino acid residues (positions 30 and 34) in the short hydrophilic stretch separating the two hydrophobic domains in addition to two basic (positions 69 and 77) and one acidic residue (position 71) in the hydrophilic carboxyl-terminal region.

A computer search revealed a potential glycosylation site, NET, at positions 70–72 (Fig. 4*A*), but glycopeptidase F (Sigma) had no effect on M9.2, whereas M45 was deglycosylated in a parallel experiment (data not shown).

A sequence motif CSVCC (positions 44–48), similar to those of potential metal-binding proteins (54), is conserved in the protein from all known mammalian sources. It is located at the center of the second hydrophobic stretch.

Apart from a partial cDNA sequence for the rat protein (GenBank accession no. H32025), homologous sequences in unidentified reading frames on chromosome IV of *Caenorhabditis elegans* (accession no. Z68227) and on chromosome III of *Drosophila melanogaster* (accession no. L07835) were also found by computer searching (Fig. 4*B*). The presumed protein of *C. elegans* was deduced by translation of the joined segments 27704–27827, 27876–27934, and 28058–28165 as indicated in the annotations to the sequence with accession no. Z68227. The presumed protein of *D. melanogaster* was deduced by translating the DNA-sequence from position 7957–8223 in reverse direction from the sequence with the accession no. L07835. The deduced *C. elegans* and *D. melanogaster* protein sequences share 44% and 35% identity, and 72% and 62% similarity, respectively, with the human protein. Transmembrane regions and hydrophobicity distribution (25, 28, 55) predicted for the deduced translation products of the unidentified reading frames from *C. elegans* and *D. melanogaster* are very similar to those for the mammalian M9.2 proteins (data not shown).

In the *C. elegans* and *D. melanogaster* sequences, a stretch with high similarity to the human CSVCC sequence motif is present. It comprises a doublet of cysteines, but cysteine 44 is not retained.

Data base searching using the TFASTA program revealed no further significant homologies; however, direct comparison with all known subunits of the bovine and yeast F and V-ATPases and with assembly factors for yeast V-ATPase led to

