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The Plasma Membrane H^+-V-ATPase from Tobacco Hornworm Midgut

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The midgut plasma membrane V-ATPase from larval *Manduca sexta*, the tobacco hornworm, is the sole energizer of any epithelial ion transport in this tissue and is responsible for the alkalinization of the gut lumen up to a pH of more than 11. This mini-review deals with those topics of research on this enzyme which may have contributed or are expected to contribute novel and general aspects to the field of V-ATPases. Topics dealt with include novel subunits or the quaternary structure of the V_1 complex, as well as the regulation of the enzyme's function by reversible dissociation of the V_1 from the V_0 complexes and by genetic control on the transcriptional and posttranscriptional level.

KEY WORDS: Vacuolar ATPase; insects; *Manduca sexta*; midgut; V_1V_0 dissociation; transcriptional regulation; antisense RNA; quaternary structure.

INTRODUCTION

H^+-V-ATPases are found in two principal locations, endomembranes and plasma membranes (see Harvey and Nelson, 1992). The larva of the moth *Manduca sexta* (Lepidoptera, Sphingidae), commonly called the tobacco hornworm, is a rich source of plasma membrane V-ATPase (Wieczorek, 1992). The enzyme can be isolated and highly purified from the larval midgut in rather large amounts. Twenty larvae provide up to 0.5 mg of pure holoenzyme, and soluble, cytosolic V_1 complexes can be purified in even higher amounts—up to 2 mg. Thus, the easy accessibility of pure enzyme makes the tobacco hornworm an appropriate model organism for research on general aspects of the V-type family of ion-transporting ATPases (Fig. 1). After reviewing earlier work on the physiology and biochemistry of the insect plasma membrane V-ATPase, we will discuss recent work on its structure and regulation.

THE ROOTS OF THE INSECT V-ATPase: PHYSIOLOGY AND BIOCHEMISTRY OF THE CATERPILLAR MIDGUT

Textbooks of physiology and of biochemistry tell us that animal cell membranes are energized by the sodium motive force established by a Na^+/K^+-ATPase. The enzyme is weakly electrogenic, pumps K^+ into cells and Na^+ out of them, and is said to be located in the basal membrane of all animal epithelia. Many insect epithelia violate this widely accepted dogma. Their cell membranes are energized by the potassium motive force established by a strongly electrogenic, primary uniporter that pumps K^+ out of the cells. The first paper on this type of ion transport appeared nearly half a century ago, when Ramsay (1953) demonstrated active K^+ secretion by insect Malpighian tubules. A decade later, the short-circuit current in the isolated caterpillar midgut was shown to be carried almost exclusively by K^+ (Harvey and Nedergaard, 1964).

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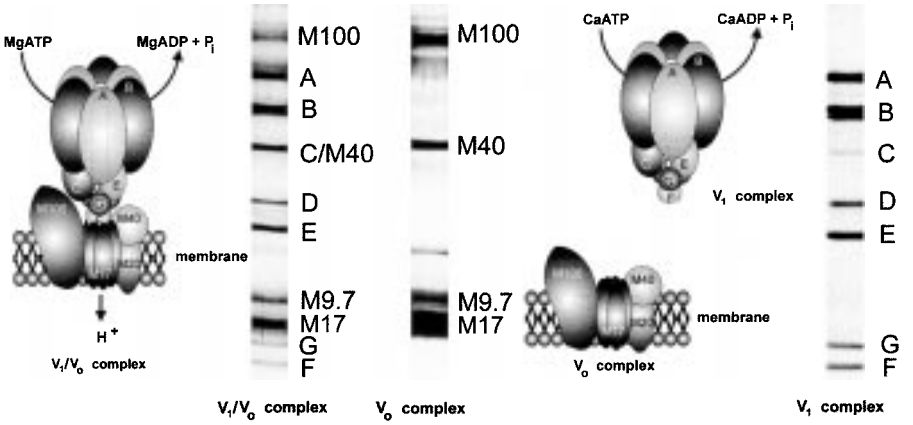

Fig. 1. Subunit composition of the *M. sexta* V-ATPase. V-ATPase holoenzyme and V_1-ATPase were prepared as described previously (Schweikl *et al.*, 1989; Graf *et al.*, 1996). The V_0 complex was partially purified (M. Huss and H. Wieczorek, unpublished data), starting with the posterior midgut of starving fifth instar larvae. The lower 10% and the upper 20% fractions of the sucrose density gradient, being enriched with V_0 complex, were subjected to anion-exchange FPLC (Mono Q). Fractions from 220 to 320 mM NaCl were further purified by gel permeation chromatography on Superdex. Proteins were separated by SDS–polyacrylamide gel electrophoresis and stained with silver. Data obtained from biochemical analysis are summarized in a structural model of the V_1V_0-holoenzyme and its subcomplexes.

