The Peripheral Complex of the Tobacco Hornworm V-ATPase Contains a Novel 13-kDa Subunit G*

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A prominent 16-kDa protein copurifies with the V-ATPase isolated from both posterior midgut and Malpighian tubules of Manduca sexta larvae and thus was believed to represent a V-ATPase subunit. [14C]N,N'-dicyclohexylcarbodiimide labeling and its position on SDS-electrophoresis gels revealed that this protein was different from the 17-kDa proteolipid. A cDNA clone encoding a highly hydrophilic protein with a calculated molecular mass of 13,692 Da was obtained by immunoscreening. Monospecific antibodies, affinity-purified to the 13-kDa recombinant protein expressed in Escherichia coli, specifically recognized the 16-kDa protein of the purified V-ATPase, confirming that a cDNA encoding this protein had been cloned. In vitro translation of the cRNA showed that the cloned 13-kDa subunit behaved like a 16-kDa protein on SDS-electrophoresis gels. The cloned protein showed 37% amino acid sequence identity to the 13-kDa V-ATPase subunit Vma10p recently cloned from yeast and some similarity to subunit b of bacterial F-ATPases. In contrast to the Vma10p protein, which behaved like a V[sub]o subunit, the M. sexta 13-kDa protein behaved like a V[sub]1 subunit, since it could be stripped from the membrane by treatment with the chaotropic salt KI and by cold inactivation. When KI dissociated V-ATPase subunits were reassOCIated by dialysis that removed the KI, the soluble, 450-kDa complex of the M. sexta V-ATPase could be purified by gel chromatography. This V[sub]1 complex consisted of subunits A, B, E, and the 13-kDa subunit, confirming that the cloned protein is a new V-ATPase subunit and a member of the peripheral V[sub]1 complex of the V-ATPase. We designate this new V[sub]1 component subunit G.

H⁺-translocating vacuolar-type ATPases (V-ATPases) occur in endomembranes as well as in various plasma membranes of eukaryotic cells (see Harvey (1992)). The tobacco hornworm (Manduca sexta) midgut V-ATPase is highly concentrated in the apical plasma membrane of the goblet cells (Wieczorek et al., 1986; Schwegl et al., 1989; Klein et al., 1991). In contrast to most other V-ATPases, it does not drive acid or fluid transport, but energizes electrophoretic K⁺/2H⁺-antiport by generating a transmembrane voltage of more than 200 mV (Wieczorek et al., 1991; Wieczorek, 1992; Azuma et al., 1995). The resulting K⁺-electrochemical potential drives the absorption of amino acids by K⁺ coupled symport (Giordana and Parenti, 1994; Martin and Harvey, 1994). Plasma membrane V-ATPases have also been found in other insect organs, such as Malpighian tubules (Drosophila hydei: Bertram et al., 1991; M. sexta: Klein et al., 1991; Formica polyctena: Van Kerkhove, 1994), where they are involved in the energization of salt and fluid secretion (for review, see Nicolson (1993)). V-ATPases are heteromultimeric enzymes composed of peripheral V[sub]1 and membrane integral V[sub]0 complexes, which together, in analogy to F-ATPases, form ball and stalk structures known as portasomes (see Harvey (1992)). The V[sub]0 part consists of at least two subunits, a 43-kDa subunit and the highly conserved 17-kDa proteolipid, subunit c, which binds DCCD (Bowman et al., 1986) and which, probably as a hexamer, forms the proton-conducting pore (Arai et al., 1988). A 14-kDa protein, first shown to be a constitutive V-ATPase subunit in M. sexta (Gräf et al., 1994b) and subsequently found in yeast and D. melanogaster (Graham et al., 1994; Nelson et al., 1994; Guo et al., 1996), exhibits some affinity to the V[sub]1 part (Gräf et al., 1994b), but also appears to be involved in the assembly and stability of the V[sub]2 complex (Graham et al., 1994). Chaudri proteins (Rea et al., 1987) or cold treatment in the presence of ATP (Moriyama and Nelson, 1989) lead to the dissociation of various V-ATPase subunits from the membrane; hence these polypeptides were defined as constituents of the peripheral V[sub]1 complex. Among them, three subunits, A, B, and E, are major components of the V[sub]1 complex and occur in every V-ATPase, including that of M. sexta (67-kDa subunit A: Gräf et al., 1992; 56-kDa subunit B: Novak et al., 1992; 28-kDa subunit E: Gräf et al., 1994a).