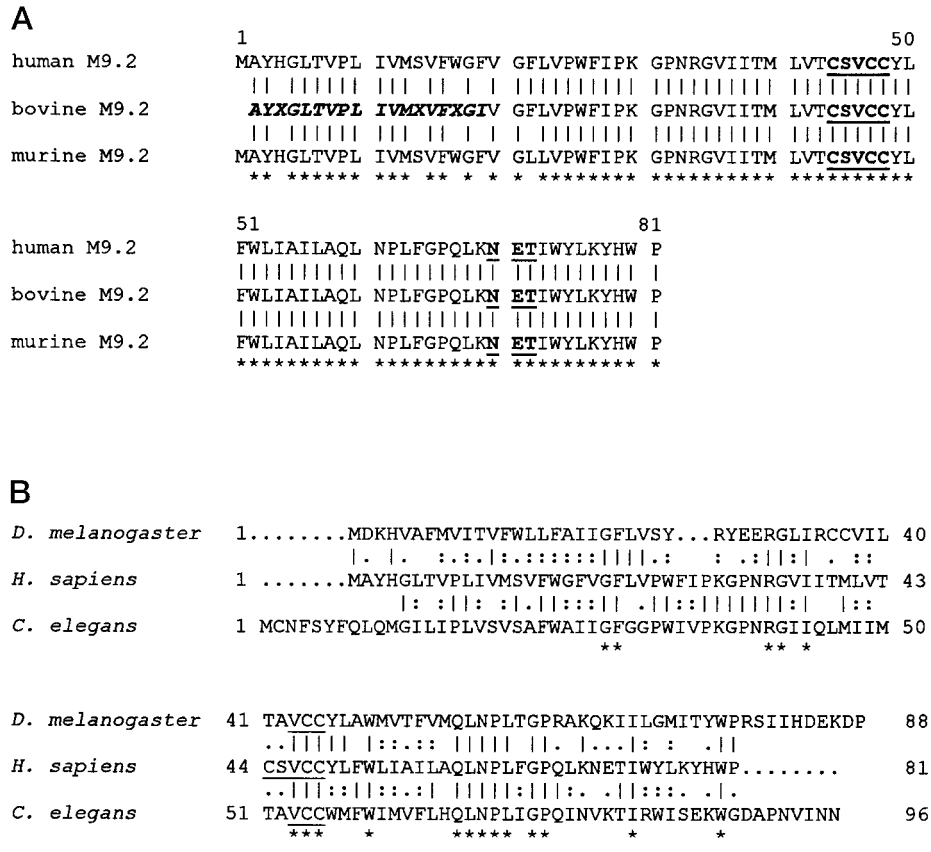

FIG. 4. **Protein sequences of mammalian M9.2 and homologous proteins.** Mammalian M9.2 protein sequences are compared in *A*. The sequences were deduced from a murine EST clone (GenBank™ accession no. W98811), from the complete human and the partial bovine cDNA sequences given in Fig. 5. According to Edman degradation (amino acids in *italic*s), the amino-terminal methionine is absent from the mature bovine protein. Potential metal binding sites, CSVCC, and potential glycosylation sites, NET, are underlined (*cf.* "Results"). Human M9.2 protein and homologous unidentified reading frames from *C. elegans* and *D. melanogaster* are compared in *B*. Identical amino acids in all three sequences are marked by *asterisks* (*).

TABLE II
Partial sequences of proteins associated with V_o

Protein	Assignment	Sample preparation	Amino acid sequences	Similarity to	Accession no.
kDa					
115	M115	Partial acidolysis[a]	PVTGDYVHKSVFIIFFQGDQLKNRVK	116-kDa polypeptide (37)	L31770
			IWNIATN		
			DLLEE		
45	M45	Direct sequencing	VPPTMNVPV	Ac45 (41, 42)	U10039
		CNBr cleavage	VTFKFIL		
39	M39[b]	Deacylation	*X*FFPELYFNVXN	32-kDa protein (43, 44)	J04204
16	M20 (50)[c]	Direct and CNBr	No sequence obtained		
14	Proteolipid c	Direct[d]	*X*EAKNGPE	16-kDa proteolipid (32)	J03835
		CNBr cleavage	AGIIAIYGLV		
		CNBr cleavage	GASAAMVFS		
13	M9.2	Direct sequencing	AY*X*GLTVPLIVM*X*VFXGI	Vma21p (56) and others (*cf.* "Results")	U09329
					P81103 (this work)
9	M8–9[e]				
8–9	Band 1	Direct sequencing	ETKQVKDPSTTYNLAYKYNF		P81134 (this work)
	Bands 2–4	Direct sequencing	DPSTTYNLAYKYNFEYPVVFNLVL		

[a] Fragments were not separated. The initial triple sequence could be arranged according to the fragments expected after cleavage of the 116-kDa polypeptide (37) at three aspartic acid-proline bonds.
[b] The amino-terminal sequence confirms that the coding region extends 231 nucleotides upstream from the initially reported start codon (43) as suggested by Bauerle *et al.* (44) by comparison with the yeast homologue Vma6p.
[c] The 16 kDa protein was tentatively assigned to M20 (*cf.* "Results").
[d] Less than 10% of proteolipid c was accessible to direct Edman degradation (*cf.* "Results").
[e] Band 1 is the largest of the four M8–9 protein bands. The sequence stretch shared by bands 1–4 is underlined.

the detection of structural similarity between human M9.2 and yeast Vma21 protein (56), expressed by a score of 45% sequence similarity and 19% identity between the two proteins (Fig. 6*A*), and by the similarity of predicted transmembraneous helices (Fig. 6*B*). When the Vma21p sequence in turn was used for a data base search, no human cDNA clones were found, although a huge number of human EST-sequences are deposited in the data bases. Therefore, it seems likely that there is no human protein with significantly higher homology to the yeast Vma21p than M9.2.