The midgut, which lacks a Na^+/K^+-ATPase (Jungreis and Vaughan, 1977), has become a model system for electrogenic cation transport. The transport activity was directly correlated with the intracellular ATP concentration (Mandel *et al.*, 1980) implying that it was mediated by a K^+-coupled ATPase. The active step of K^+ transport was localized in the apical membrane of goblet cells using microelectrodes and electron microscopy (Harvey *et al.*, 1983), a result that was confirmed by X-ray microanalysis of potassium concentrations (Dow *et al.*, 1984). Based upon tissue organization and ultrastructure, novel methods were developed for the isolation of goblet cell apical membrane, along with three other midgut plasma membrane regions in high purity (Cioffi and Wolfersberger, 1983; Harvey *et al.*, 1983) and the field was opened for straightforward biochemical investigations.

In contrast to the other three midgut plasma membrane regions, the goblet cell apical membrane exhibited a K^+-stimulated, Mg^{2+}-dependent ATPase activity that was insensitive to the Na^+/K^+-ATPase inhibitors ouabain and vanadate, as well as to the inhibitors of mitochondrial F-ATPases, oligomycin and azide (Wieczorek *et al.*, 1986). On the other hand, enzyme activity could be inhibited by *N*,*N*′-dicyclohexylcarbodiimide, by *N*-ethylmaleimide and nitrate. While the enzyme thus exhibited characteristic features distinguishing it from P- and F-ATPases, its properties resembled those of ATPases from various acidic organelles and from certain kidney plasma membranes. Solubilization of the goblet cell apical membranes, using the nonionic detergent $C_{12}E_{10}$, followed by zonal centrifugation of the supernatant in a sucrose density gradient and by anion-exchange FPLC on a Mono Q column, led to the isolation of an ATPase activity that displayed the same substrate and inhibitor sensitivity as the membrane-bound ATPase (Schweikl *et al.*, 1989). Its polypeptide pattern, as revealed by SDS polyacrylamide gel electrophoresis (Schweikl *et al.*, 1989), and the sensitivity of both the membrane-bound and the solubilized forms to submicromolar concentrations of bafilomycin A_1 (Wieczorek *et al.*, 1991) established the enzyme unequivocally as a member of the V-ATPase family.

Studies with vesicles derived from the apical membranes of goblet cells demonstrated that the insect plasma membrane V-ATPase, like all other eukaryotic V-ATPases, is an exclusive proton pump and that the voltage generated by primary H^+ transport energizes secondary K^+/H^+ antiport (Wieczorek *et al.*, 1989, 1991). Thus, net active potassium ion transport in the lepidopteran midgut is the consequence of an H^+-V-ATPase, K^+/H^+ antiporter couple in the goblet cell apical membrane. The high transmembrane voltage of more than 240 mV (Dow and Peacock, 1989) that is produced by the H^+-V-ATPase, drives the antiport

which is electrophoretic, with 2 H^+ being exchanged for 1 K^+ (Azuma *et al.*, 1995). The absorption of protons coupled to the secretion of potassium ions leads to alkalinization of the midgut lumen resulting in a pH of more than 11 (Fig. 2). The resulting potassium motive force is used to drive electrophoretic, K^+-coupled uptake of nutrients such as amino acids (Castagna *et al.*, 1998).

NOVEL SUBUNITS

cDNA's encoding eight subunits of the tobacco hornworm V-ATPase have been cloned and sequenced. The deduced amino acid sequences of the V_1 subunits A, B, and E (Graf *et al.*, 1992, 1994a; Novak *et. al.*, 1992) and the V_0 subunits c (M17) and M40 (Dow *et al.*, 1992; Merzendorfer *et al.*, 1997) exhibited significant similarities to subunits previously described in yeast vacuoles, chromaffin granules, and other endomembranes. Three novel polypeptides, the V_1 subunits F and G and a membrane bound polypeptide, M9.7, have been established as constitutive V-ATPase subunits.

