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

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Vacular 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 
vacular 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) 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 sub-
unit 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 V₀ and V₁, 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 (V₁V₀) and free membrane sector (V₀) 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. Aminoacyltransfer RNA, oligodeoxynucleotides, 

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL 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 protein database with accession numbers P81130 (bovine M9.2 protein) and P81134 (bovine M8-9 protein).

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‡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; V₁V₀, V-ATPase, vacuolar proton pumping ATPase (holoenzyme); F₁F₀, complex V, mito-
chondrial proton pumping ATPase (holoenzyme); V₀ and V₁, hydrophilic (catalytic) and hydrophobic (transmembrane) sectors of V-ATPase, re-
spectively; 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-mor
pholino)propanesulfonic acid.

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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 of 1 M 6-aminohexanoic acid, 50 mM BisTris/ HCl, pH 7.0, and 21 µl of 10% deoxy maltoside, and centrifuged at 100,000 × g. After addition of 90 µl of 5% Serva Blue G in 500 mM 6-aminohexanoic acid, 50 µl were applied to 10-ml 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<sub>c</sub> and V<sub>c</sub>V<sub>o</sub> complexes were electropholated 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% iodoobesin 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<sub>2</sub>-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 MM5SD12 (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'-ATC/C(T/G) AGT ATG AGC GTG TGC GGT GGC-3' and VATPB9.2n, 5'-GCC AAA AIA GAT GAT AGC ACA C-3'. PCR was performed in 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 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 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 derived from this sequence: VATPB9.2a, 5'-GGG GCA TCG TC GCT GTC GCC 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 cycling as above. Concluding a pool containing 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.

Sequence of Human and Bovine Clones—The insert from clone BVATPM9.2 was cut with BamHI and cloned into pBlueScript™ II SK (−). The new clone pBBM9.2 was subcloned by using the BstEII site at nt 246. The insert of human cDNA clone 143553 was cut out with EcoRI and HindIII and cloned into pBlueScipt™ II SK (−). Clones pBBM9.2 and pBBHM9.2 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 [32P]dCTP by random priming, and QuikHyb™ solution from Stratagene was used for hybridization. A 900-bp cDNA was excised from pBBM9.2 with BamHI and used as a probe for M9.2. The probe against bovine V<sub>c</sub>V<sub>o</sub>ATPase subunit c (proteolipid c), GenBank™ accession number J09385 (32), was made by PCR using primers Bvch 5'-TCA GCC GGC ATG GTC TTC AG-3' and Bvcr 5'-CGG CAG AAG TGA GGA GTA GGA-3'. Using the bovine adrenal medulla cDNA library as a template, a 358-bp fragment corresponding to positions 190–547 of V<sub>c</sub>V<sub>o</sub>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<sub>1</sub>V<sub>o</sub> holocomplex and free V<sub>o</sub>-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<sub>o</sub>-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<sub>c</sub>V<sub>1</sub>V<sub>0</sub>-ATPase. The position of free V<sub>1</sub> sector is also indicated in Fig. 1, although the amounts were too low for detection in BN-PAGE (see below).

Second-dimensional SDS-PAGE of lane M from BN-PAGE (cf. Fig. 1) revealed the characteristic polypeptide patterns of V<sub>c</sub>V<sub>o</sub> holoenzyme and V<sub>o</sub> membrane sector. Additionally, minor amounts of free V<sub>1</sub> sector and some contaminating mitochondrial F<sub>1</fsub>F<sub>0</sub>-ATP-synthase could be detected (Fig. 2, upper panel). F<sub>1</fsub>F<sub>0</sub>- was removed by Triton X-114 extraction/precipitation (Fig. 2, lower panel). Using the migration distances of V<sub>c</sub>V<sub>0</sub> (~1000 kDa), F<sub>1</fsub>F<sub>0</sub> (≈550 kDa), and V<sub>o</sub> (~440 kDa) for calibration, the apparent mass of free V<sub>1</sub> 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<sub>c</sub>V<sub>0</sub> and the major band of V<sub>o</sub> indicated the positions of minor amounts of V<sub>o</sub> sector in higher oligomeric states.

The molar ratio of V<sub>c</sub>N/V<sub>c</sub>V<sub>0</sub> in isolated chromaffin granule membranes was deduced from Coomassie Blue-stained twodimensional gels by densitometric quantification of proteolipid c and 115- and 39-kDa proteins (data not shown). A 5-fold molar excess of free V<sub>1</sub> 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<sub>c</sub>N/V<sub>c</sub>V<sub>0</sub> 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).
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 V1V0 holoenzyme could therefore be detected only with prolonged silver staining (data not shown). However, because subunits of V1V0 and V0 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 (lower panel, left side). In addition to a major form of the V0 membrane sector, the positions of minor forms with higher oligomeric states are indicated.

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 V0 membrane sector (right side), to V1 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 V0 membrane sector, the positions of minor forms with higher oligomeric states are indicated.

FIG. 3. Subunit composition of V1V0 ATPase and V0 membrane sector. The multiprotein complexes recovered by electrophoresis from preparative blue native gels were resolved by Tricine-SDS-PAGE using a 16.5% acrylamide gel. Bovine heart bc1 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”).

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 G1 antibody reacted with the 15-kDa band of V1V0, but did not detect any protein of the V0 sector (cf. “Discussion”). The unidentified 16-kDa band of the V0 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 V0 and V1V0 subunits, and for determination of their staining ratios relative to the M39 subunit (Table I). The stain intensities of
The data were obtained by densitometric quantification of different V₀ and V₁V₀ 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").