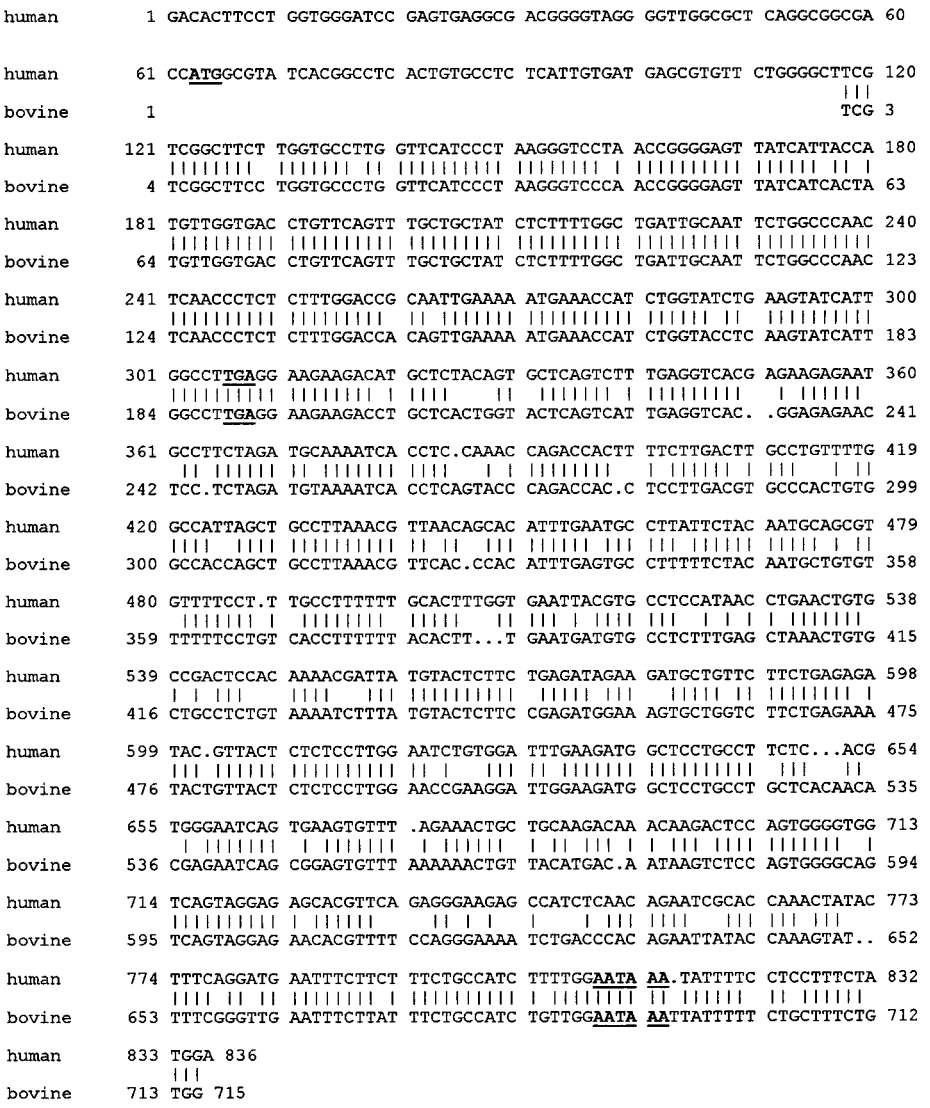

FIG. 5. **Bovine and human M9.2 cDNA.** The complete human M9.2 cDNA (GenBank™ accession number Y15286) and the bovine M9.2 cDNA (GenBank™ accession number Y15285) with incomplete 5′ terminus were aligned using the "multalign" program. The presumed initiator and terminator codons ATG and TGA, respectively, and the polyadenylation signal AATAAA are *underlined*.

Tissue Distribution of M9.2 mRNA—A Northern blot using RNA from various bovine tissues was hybridized with a ^{32}P-labeled 900-bp cDNA probe against bovine M9.2, which was excised from pBBM9.2 with *Bam*HI. A ≈900-bp transcript was present in all tissues, but in low concentrations in skeletal muscle, heart muscle, and cortex (Fig. 7*A*). The same blot was rehybridized with a probe against human glyceraldehyde-3-phosphate dehydrogenase, which is present in every tissue (Fig. 7*B*), and with a probe against the bovine V_1V_0-ATPase proteolipid c (Fig. 7*C*). Comparable M9.2/proteolipid c signal ratios were observed in most tissues, including skeletal and heart muscle with weak hybridization signals, but not in brain. The proteolipid c signal in brain was strong, whereas the M9.2 signal was hardly detectable. Quantification indicated an approximately 100-fold lower M9.2/proteolipid c signal ratio in brain.

DISCUSSION

Two-dimensional electrophoresis (BN-PAGE/Tricine-SDS-PAGE) was used to identify the proteins associated with V_1V_0 holocomplex and V_0 membrane sector. The novel M9.2 and M8-9 proteins were identified as proteins associated with the V_0 membrane sector for the following reasons. (i) Because BN-PAGE separates membrane proteins according to their molecular masses (17), contaminants of V_1V_0 and V_0 complexes should also be multiprotein complexes or oligomeric forms of smaller complexes. However, in the 29–115-kDa range, the only proteins detectable have already been identified in different V-ATPase preparations (3–6), which makes the presence of significant amounts of contaminating protein complexes unlikely. (ii) The M9.2 and M8-9 proteins were found both in the membrane sector and in the holoenzyme. Their staining intensities relative to the M39 subunit were almost identical in the membrane sector and in the holoenzyme (Table I). (iii) Proteins that precipitate during BN-PAGE could contaminate the V-ATPase complexes. However, in two-dimensional gels, these contaminants would appear as smearing bands crossing the polypeptide columns of the complexes as was found with dopamine-β-monooxygenase. This is not the case for the M9.2 and M8-9 proteins, as they are found only as discrete spots at the positions of the V_0 and V_1V_0 complexes.