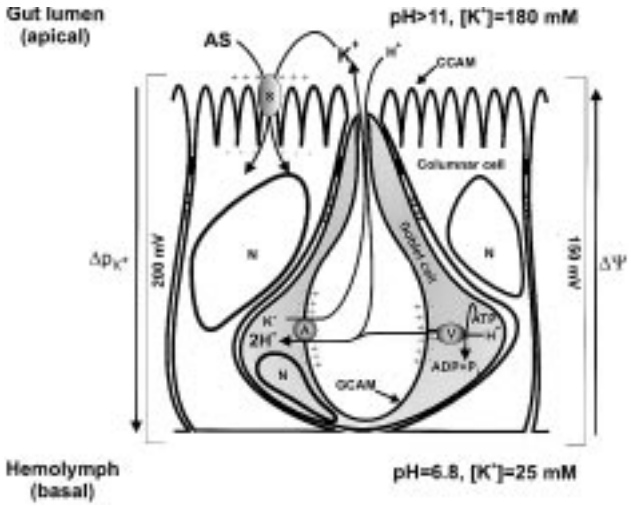

Fig. 2. Energization of the lepidopteran midgut epithelium by the plasma membrane H^+-V-ATPase. Goblet cell apical membranes (GCAM) in the larval midgut of *M. sexta* are the site of active and electrogenic K^+ secretion. The only primary pump in this system is the H^+-V-ATPase (V) that energizes electrogenic $K^+/2H^+$ antiport by the electrical component of the proton motive force, resulting in a net efflux of K^+ into the gut lumen. The combined action of the H^+-V-ATPase and the $K^+/2H^+$-antiporter (A) explains the generation of the highly alkaline pH measured in the gut lumen. The established transapical voltage generated by primary H^+ transport drives amino acid/K^+ symport (S) across the columnar cell apical membranes (CCAM). Measured values are given for the transepithelial voltage ($\Delta\Psi$), the transepithelial electrochemical potential of K^+(Δpk^+), the K^+ concentrations and the pH values. Nuclei of goblet and columnar cells are marked with N.

Subunit F was first cloned from *M. sexta* midgut (Graf *et al.*, 1994b). Subsequently, it was also found in yeast (Nelson *et al.*, 1994; Graham *et al.*, 1994) and mammals (Peng *et al.*, 1996). Both ATP hydrolysis and ATP-dependent H^+ transport are inhibited by monospecific antibodies to the insect F subunit; since inhibition depends on the presence of ATP, antibody binding appears to be the result of conformational changes and/or subunit rearrangements in the V-ATPase. Experiments using chaotropic KI for stripping of peripheral V_1 components from the membrane indicated that subunit F is not as strictly associated with the V_1 complex as the other four V_1 subunits A, B, E, and G (Graf *et al.*, 1996): the V_1 complex obtained after stripping and reconstitution from the supernatant does not contain subunit F. However, if recombinant subunit F is added during reconstitution, it incorporates into the resulting V_1 complex.

Subunit G was initially cloned from yeast (Supekova *et al.*, 1995) and first assigned to the V_0 complex since stripping of V_1 subunits by cold inactivation in the presence of ATP left a significant amount of this subunit in the membrane. Indeed, it exhibits some sequence similarity to the soluble domain of the F_0 subunit b, although it lacks the membrane anchor that is characteristic for the *N*-terminal region of this F-ATPase subunit. Subsequent cloning of the *M. sexta* subunit G and stripping experiments using KI and cold inactivation located the subunit in the V_1 complex (Lepier *et al.*, 1996). This location was corroborated by the analysis of subunit G in bovine clathrin-coated vesicles (Crider *et al.*, 1997). Evidently, subunit G plays some role in the connection of the V_1 and the V_0 complex and, like subunit F, may be a member of the stalk that connects the catalytic V_1 subunits A and B with the membrane-bound V_0 sector.