Taken together, up to six genuine V-ATPase subunits have now been identified at the molecular level. However, although recent years have seen considerable progress in elucidating the molecular structure of V-ATPases, we are still far from knowing the actual number or the proper function of V-ATPase subunits, irrespective of their origin. Several subunits, which may not be universal constituents of V-ATPases, have been described (see Nelson (1992)); moreover, several unidentified polypeptides copurify with the holoenzyme in many V-ATPase preparations (Adachi et al., 1990; Gluck and Caldwell, 1987; Perez-Castineira and Apps, 1990; Ward and Sze, 1992). In particular several polypeptides have been detected in the range of 10–20 kDa. For instance, a 16-kDa polypeptide was released, together with known V[sub]1 subunits, by chaotropic treatment of Neurospora crassa vacuolar membranes (Bowman et al., 1989).

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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) X92805.

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1 The abbreviations used are: DCCD, N,N'-dicyclohexylcarbodiimide; MOPS, 4-morpholinepropanesulfonic acid; TBS, Tris-buffered saline.
Again, protein bands in the range of 10–15 kDa, obtained by SDS-polyacrylamide gel electrophoresis, have been discussed as putative peripheral subunits of the V-ATPase from bovine clathrin coated vesicles (Puopolo et al., 1992). Very recently, a 13-kDa subunit Vma10p was identified as a putative constituent of the V_o complex in the yeast V-ATPase (Supekova et al., 1995). Here we report the identification of a V-ATPase subunit, which has a calculated molecular mass of 13 kDa and an apparent molecular mass of 16 kDa. This novel subunit is a major V_o component of the M. sexta V-ATPase and we designate it subunit G.

**EXPERIMENTAL PROCEDURES**

**Insects—Larvae of M. sexta (Lepidoptera, Sphingidae) were reared under long day conditions (16 h of light) at 27°C using a synthetic diet (modified according to Bell and J oachim, 1974).**

**[14C]DDC Labeling—Labeling reactions were performed in a volume of 500 μl containing 5-10 μg of purified V-ATPase in 50 mM Tris-MOPS (pH 8.0), 1 mM MgCl₂, 1 mM β-mercaptoethanol, 0.1 mM EDTA, 0.003% C₁₂E₁₀, and 10 mM [14C]DDC (50-60 Ci/mmol). Samples were incubated for 30 min at 30°C. Labeled protein was collected by trichloroacetic acid precipitation; the pellet was washed twice with ice-cold acetone, air-dried, resuspended in 15 μl of SDS buffer (125 mM Tris-HCl, pH 6.8, 5% sucrose, 2% SDS, 2% β-mercaptoethanol, 0.005% bromphenol blue) and incubated for 1 h at 37°C. After SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining, the gel was incubated in enhancer solution EN’HANCE, DuPont) for 1 h, washed in cold water for 1 h, and dried on Whatman filter paper. The dried gel was exposed to Kodak x-ray film at –70°C for a minimum of 3 days.

**Purification of Monospecific Antibodies to the 13-kDa Protein—Purified V-ATPase (0.5 mg) was electrophoresed on a SDS-agarose gel (Pharmacia Fine Chemicals) with a 1% gel concentration. The 16-kDa band was excised, and the protein was extracted from the agarose matrix by repeated freeze-thawing in 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 50% methanol, 10% acetic acid, until opaque bands were visible in the clear gel. This plasmid was purified by a conventional miniprep procedure (Sambrook et al., 1989). To destroy residual RNAse the plasmid DNA preparation was treated with proteinase K. After phenol/chloroform extraction and ethanol precipitation the plasmid DNA was used for coupled in vitro transcription/translation (TNT-Rabbit reticulocyte system from Promega). Reactions were performed according to the manufacturer by adding [35S]methionine (20 μCi/ml, 1 Ci/m mole) and [32P]dCTP (1 Ci/m mole) to the translation mixture. The labeled proteins were separated on a 15% SDS-polyacrylamide gel.**