The assignment of subunit G to V_0 or V_1 is still a matter of debate. A protein homologous to subunit G was first discovered as a component of the yeast V-ATPase, encoded by the VMA10 gene (57). It was named M16, and was suggested to belong to V_0 on the basis of its sequence homology with subunit b of F-ATPase, from the characteristics of VMA10 knockouts, and from cold inactivation of the V-ATPase, which failed to release it from the membrane. Similar results were obtained on cold inactivation of the chromaffin granule V-ATPase (49); however, Tomashek *et al.* (58) have shown recently that Vma10p inter-

A

```
human M9.2        1 MAYHG.....LTVPLIVMSVFWGFVGFLVPWFIPKGPNRGVIITM 40
                    || ..          :|.:::|:.|   :. |::. |.|..  .|.: .
yeast Vma21p      1 MAVDVPRAVINKLMLFTAAMVVLPV...LTFFIIQQFTPNTLISGGLAAA 47

human M9.2       41 LVTCSVCCYLFWLIAILAQLNPLFGPQLKNETIWYLKYHWP 81
                     :... :..|:  ::|: .: :.  ... |.|.
yeast Vma21p      48 MANVVLIVYI..VVAFREDTEDHKVDGNKKED..... 77
```

B

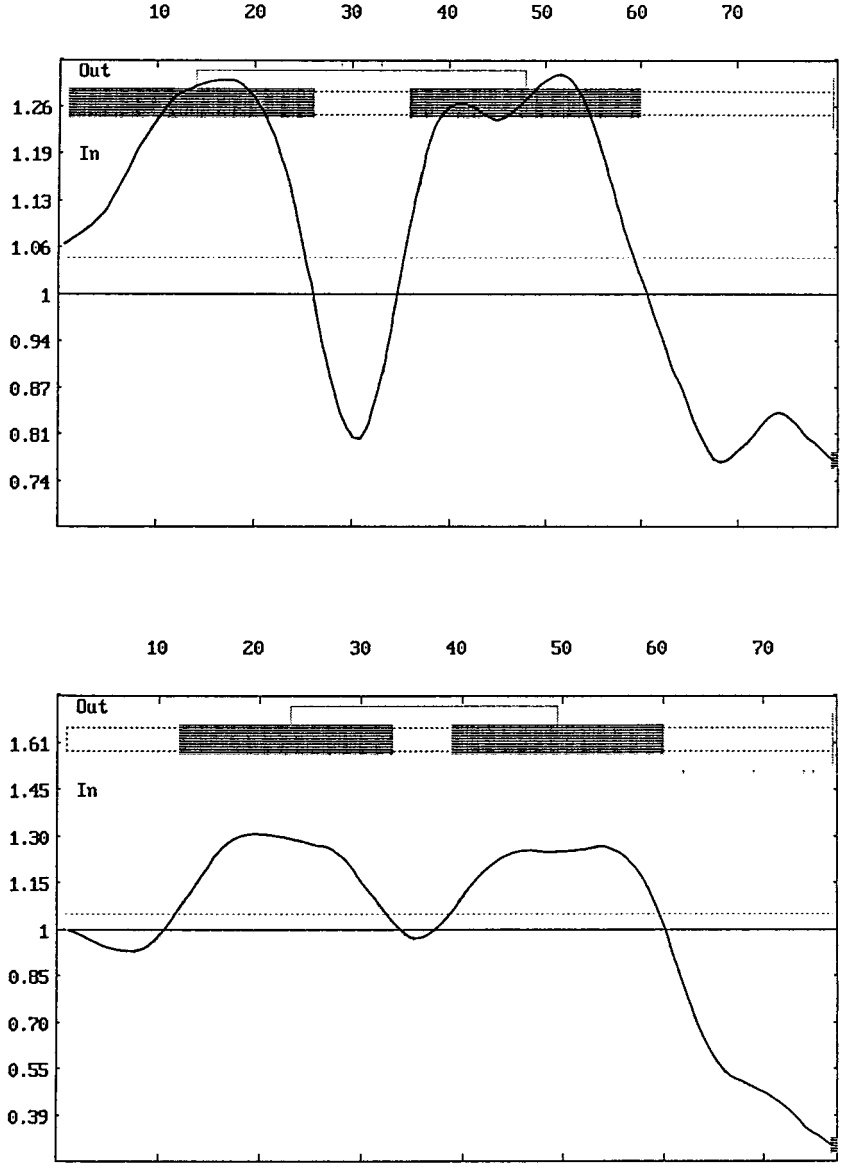

FIG. 6. **Comparison of human M9.2 and yeast Vma21 protein.** The GAP program was used for the alignment in *A*. Helical profiles of human M9.2 protein (*B, top panel*) and yeast Vma21p (*B, lower panel*) according to Mohana Rao and Argos (25) were computed with the ANTHEPROT program (26, 27).

acts with subunit E and classified it as a stalk subunit, belonging to V_1. Subunit G in the midgut V-ATPase of *Manduca sexta* could be released from the membrane by cold inactivation or by treatment with chaotropic anions (59). Cold inactivation studies suggested also that subunits G and H from bovine brain clathrin-coated vesicles (60), which were later shown