Subunit 9.7 was cloned recently (GenBank/EMBL accession No. AJ006029; Merzendorfer, 1998) and shows appreciable amino acid identity to the novel 9.2-kDa membrane-associated protein of bovine chromaffin granules (Ludwig *et al.*, 1998). It also exhibits some similarity to the yeast protein vma21p, which is a constituent of the endoplasmic reticulum where it is involved in V-ATPase biogenesis without being a subunit (Hill and Stevens, 1994). Because of its extensive glycosylation, accounting for half of its molecular mass, the *M. sexta* 9.7-kDa polypeptide has an electro-

phoretic mobility of 20 kDa. Since, after detergent solubilization of the highly purified goblet cell apical membrane, it copurifies with all other V-ATPase subunits and since significantly more than 90% of the whole midgut immunoreactivity resides in the goblet cell apical membrane, M9.7 has to be a constitutive V-ATPase subunit. Although its role remains enigmatic, it appears to differ from that of the yeast vma21p protein, whose function is restricted to the endoplasmic reticulum.

REGULATION OF V-ATPase ACTIVITY BY V_1/V_0 DISSOCIATION

The larval stage of lepidopteran insects such as *M. sexta* is characterized by periods of feeding, which are interrupted several times by molts that allow the caterpillar to grow and to increase its weight approximately 10,000-fold within 3 weeks. This rapid and enormous growth requires that the uptake of nutrients, such as amino acids, be energized very effectively. Indeed, 10% of a tobacco hornworm's total ATP production is used for active K^+ transport in the midgut (Dow, 1984) and thus for the plasma membrane V-ATPase which ultimately energizes nutrient absorption. Therefore one would expect, for reasons of economy, that V-ATPase activity would be strongly regulated, especially in periods of low energy consumption. Those periods should include larval/larval molts, the larval/pupal molt, and starvation. In all these cases, the hornworms do not feed and, therefore, they would have no use for the voltage, generated by the V-ATPase, to drive K^+-coupled secondary amino acid uptake.

Indeed, the voltage of more than 100 mV across the midgut epithelium, indicating net active K^+ transport and thus activity of the V-ATPase, was abolished completely during the larval/larval molt (Sumner *et al.*, 1995). Simultaneously, ATP hydrolysis and ATP-dependent H^+ transport in vesicles derived from the goblet cell apical membrane were found to be reduced more than 85%. After the molt, both ATPase activity and proton transport were restored to their normal levels. Immunocytochemistry of midgut cryosections, using monoclonal antibodies to the peripheral catalytic subunits A and B, revealed a reversible loss of antibody labeling during the molt. Immunoblots after SDS polyacrylamide electrophoresis of the goblet cell apical membrane confirmed the lack of immunoreactivity during the molt. In addition, the gels clearly showed that the amount of all V_1 subunits was strictly reduced, not just that of the A and B subunits. By contrast, the V_0 subunits M17 and M40 remained in the membrane.

Although dissociation of V_1 subunits from the membrane is well known from *in vitro* experiments with chaotropic reagents or with ATP at low temperatures (see Nelson, 1992), this was the first demonstration that V-ATPase activity can be regulated *in vivo* by the disassembly of V_1 and V_0 complexes. Disassembly of V_1 and V_0 subunits was also shown to play a role in yeast (Kane, 1995); if yeast cells are deprived of glucose, a rapid dissociation of V_1 subunits occurs, followed by a rapid reassembly upon restoration of glucose, with no evidence of new subunit biosynthesis. These similar findings from two organisms so distant in evolution as insects and yeast may point to a general regulatory mechanism.

After regulation of V-ATPase activity in the tobacco hornworm midgut had been shown convincingly to happen by dissociation of the V_1 subunits from the V_1/V_0 holoenzyme, it remained unclear whether the components dissociated as single, isolated subunits, as subcomplexes, or as a whole V_1 complex. The last alternative was shown to be correct: the loss of membrane-bound V-ATPase activity is strictly correlated with the increase of soluble cytosolic V_1 complexes (Graf *et al.*, 1996). During the larval/larval molt as well as after starvation and, as detected quite recently, during the larval/pupal molt as well (M. Huss and H. Wieczorek, unpublished results), the amount of cytosolic V_1 complexes increases to the unexpectedly high level of 2% of the total cytosolic protein. By contrast, the cytosolic V_1 pool was reduced drastically after the larval/larval molt or in larvae that had been refed after starvation. The restoration of the holoenzyme does not require biosynthesis of new subunits since translational inhibition by cycloheximide has no effect on the reassembly of V_1 and V_0 subunits (Jager and Klein, 1996).