**Labeling reactions were performed in a volume of 300 μl containing 5–10 μg of protein in 0.6 ml was coupled to CNBr-activated Sepharose 4B, following the manufacturer’s instructions.** The slurry was filled into a MicroSpin column (Pharmacia Bio-tech Inc.) resulting in a final bed volume of approximately 0.3 ml, and filtered rabbit polyclonal antiserum to the purified V-ATPase holoenzyme (Wieczorek et al., 1992), using a 1:10 dilution of the monospecific antibodies. For immunoprecipitation of the translated protein, 0.5 μl of blocking buffer (0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), 0.05% Tween 20). For SDS-gel electrophoresis, the translated protein that was bound to the beads was eluted by boiling in 100 μl of SDS buffer for 3 min. The whole sample was used for one lane of an SDS-polyacrylamide gel electrophoresis gel. The dried gel was exposed to Kodak XAR 5 film for 5 h at –80°C.

**Preparation of V-ATPase from Malpighian tubes—For one preparation, Malpighian tubules from 20 fifth instar larvae were pooled in 6 ml of ice-cold M buffer (0.3 M mannitol, 17 mM Tris-HCl (pH 7.5), 5 mM EGTA). Brush border membranes from Malpighian tubules were isolated by a modification of the protocol of Pringle and spiralin (1974; Wolbersberger et al., 1987). In brief, after homogenization of the tissue for 45 s at 20,000 rpm using an Ultraturrax homogenizer, 6 ml of ice-cold 24 mM MgSO₄ was added. After 15 min on ice, the sample was centrifuged for 15 min at 2,500 g and 4°C, and the supernatant containing the brush border membranes was transferred into a new tube and centrifuged for 10 min at 30,000 g and 4°C. For further purification the entire procedure was repeated after resuspending the 30,000 g pellet in 2 ml of M buffer and adding an equal amount of 24 mM MgSO₄. The resulting brush border membrane pellet was frozen in liquid nitrogen and stored at −20°C. The V-ATPase was solubilized and purified as published previously (Schwelker et al., 1988; Wieczorek et al., 1990).**

**Isolation of the V_o Complex of the V-ATPase—V_o subunits were stripped from V₅ by incubating 0.6 ml of Malpighian tubule brush border membranes, suspended in M buffer (approximately 1 mg of
protein), in the presence of 0.8 M KI for 1 h on ice. After centrifugation at 100,000 × g for 1 h at 4 °C, the dissociated V₁ subunits in the supernatant were reassociated by dialysis against 500 ml of a buffer (pH 7.0) consisting of 50 mM KCl, 10 mM Tris-MOPS, 3 mM β-mercaptoethanol, 0.5 mM EGTA. The sample was concentrated to approximately 0.1 ml on a Centricon 10 concentrator and fractionated by fast protein liquid chromatography on a Superdex 200 HR 10/30 gel chromatography column using the dialysis buffer. The V₁ complex was found in the fraction containing proteins of approximately 450 kDa (using ferritin as standard).

Other Methods—Isolation and purification of the V-ATPase from Manduca midgut goblet cell apical membranes, protein determination with Amido Black, standard SDS-polyacrylamide gel electrophoresis, Western blotting on nitrocellulose membranes (BA85), and immunostaining were performed as described previously (Schweikl et al., 1989; Wieczorek et al., 1990, 1991; Gräf et al., 1994a). For preparation of the crude membrane pellet from mouse kidney, one kidney was homogenized for 45 s at 20,000 rpm using an Ultraturrax homogenizer. The homogenate was cleared by centrifugation at 100,000 × g for 5 min at 4 °C, and the supernatant was centrifuged again at 100,000 × g for 30 min at 4 °C. The membrane pellet was resuspended in M buffer.