to be isoforms and renamed G_1 and G_2, belong to V_1 rather than to V_0 (48). In the present work, we could identify subunit G in the holo-V-ATPase, but not in the V_0 membrane sector, by using an anti-G_1 antibody (Western blot not shown). This direct approach again suggests that subunit G is a V_1 component.

The electrophoretic separation of the holoenzyme (V_1V_0) from its subcomplexes (V_1 and V_0) allowed the determination of the molar ratio of the various species. We found a V_0/V_1V_0 ratio of 5 after solubilization of chromaffin-granule membranes and resolution by BN-PAGE. It is hard to exclude the loss of V_1

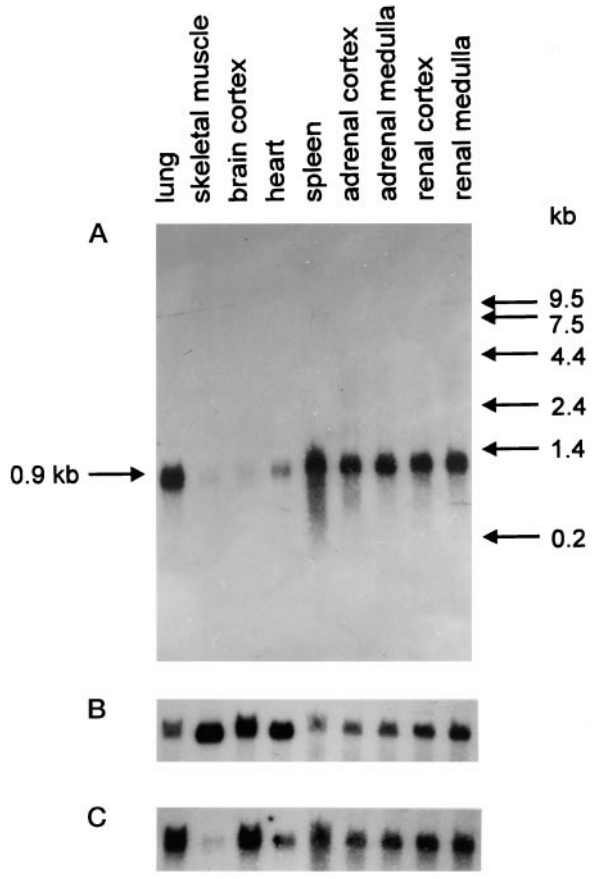

FIG. 7. **Northern blot analysis of M9.2 from various tissues.** A Northern blot with total RNA from different bovine tissues was hybridized with probes against bovine M9.2 (*A*), human glyceraldehyde-3-phosphate dehydrogenase (*B*), and proteolipid c from bovine V-ATPase (*C*). A 0.9-kilobase pair M9.2 transcript was detected in all tissues, but with strongly differing signal intensities. In all tissues except brain, the ratios of the M9.2/proteolipid c hybridization signals were comparable. The M9.2/proteolipid c ratio in brain was about 100-fold lower, but the strong signal of proteolipid c indicated the presence of substantial amounts of V-ATPase.

subcomplexes during membrane isolation, particularly as dissociation of V_1V_0 is promoted by MgATP at low temperatures. Nevertheless, we consider this unlikely for the following reasons: 1) 2 mM EDTA was included in all buffers during membrane isolation; 2) release of subunit B, a component of V_1, was not detectable by immune blotting of soluble fractions obtained during membrane isolation.