The cytosolic V_1 complex lacks the Mg^{2+}-dependent ATPase activity that is characteristic of the holoenzyme, but it exhibits Ca^{2+}-dependent activity which switches back to Mg^{2+}-dependent ATPase activity in the presence of 25% methanol; in these properties it resembles certain F_1-ATPases (Sakurai *et al.*, 1981; Hicks and Krulwich, 1986). The yield of $\sim$0.5 mg/g of tissue, which is approximately the same as that of the preparation of chloroplast F_1-ATPase from spinach leaves, allows the preparation of milligram quantities from the midguts of less than 20 tobacco hornworms in less than 10 h. These amounts were sufficient to undertake the detailed

investigations into the structure of the enzyme that are discussed below.

TRANSCRIPTIONAL REGULATION OF V-ATPase BIOSYNTHESIS

Little is known about the control of V-ATPase subunit biosynthesis during transcription, except for a few investigations that have dealt with control by the transcriptional machinery. In *Neurospora crassa*, V-ATPases are thought to play a housekeeping role because they appear to be expressed continuously in the membranes of acidic organelles. Thus specific control of V-ATPase gene transcription may not occur. In support of this view, several promoters of *Neurospora* V-ATPase genes resemble those of mammalian housekeeping genes, since they lack TATA elements and exhibit a high GC content (Wechser and Bowman, 1995).

In more complex organisms, cell-specific demands require a particular control of V-ATPase expression that allows regulation of the amounts of protein present in the target membranes. For instance, transcription of V-ATPase genes in the slime mold, *Dictyostelium discoideum*, may be regulated during development. In Northern blots, the amounts of *vatB*-transcripts encoding subunit B were shown to be highest during growth and to decrease during starvation, a signal that triggers the transition from solitary amebae to multicellular fruiting bodies (Bracco *et al.*, 1997). Levels of *vatB*-mRNA also increased during phagocytosis and under alkaline conditions. In the mammalian hematopoietic system, increased V-ATPase activity in the lysosomal membranes during monocyte to macrophage differentiation may be met by increased transcription rates of the corresponding genes. In particular, one subunit, the B2-isoform, is amplified transcriptionally by a cAMP-dependent pathway that involves both multiple AP2 sites in the TATA-less and GC-rich promoter and a novel AP2-binding transcription factor (Lee *et al.* 1995, 1997).

As reported above, V-ATPase activity in goblet cell apical membranes of molting or starving tobacco hornworms is downregulated by the dissociation of the catalytic V_1 complex from the holoenzyme (Sumner *et al.*, 1995; Graf *et al.*, 1996). Since continued synthesis of V-ATPase transcripts during these periods would waste energy, the decrease in enzyme activity may be accompanied by decreased transcription rates of the corresponding genes. To investigate this hypothesis we sequenced the 5′ regions of the genes *mvB*, *mvG* and *mvM40* encoding the *M. sexta* V-ATPase subunits B, G, and M40, respectively (Merzendorfer, 1998). The nucleotide sequences of these genes were compared over a region of approximately 1 kb upstream of the translational start codon. Although dissimiliar in sequence, the promoters of *mvB* and *mvG* shared features common with inducible or tissue-specific promoters of vertebrates. They showed canonical TATA boxes and a low overall GC content of approximately 30%, with a similiar allocation in the proximal region. In addition, at the same position, both promoters contained a motif similiar to the consensus sequence of the cAMP-responsive element (CRE, Roesler *et al.*, 1988). By contrast, the promoter of *mvM40* lacked apparent TATA boxes and CREs and exhibited a different GC-distribution pattern, although the averaged GC content of approximately 35% was only negligibly higher. Overall, the *mvM40* gene resembled housekeeping genes of other organisms. The observed differences in structure may indicate that gene expression of peripheral and membrane-bound V-ATPase subunits are regulated in distinct ways. However, a coordinated regulation of all three promoters remains possible, since the 5′ regions of *mvB*, *mvG*, and *mvM40* all contain an ecdysone-responsive element (EcRE) corresponding to the consensus sequence KNTCANTNNMM (Luo *et al.*, 1991). To test the effect of cAMP and ecdysteroids on the promoter activity of *mvB*, we performed luciferase assays in *Sf*9 cells with a series of plasmids containing different 5′regions of *mvB* (Merzendorfer, 1998). The data suggest that the essential region for transcriptional activity lies within approximately 400 bp upstream of the protein coding region. Incubation of the cells with 250 μM of the membrane permeant cAMP derivative, 8-chlorophenylthio-cAMP, led to a 70% increase of transcriptional activity as compared with control cells, whereas treatment with 160 n*M* of the protein kinase C (PKC) activator, tetradecanoylphorbol-13-acetate, had no effect. Together with the presence of a CRE-like motif in the *mvB* promoter, these results suggest that cAMP and protein kinase A (PKA), but not PKC, are involved in the up-regulation of *mvB* transcription. 20-Hydroxyecdysone induced a nearly 70% decrease of transcriptional activity. The ecdysone-mediated inhibition may prevent the expression of subunit B during the molt when V-ATPase activity is down-regulated and

ecdysteroid titers of the hemolymph reach high levels (Bollenbacher *et al.*, 1981).