RESULTS

The Purified M. sexta V-ATPase Contains Two Proteins in the 16-kDa Range—A prominent protein band with an apparent molecular mass of 16 kDa, appearing in SDS-polyacrylamide gels of the purified M. sexta midgut V-ATPase with a highly reproducible staining intensity relative to the other protein bands (Fig. 1), had already been noticed by Schweikl et al. (1989). Since a plasma membrane V-ATPase had been demonstrated previously immunocytochemically in Malpighian tubules of M. sexta (Klein et al., 1991; Russell et al., 1992), we purified the enzyme from brush border membranes and found a strongly stained 16-kDa band in SDS gels of this preparation, too (Fig. 1). The enrichment of the 16-kDa protein in the same fraction as all other established V-ATPase subunits, together with the fact that it is one of the major proteins in the preparation of the purified V-ATPase, argue strongly that it is a constitutive subunit of the insect V-ATPase.

Initially, Schweikl et al. (1989) had assumed that the 16-kDa band represented the proteolipid that forms the proton channel and is a genuine and universal V-ATPase subunit. However, several findings contradicted this assumption. First, the 16-kDa protein was stripped from the membrane by treatment with chaotropic iodide as well as by the gentler method of cold inactivation (Fig. 2; see also Fig. 5 in Gräf et al. (1994b)), and therefore it could not be a membrane protein; although the release of peripheral subunits by cold inactivation was less efficient than by iodide stripping, the 16-kDa protein was stripped to the same extent as the established V₁ subunits A, B, and E. Second, [¹³C]DCCD labeling showed that, in SDS-polyacrylamide gel electrophoresis, the proteolipid exhibited an apparent molecular mass of 17 kDa (Fig. 3). The 17-kDa band was only weakly stained by Coomassie Blue in ordinary SDS gels and was not stripped from the membrane by treatment with chaotropic iodide (Fig. 2, lanes 2 and 3; see also Fig. 5 in Gräf et al. (1994b)). Third and finally, the strong staining of the 16-kDa band observed with Coomassie Blue would not be expected for a highly hydrophobic protein such as the proteolipid, since it possesses only a very low content of positively charged amino acids (Dow et al., 1992).

Isolation and Sequencing of cDNA Encoding a 13-kDa Protein—The antiserum against the purified V-ATPase was very rich in 16-kDa-specific antibodies, enabling us to purify monospecific antibodies by affinity chromatography on a Sepharose column that had been conjugated with the 16-kDa protein eluted from SDS–agarose gels (not shown). The monospecific antibodies were used for immunoscreening of a M. sexta larval posterior midgut cDNA library (Gräf et al., 1992). One positive clone was isolated after three screening steps. The 808 base pairs of cDNA sequence obtained included an open reading frame (base position 45–395) encoding a very hydrophilic protein with 117 amino acids, a calculated molecular mass of only 13,692 Da (Fig. 4), and an isoelectric point at pH 9.88 (DNAsis). The initiator ATG was chosen due to its sequence environment which is similar to other cloned cDNAs encoding M. sexta V-ATPase subunits (Gräf et al., 1992, 1994b; Novak et al., 1992; Dow et al., 1992) and matches closely the consensus sequence for the translation start site in eukaryotes (Kozak, 1989). Using the putative coding region of the cDNA for hybridization screening of a cDNA library from the silk gland of Bombyx mori, we detected a clone exhibiting an almost identical DNA.
sequence; the identity of the deduced amino acid sequence was 94\%.

The deduced M. sexta amino acid sequence was 37\% identical and 63\% similar to the 13-kDa protein Vma10p, which had been identified very recently as a V-ATPase subunit from the fly (Fig. 5a; Supekova et al., 1995); most of the conserved amino acids occur in the N-terminal half of the protein (48\% identity, residues 1–59). Some similarity appeared to exist to subunit b of bacterial F-ATPases (Fig. 5a; 26\% identity in a 100-amino acid overlap to the F-ATPase subunit b of Vibrio alginolyticus; Krumholz et al., 1989), but also to the N-terminal part of tropomyosin from African clawed frog; Hardy et al., 1991).