A large excess of V_0 over V_1V_0 has been reported before in chromaffin granule membranes (61), although in this case the ratio was determined after a prepurification step, which may have selected for the membrane sector. There have, however, been several other reports of the occurrence of free V_0 and V_1: After solubilization of stripped bovine brain clathrin-coated vesicles with the nonionic detergent $C_{12}E_9$, a V_0/V_1V_0 ratio of about 2 was found by glycerol-gradient velocity centrifugation (62), and free V_1 was detected in cytosol from bovine brain and from Madin-Darby bovine kidney cells (63). Convincing evidence for the regulation of V-ATPase activity by the reversible dissociation of V_1V_0 has been presented. This occurs in the vacuoles of *S. cerevisiae* in response to glucose deprivation (64), and in goblet cell apical membranes of *M. sexta* during moulting or starving of the larvae (65, 66). Whether reversible dissociation of V_1V_0 might also have a regulatory role in chromaffin cells, or whether V_0 itself might have an independent function, for example in exocytosis, is still a matter for speculation (67, 68). It is noteworthy that in synaptic vesicles V_0 appears to exist in a complex with the vesicle membrane proteins synaptobrevin and synaptophysin (69).

Coomassie staining intensities of V_0-subunits (Table I) did not indicate a high copy number for any V_0 protein except for proteolipid c. Assuming 1:1 stoichiometries for all V_0 proteins except six copies of proteolipid c as determined by Arai *et al.* (70), and neglecting the extent of glycosylation, a total mass of 288 kDa was calculated from the masses of the proteins listed in Table I. It was impossible to assign a monomeric or dimeric state to the major band of the V_0 membrane sector, because it had an apparent mass of 440 kDa in BN-PAGE, which was between the calculated masses of a monomeric (288 kDa) and a dimeric state (576 kDa). There are no data at present on the effects of protein glycosylation on the apparent masses in BN-PAGE. However, we speculate that the major V_0 form was the monomeric form, because glycosylation of M115 and M45 subunits should increase the Stokes radius and the apparent mass.

The holoenzyme seemed to be present in monomeric form, inasmuch as the calculated mass of 815 kDa was close to the apparent mass of around 1000 kDa in BN-PAGE, assuming 3 copies each of subunits A and B (11, 70). Furthermore, 3 copies of subunit G were assumed for calculation, because the normalized staining intensity of subunit G was about 3–4 times higher than that of M39 (cf. Table I).

In the mammalian M9.2 protein a CSVCC sequence resembles potential metal-binding motifs (54), but only a cysteine doublet is retained at corresponding positions in the *C. elegans* and *D. melanogaster* sequences. If the *C. elegans* and *D. melanogaster* sequences were equivalent to the mammalian sequences, this would argue against the presence of a functional metal binding site.

The sequence similarity of human M9.2 and the yeast Vma21 proteins is not very high (45% similarity, 19% identity), but corresponding proteins of yeast and mammalian origin can have low sequence similarity, as shown by comparison of the 6.4-kDa protein of bovine bc_1 complex and the homologous yeast 8.5-kDa protein (71). The sequence and structural similarities of M9.2 and Vma21p indicate that the two proteins are potential homologues, and that assembly of mammalian V-ATPase might follow a pathway similar to that of the yeast V-ATPase. However, yeast Vma21p, which is required for assembly of V-ATPase, is not a subunit of V-ATPase, but instead localizes to the endoplasmatic reticulum membrane (56), whereas M9.2 protein was found to be associated with V_0 and V_1V_0 complexes in adrenal glands. Because antibodies against M9.2 are not yet available, we cannot exclude that M9.2 additionally or mainly localizes to the endoplasmic reticulum membrane. It seems conceivable that the mammalian protein is integrated into the complex after exerting its function in assembly, whereas yeast Vma21p is not.

M9.2 mRNA was detected in all tissues, but the M9.2/proteolipid c transcript level was about 100-fold lower in brain than in other tissues. This tissue-specific variation is not yet understood, but could indicate altered translational control, or decreased M9.2 protein degradation in brain. Alternatively, one could speculate that an undetected brain-specific analogue of M9.2 exists.

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