ANTISENSE RNA-MEDIATED REGULATION OF V-ATPase BIOSYNTHESIS

Large amounts of V-ATPase are found in apical membranes of goblet cells, whereas it is barely detectable in the apical membranes of the columar cells or in the lateral and basal membranes and endomembranes of both cell types (Wieczorek *et al.*, 1986; Klein *et al.*, 1991). Unexpectedly, *in situ* hybridization using midgut cryosections indicated high levels of mRNA encoding the V-ATPase subunits A and M17 in the columnar cells (Jager *et al.*, 1996). The presence of mRNA for V-ATPase subunits without corresponding amounts of protein may be the result of a posttranscriptional control mechanism that regulates the translation of V-ATPase transcripts in the columnar cells down to a level that just guarantees the housekeeping function of V-ATPase in endomembranes.

Beside protein–mRNA interactions in the 5′- or 3′-untranslated region (Standart and Jackson, 1994), translational control may also be mediated by the expression of antisense mRNA, as it has been demonstrated for the *lin-4*/*lin-14* transcripts of *Caenorhabditis elegans* (Lee *et al.*, 1993). Control by antisense RNA may occur in *M. sexta* since cDNA cloning and anchored reverse transcriptase—polymerase chain reaction (RT-PCR) disclosed evidence for a naturally occurring antisense transcript in the midgut that is complementary to the spliced mRNA of the membrane-associated V-ATPase subunit M40 (Merzendorfer *et al.*, 1997).

In contrast to prokaryotes, where antisense-mediated regulation of gene expression is well documented (Zeiler and Simons, 1996), the role of antisense RNA in eukaryotes remains enigmatic (Vanhee-Brossollet and Vaquero, 1998). Beside some reports suggesting its involvement in the control of translation, antisense RNAs are mainly thought to take part in the regulation of sense–mRNA stability mediated by the formation of RNA–RNA hybrids and their subsequent degradation by cellular dsRNases (Nellen and Lichtenstein, 1993). The formation of double-stranded RNA (dsRNA) may also play a role in *M. sexta*, since nuclease protection assays showed that midgut polyA–RNA contains a significant fraction of dsRNA (Merzendorfer, 1998).

STRUCTURE OF THE V_1-ATPase

Despite the wealth of biochemical, biophysical, and molecular biological information on V-ATPases, the mechanism by which proton transport is coupled to ATP hydrolysis remains an unsolved mystery. This deficiency is largely due to the lack of detailed information about the three-dimensional structure of the enzymes. The first evidence that V_1 complexes form ball and stalk structures, portasomes, goes back to the 1960s when 10-nm particles, detected by conventional transmission electron microscopy in sections of insect ion-transporting epithelia, were first implicated in active transport (Gupta and Berridge, 1966; Anderson and Harvey, 1966). At that time, V-ATPases had not yet been discovered, but today there is a general agreement that portasomes represent V_1 complexes (Harvey, 1992). Several years ago, negatively stained preparations of vacuolar membranes and of purified V-ATPase from *Neurospora crassa* provided more detailed electron microscopical information (Dschida and Bowman, 1992) and, recently, a model was presented based on examination of negatively stained single particles of the V-ATPase from *Clostridium fervidus* (Boekema *et al.*, 1997). This model showed a two-domain structure, in which the membrane-integrated part, V_0, and the peripheral part, V_1, are linked by a central and a peripheral "stalk."