The secondary structure of the deduced M. sexta 13-kDa protein may be helpful in deducing its function. The sequence of the N-terminal half predicts that it forms a continuous, highly hydrophilic \( \alpha \)-helix, followed by two further \( \alpha \)-helices covering approximately 20\% of the whole sequence (Fig. 5b).

Expression of the 13-kDa Protein—To confirm that the cDNA encoding the protein corresponding to the 16-kDa band of SDS-electrophoresis gels had been cloned and that it contained the complete coding sequence, the recombinant protein was expressed using two different strategies. First, the postulated coding sequence was cloned into the E. coli expression vector pMAL-c2. Periplasmic expression in a protease-deficient strain (UT5600) rather than cytoplasmic expression was chosen because we had observed strong degradation when we used pMAL-c2 and standard E. coli strains in preliminary experiments. Although the fusion protein was the main protein constituent of the periplasmic fraction (Fig. 6), it was partially degraded. Therefore it was not appropriate to cleave the fusion protein at the fusion site with factor Xa protease in order to determine the apparent molecular mass of the recombinant 13-kDa protein by SDS-polyacrylamide gel electrophoresis. However, the periplasmic fraction seemed to be pure enough to be used for the purification of antibodies to the recombinant protein from the anti-holoenzyme serum by affinity chromatography. Western blots with purified V-ATPase showed that the resulting 13-kDa specific antibody preparation was highly specific for the 16-kDa protein (Fig. 7, lanes 2 and 3). Only after long incubation times for the alkaline phosphatase reaction or at high antibody concentrations could a weak cross-reaction with the 28-kDa subunit E be observed (Fig. 7, lane 3). These results clearly indicate that the 13-kDa recombinant protein used for antibody purification corresponds to the 16-kDa protein of the purified V-ATPase.

However, it was still not clear whether the remarkable difference between the calculated and the apparent molecular masses of 13 and 16 kDa, respectively, was due to intrinsic properties of the protein or to an incomplete open reading frame in the cloned cDNA. Therefore the opened reading frame, starting at base position 45, together with the 3\'-untranslated region, was cloned into the translation vector pSPUTK which contained the S\'-untranslated sequence of \( \beta \)-globin to achieve efficient in vitro transcription/translation by using SP6 polymerase and rabbit reticulocyte lysates. Since the crude lysate contained an enormous amount of low molecular mass proteins that prevented any exact size determination of the \([\text{[35S]}\text{Met}\text{]}\) labeled, translated protein on SDS-electrophoresis gels, we immunoprecipitated the translated protein using the anti-holoenzyme antiserum. Subsequent SDS-polyacrylamide gel electrophoresis, followed by autoradiography, clearly indicated an apparent molecular mass of 16 kDa. This result confirmed that the cDNA sequence, which predicted a 13-kDa protein, included the complete open reading frame for the 16-kDa protein (Fig. 8). The unexpectedly high apparent molecular mass of this protein in SDS-polyacrylamide gel electrophoresis may be a consequence of its high, almost 41\%, content of charged amino acids. By contrast, subunits A and B, whose apparent molecular masses match closely their calculated molecular masses, have a content of charged amino acids of less than 25\%.