### Table I

| Subunit | kDa | Staining ratios of subunits of V₀ (n = 5) | Staining ratios of subunits of V₁V₀ (n = 5) |
|---------|-----|----------------------------------------|----------------------------------------|
| M115    | 96.3| 0.72 ± 0.14                            | 0.40 ± 0.09                            |
| A       | 68.5| 1.95 ± 0.13                             | 0.67 ± 0.09                            |
| B       | 56.6| 2.69 ± 0.07                             | 1.17 ± 0.28                            |
| M45     | 25.4| 0.94 ± 0.11                             | 0.88 ± 0.04                            |
| C       | 44  | 1.00                                     | 0.88 ± 0.04                            |
| M39     | 39  | 1.00                                     | 0.88 ± 0.04                            |
| D+E     | 26.1+28.3| 1.68 ± 0.08 | 0.97 ± 0.19 |
| M20*    | 16  | 1.42 ± 0.10                             | 0.67 ± 0.07                            |
| G       | 13.7| 3.48 ± 0.52                             | 0.92 ± 0.05                            |
| Proteolipid c | 15.7 | 3.48 ± 0.52 | 0.92 ± 0.05 |
| F*      | 12.5| 1.10 ± 0.14                             | 0.88 ± 0.07                            |
| M9.2    | 9.2 | 0.41 ± 0.05                             | 0.92 ± 0.05                            |
| M8-9    | 9   | 0.67 ± 0.10                             | 1.10 ± 0.14                            |

M9.2 and M8-9 relative to M39 were almost identical in the V₀ membrane sector and in the V₁V₀ holoenzyme which indicates almost identical stoichiometries in V₀ and V₁V₀. 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 decylating conditions. The sequences obtained from V₀ 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 electrophoretic transfer. 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. 4A), 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 Vanderkoor (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. 4A and 6B, 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. 4A), but glycopetidase 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. 4B). 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
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. 6A), and by the similarity of predicted transmembrane helices (Fig. 6B). When the Vma21p sequence in turn was used for a database search, no human cDNA clones were found, although a huge number of human EST-sequences are deposited in the databases. Therefore, it seems likely that there is no human protein with significantly higher homology to the yeast Vma21p than M9.2.

**TABLE II**

| Protein Assignment | Sample preparation | Amino acid sequences | Similarity to Accession no. |
|-------------------|--------------------|----------------------|---------------------------|
| **kDa**           |                    |                      |                           |
| 115               | M115               | Partial acidolysis<sup>a</sup> | PVTGDYHVSFIVIIFQSDLKVRK | 116-kDa polypeptide (37) | L31770 |
| 45                | M45                | Direct sequencing | VFTKIF | Ac45 (41, 42) | U10039 |
| 39                | M39<sup>b</sup>   | Deacylation        | XPEFELPKXN | 32-kDa protein (43, 44) | J04204 |
| 16                | M20 (50)<sup>c</sup> | Direct and CNBr cleavage | No sequence obtained | 16-kDa proteolipid (32) | J03835 |
| 14                | Proteolipid c      | Direct<sup>d</sup> | XEAAGKPE | 16-kDa proteolipid (32) | J03835 |
| 13                | M9.2               | Direct sequencing | AYXGLTVPLIVMWMFXGI | Vma21p (56) and others (cf. “Results”) | U09329 |
| 9                 | M8–9<sup>e</sup>   | Band 1             | ETKQVRKDPETYNLAKYNE | Vma21p (56) and others (cf. “Results”) | P81103 (this work) |
| 8–9               | Band 2–4           | Direct sequencing | DESYYNVLKSYENYEPVFLNLVL | Vma21p (56) and others (cf. “Results”) | P811134 (this work) |

<sup>a</sup> 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.

<sup>b</sup> 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.

<sup>c</sup> The initial sequence extends 231 nucleotides downstream from the initially reported start codon (43) as suggested by Bauerle et al. (44) by comparison with the yeast homologue Vma6p.

<sup>d</sup> Less than 10% of proteolipid c was accessible to direct Edman degradation (cf. “Results”).

<sup>e</sup> Band 1 is the largest of the four M8–9 protein bands. The sequence stretch shared by bands 1–4 is underlined.
Tissue Distribution of M9.2 mRNA—A Northern blot using RNA from various bovine tissues was hybridized with a 32P-labeled 900-bp cDNA probe against bovine M9.2, which was excised from pBBM9.2 with BamHI. A 900-bp transcript was present in all tissues, but in low concentrations in skeletal muscle, heart muscle, and cortex (Fig. 7A). The same blot was rehybridized with a probe against human glyceraldehyde-3-phosphate dehydrogenase, which is present in every tissue (Fig. 7B), and with a probe against the bovine V1V0-ATPase proteolipid c (Fig. 7C). 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 V1V0 holocomplex and V0 membrane sector. The novel M9.2 and M8-9 proteins were identified as proteins associated with the V0 membrane sector for the following reasons. (i) Because BN-PAGE separates membrane proteins according to their molecular masses (17), contaminants of V1V0 and V0 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 V0 and V1V0 complexes.

The assignment of subunit G to V0 or V1 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 V0 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-
acts with subunit E and classified it as a stalk subunit, belonging to V₁. 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 G₁ and G₂, belong to V₁ rather than to V₀ (48). In the present work, we could identify subunit G in the holo-V-ATPase, but not in the V₀ membrane sector, by using an anti-G₁ antibody (Western blot not shown). This direct approach again suggests that subunit G is a V₁ component.

The electrophoretic separation of the holoenzyme (V₁V₀) from its subcomplexes (V₁ and V₀) allowed the determination of the molar ratio of the various species. We found a V₁/V₀ ratio of 5 after solubilization of chromaffin-granule membranes and resolution by BN-PAGE. It is hard to exclude the loss of V₁.

![Comparison of human M9.2 and yeast Vma21 protein](image)

**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).
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$_{8}$, a $V_1V_0/V_1$ 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 endoplasmic 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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