The development of a purification procedure for the V_1 complex from *M. sexta* (Graf *et al.*, 1996), that was discussed above, allowed us to investigate the gross structure of this complex in solution by X-ray small-angle scattering (Svergun *et al.*, 1998). The radius of gyration, R_g, has been calculated to be 6.2 $\pm$ 0.1 nm and the maximal particle dimension, D_{max}, is 22 $\pm$ 0.1 nm. The V_1 ATPase is thus a highly elongated molecule under the solvent conditions that were used. The molecular mass, as calculated from forward scattering of the V_1 ATPase and a reference solution of bovine serum albumin, was 550 $\pm$ 20 kDa, in agreement with the determined Porod volume of 970 $\pm$ 30 nm^3. Based upon molecular mass of V_1 and the apparent molecular masses of the individual subunits, 67, 56, 40, 32, 28, 14, and 16 kDa, the proposed stoichiometry of the V_1 ATPase is A_3:B_3:C:D:E:F:G_3 (Svergun *et al.*, 1998).

The shape of the V_1 complex, determined *ab initio* from the experimental data is rather anisometric (see Fig. 3). With a hexagonal headpiece that is approximately 14.5 nm in diameter and a stalk at a right angle to that hexameric mass, the shape of the molecule from

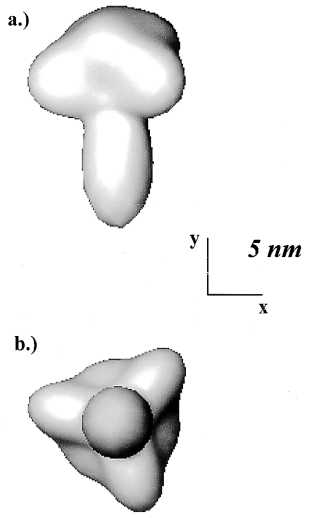

Fig. 3. Low-resolution envelope of the V_1-ATPase from *M. sexta*: (a) Side view of the molecule; (b) view from the bottom of the V_1-ATPase.

the side resembles that of a mushroom. The six masses at the top of the molecule, assumed to be the subunits A and B, are arranged in an alternating manner, consistent with recent images of negatively stained single particles of this V_1 complex, which show a ring of six major masses surrounding a central seventh mass (M. Rademacher, T. Ruiz, H. Wieczorek, and G. Gruber, unpublished data). The stalk, approximately 11 nm high and 6.4 nm wide, would comprise subunits C, D, E, F, and G (assuming a stoichiometry of C:D:E:F:G_3) and have a total mass of approximately 162 kDa.

A key remaining question is how catalytic-site events are coupled to ion translocation. Direct ion transfer from the hexagonal headpiece, in which ATP hydrolysis takes place, to the V_0 portion seems unlikely, because of the significant length of the stalk. As in the F-ATPase (Cross and Duncan, 1996; Junge *et. al.*, 1997; Kinosita *et al.*, 1998; Capaldi *et al.*, 1996), the more likely explanation is that the V_1 headpiece and the V_0 sector communicate through conformational changes in the stalk subunits.

PERSPECTIVES

One topic of future V-ATPase research will be a sufficient resolution of its structure in order to understand the mechanism of coupling. Many bioenergetics afficionados would agree that V-ATPases probably couple ATP hydrolysis to proton translocation by a rotatory mechanism, like that suggested for F-ATPases. This agreement is based on many similarities between the two enzymes, especially regarding the catalytic subunits. Recall that V- and F-ATPases are genetically related, with ~25% sequence identity between subunits A_V and β_F and between subunits B_V and α_F. The structural data presented in this paper support the hexagonal arrangement of the A and B subunits that is familiar from the arrangement of β and α subunits in F-ATPases. In the near future, averaged electron microscopical images of V-ATPases labeled with antibodies to A and B subunits may confirm their alternating arrangement in the enzyme and labeling with antibodies to C, D, E, F, and G subunits may reveal the spatial arrangement of these stalk subunits and thus provide the basis for exploring their function in the stalk.

Another topic may be that of the enzyme's regulation in the cellular context. Both the multitude of locations and the heteromultimeric structure make it likely that V-ATPases are regulated in various ways. Regulation encompasses, for instance, the control of gene expression or the control of enzyme activity, such as the reversible disassembly of V_1 and V_0 complexes. Beyond these two examples, a bewildering array of mechanisms by which V-ATPases might be regulated have already been identified. In the course of evolution, these ancient enzymes have adapted to a broad array of cellular roles, which range from vacuolar acidification to the generation of voltages which drive the movement of ions through symporters, antiporters, and ion channels. Understanding the regulatory mechanisms and their role in the context of cell physiology is a challenge for the coming years.

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