Identification of the 13-kDa Protein as a Subunit of the V\(_1\) Complex of the V-ATPase—Although the 13-kDa protein copurified with established V-ATPase subunits and appeared as a prominent band in SDS-polyacrylamide gels of the purified V-ATPase preparation, it was desirable to obtain further evidence that it is a genuine subunit of the V-ATPase holoenzyme. First we tried to inhibit ATP-dependent proton transport by the monospecific antibodies, in experiments analogous to those reported for the 14-kDa subunit (Gräf et al., 1994b). However, the antibodies did not influence the reaction. Although this result was disappointing, it was not unexpected, since subunit-specific antibodies do not necessarily inhibit the function of a complex holoenzyme (see also Nelson et al., 1994). If the 13-kDa protein is a genuine V-ATPase subunit and, moreover, a constituent of the V\(_1\) complex, it should be present in the isolated V\(_1\) complex in quantities similar to those in the purified V-ATPase. Therefore we stripped peripheral membrane proteins,
including the \( V_4 \) subunits, from \( V \)-ATPase-containing brush border membranes of Malpighian tubules by treatment with a high concentration of chaotropic iodide. After centrifugation, the supernatant was dialyzed again against iodide-free buffer and, thereafter, subjected to gel chromatography. Analysis by SDS-polyacrylamide gel electrophoresis revealed \( V_1 \) subunits in low molecular mass fractions, but also in a fraction corresponding to an apparent molecular mass of 450 kDa, the approximate calculated molecular mass of the \( V_1 \) complex (Fig. 9). Fractions corresponding to higher molecular masses contained no protein. Since no protein could be detected in the 450-kDa fraction without dialysis (Fig. 9), we concluded that the \( V_4 \) subunits had been dissociated by the iodide treatment to subcomplexes and monomers and that a small portion (approximately 2% of total protein in the stripping supernatant) of \( V_1 \) proteins had reassembled during dialysis, forming large complexes of approximately 450 kDa. This dissociation/reconstitution was similar to that which Puopolo et al. (1992) obtained with \( V \)-ATPase from bovine clathrin-coated vesicles. However, the composition of the reconstituted \( M. sexta \) 450-kDa \( V_1 \) complex was unique.

The main proteins constituting the complex were the 67-kDa subunit A, the 56-kDa subunit B, the 28-kDa subunit E, and the 13-kDa (apparent 16-kDa) protein (Fig. 9). Furthermore, the staining intensity of the 16-kDa band was highly reproducible relative to the three other \( V_1 \) subunits and was similar to that observed for the purified \( V \)-ATPase. Other proteins seemed to be present only in substoichiometric amounts and therefore were probably impurities.

Cross-reaction of the 13-kDa Subunit-specific Antibodies with \( V \)-ATPase-containing Membrane Preparations of Xenic Origin—The 13-kDa specific antibodies, originally directed to the plasma membrane \( V \)-ATPase of \( M. sexta \) midgut, cross-reacted not only with the 16-kDa band of the Malpighian tubule \( V \)-
DISCUSSION

The cDNA encoding a novel subunit of the M. sexta V-ATPase was cloned by immunoscreening. The hydrophilic protein consisted of 117 amino acids with a calculated molecular mass of 13,692 Da. It showed 37% sequence identity to the recently published 13-kDa subunit Vma10p of the yeast V-ATPase (Supekova et al., 1995) as well as some similarity to subunit b of bacterial F-ATPases. In Western blots, monospecific antibodies to the 13-kDa protein cloned from M. sexta midgut specifically recognized the 16-kDa band of the purified V-ATPase from M. sexta midgut and Malpighian tubules. Furthermore, upon dialysis of the stripping supernatant from the chaotropic treatment, the 13-kDa subunit as well as the A, B, and E subunits of the V-ATPase, all present in the complex mixture of dissociated proteins, may also be represented by other low molecular mass proteins copurifying with the respective V-ATPases.

The 13-kDa Subunit Shares an Epitope with Subunit E—Western blots of both midgut and Malpighian tubule V-ATPase from M. sexta which were probed with the 13-kDa specific antibodies showed a cross-reaction with a band in the range of 30 kDa (Fig. 7). This result was not surprising, since our monoclonal antibody 47-5 (Klein et al., 1991) binds to the 13-kDa subunit and to the 28-kDa subunit E of the purified midgut V-ATPase (Fig. 7, lane 6), suggesting that both proteins share a common epitope. Indeed, a five-amino acid sequence, EARKR (boxed in Fig. 4), is found in both the 13-kDa subunit and subunit E (Gräf et al., 1994a). Thus, the epitope reacting with the monoclonal antibody appears to be clearly defined. The 13-kDa specific antibodies also cross-reacted with a protein in the 30-kDa range of a membrane preparation from crab gills but not from mouse kidney (Fig. 7). This epitope may be common to invertebrates, especially since the EARKR sequence is found neither in mammalian and yeast subunits E nor in the yeast 13-kDa protein Vma10p.

The 13-kDa Subunit Is a Member of the V1 Complex—In our recent report on the 14-kDa subunit we had already shown that the 16-kDa protein of the membrane bound V-ATPase was released from midgut goblet cell apical membranes upon treatment with chaotropic iodide (Fig. 5 in Gräf et al. (1994b)). In this paper, we obtained the same result using Malpighian tubule brush border membranes. Moreover, cold inactivation experiments also indicated that the 13-kDa protein is a peripheral V-ATPase subunit. Furthermore, strong staining of the proteolipid with Coomassie Blue (see Introduction) may not represent the proteolipid as alleged. Thus, strong staining of the proteolipid with Coomassie Blue would not be expected because of its very low content of positively charged amino acids. Instead, these bands may represent mainly the novel 13-kDa subunit. However, the novel subunit may also be represented by other low molecular mass proteins copurifying with the respective V-ATPases.

The 13-kDa protein cross-reacted with a 16-kDa protein band in V-ATPase containing membrane preparations of both crab gills and mouse kidney. Finally, a fairly homologous 13-kDa protein was recently identified as a subunit of the yeast V-ATPase (Supekova et al., 1995).

We presume by analogy to our case, that some of the strongly stained bands in the 16-kDa range found in published SDS-electrophoresis gels of purified V-ATPases from other sources (see Introduction) may not represent the proteolipid as alleged. Thus, strong staining of the proteolipid with Coomassie Blue would not be expected because of its very low content of positively charged amino acids. Instead, these bands may represent mainly the novel 13-kDa subunit. However, the novel subunit may also be represented by other low molecular mass proteins copurifying with the respective V-ATPases.

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Evidence indicate that the 13-kDa protein is a genuine V-ATPase subunit. First, it copurifies in strictly reproducible amounts with the V-ATPase purified from both the midgut goblet cell apical membrane and the Malpighian tubule brush border membrane. Second, it is a major component not only of the holoenzyme, but also of the reassociated V1 complex. Third, in Western blots monospecific antibodies to the recombinant 13-kDa protein cross-reacted with a 16-kDa protein band in V-ATPase containing membrane preparations of both crab gills and mouse kidney. Finally, a fairly homologous 13-kDa protein was recently identified as a subunit of the yeast V-ATPase (Supekova et al., 1995).

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been designated as subunit F (Nelson et al., 1994), we suggest the term subunit G for the 13-kDa subunit.

Our conclusion that the 13-kDa subunit G is a member of the \(V_1\) complex seems not to be in line with results obtained in yeast, where cold inactivation experiments and the properties of the yeast null mutant suggested that the homologous 13-kDa subunit \(V\text{ma}10\) is a member of the membrane bound \(V_0\) sector of the V-ATPase (Supeková et al., 1995). However, the alignment of the two derived amino acid sequences may explain this apparent contradiction (Fig. 5a): sequence identities are clustered in the N-terminal parts (48% identity from residues 1 to 59), whereas the C-terminal parts share only 24% identical amino acids. The sequence similarity to subunit b of bacterial F-ATPases argues neither for nor against the \(V_1\) or \(V_0\) membership of the 13-kDa subunit from both M. sexta and yeast, since subunit b appears to be anchored to the membrane by its apolar N-terminal region (Deckers-Hebestreit and Altendorf, 1992), for which no equivalent exists in the 13-kDa subunits (Fig. 5b).

The function of the 13-kDa subunit is enigmatic so far, but the predicted unusual secondary structure with one continuous, highly charged \(\alpha\)-helix covering the N-terminal half of the protein, and its similarity with the F-ATPase subunit b regarding both sequence and predicted secondary structure of the N-terminal part may provide a clue to understanding its role in the V-ATPase holoenzyme. For example, the location of the 13-kDa insect subunit G in the \(V_1\) sector and the homologous 13-kDa yeast, where cold inactivation experiments and the properties of subunit G for the 13-kDa subunit.

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