Characterization of ATPases of apical membrane fractions from Locusta migratoria Malpighian tubules

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Abstract

Apical and basal membrane fractions from Locusta Malpighian tubules were prepared and were characterized by marker enzyme analysis. The apical membranes contained an azide- and orthovanadate-insensitive ATPase activity that was inhibited by bafilomycin A1 (IC 50 = 0.44 nM) and NEM (IC 50 = 2.15 μM), and thus was characterized as a putative V-type ATPase. The enzyme was stimulated by a variety of monovalent cations (Tris > K = Na > choline > Li = Rb) maximal stimulation occurring at 30-40 mM. It was also stimulated by a variety of monovalent anions (maximal activation 30-40 mM), but was strongly inhibited by nitrate and thiocyanate. SDS-PAGE separation of proteins present in the various membrane fractions was carried out. The apical membrane fraction alone contained a 28 kDa protein band that bound a monoclonal antibody specific for a 28 kDa peptide which was a component of the V-type ATPase from midgut of Manduca sexta and, in native gels, possessed ATPase activity which was also sensitive to both bafilomycin and NEM but not to azide or orthovanadate. Binding of the fluorescent monoclonal antibody was located at the apical boundary of the tubule cells. It was concluded that a V-type ATPase is present at the apical surface of Locusta Malpighian tubule cells and that it is involved in their secretory functioning. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Insect; Locust; Malpighian tubules; Membrane; V-type ATPase

1. Introduction

The Malpighian tubules of Locusta, in common with those of a number of other insects which have been studied (e.g. Calliphora, Berridge, 1968; Tipula paludosa, Coast, 1969; and Schistocerca gregaria, Maddrell and Klunsuwan, 1973), transport K⁺ against a chemical gradient over a wide range of external K⁺ concentrations. In addition, K⁺ are transported in preference to Na⁺ even when present at much lower concentrations in the bathing medium (Anstee et al., 1979). Measurements of potential difference across the tubule wall indicate that the lumen is positive with respect to the bathing medium (Anstee et al., 1980; Fathpour et al., 1983; Morgan and Mordue, 1983; Baldrick et al., 1988). On these grounds, it has been proposed that the transport of K⁺ is an active process. Nevertheless, both Na⁺ and K⁺ are necessary for maximal fluid secretion (Morgan and Mordue, 1981).

A number of models have been proposed to explain ion and water translocation across insect Malpighian tubules (see reviews by Phillips, 1981; Maddrell and O’Donnell, 1992; Nicolson, 1993; Dow, 1994; van Kerkhove, 1994; Beyenbach, 1995; Pannabecker, 1995). Almost all require that active transport occurs across the basal and apical cell membranes and, in particular, that K⁺ and/or Na⁺ are transported into the lumen by an apical electrogenic cation pump. Harvey et al. (1983) proposed that the apical pump may represent a K⁺-activated ATPase. More recently, studies on larval midgut of M. sexta have reported the presence of V-type ATPase activity associated with isolated apical membrane vesicles from goblet cells (Wieczorek et al., 1989, 1991). It was suggested that in these cells, the apical electrogenic K⁺ pump consisted of a V-type ATPase working in parallel with a cation/proton antiporter. Subsequent physiological studies on insect Malpighian tubules have produced evidence for K⁺ secretion via a nH⁺/K⁺ anti-
porter driven by the proton motive force produced by a V-type ATPase present in the membrane (Bertram, 1989; Bertram et al., 1991; Klein et al., 1991; Maddrell and O’Donnell, 1992; Weltens et al., 1992; Dow, 1994; Zhang et al., 1994). There is a growing body of evidence that supports the localization of a V-type ATPase on the apical border of insect Malpighian tubule cells, (Klein et al., 1991, M. sexta; Russell et al., 1992, Manduca; Pietrantonio and Gill, 1995; Heliothes; Garayoa et al., 1995, Formica).

Previous studies (Ansee and Bell, 1975, 1978; Ansee and Fathpour, 1979, 1981; Fogg et al., 1991) have revealed the presence of Mg$^{2+}$-dependent, Na$^+$/K$^+$-activated ATPase activity in microsomal preparations from Malpighian tubules of Locusta, and that this activity is mainly associated with the basal plasma membranes. Fogg et al. (1991) suggested that an anion-stimulated ATPase, also present, might be a characteristic of a V-type ATPase. The acknowledged dependency of fluid transport by Malpighian tubules on membrane transport proteins suggested that a combined biochemical and immunocytochemical study be carried out to determine whether a V-type ATPase is present in Malpighian tubules of Locusta; to determine its subcellular localization and to characterize the enzyme activity present. Studies on Locusta make significant contributions to the understanding of the mechanisms and control of Malpighian tubule cell function in insects. The results of this present study add to this existing body of knowledge, largely derived from structural, physiological, and biochemical work carried out in our laboratory.

2. Materials and methods

Mature adult locusts, Locusta migratoria L., were used and these were taken from a population maintained under crowded conditions at 28 ± 0.5°C and 60% relative humidity. The photoperiod was 12 h light: 12 h dark.

2.1. Rate of fluid secretion

In vitro measurements of the rate of fluid secretion were carried out following the method of Maddrell and Klunusuan (1973), modified later by Ansee and Bell (1975).

2.2. Preparation of different cell fractions

Animals were killed by decapitation and their Malpighian tubules quickly dissected out and placed in 10 ml of an ice-cold homogenization medium consisting of 250 mM sucrose in 5 mM imidazole buffer (pH 7.5). Homogenization was carried out in a glass homogenizer with a Teflon pestle (clearance 0.1–0.15 mm) with 20 passes of the plunger at 100 rev/min; the homogenization tube was surrounded by ice throughout this procedure. The resulting homogenate was centrifuged to separate the different membrane components using a method based on that described by Rodriguez and Edelman (1979) and Fogg et al. (1991). Initially, the homogenate was centrifuged at 600g for 10 min at 0°C. The pellet from this spin was discarded the supernatant (S$_1$) retained and centrifuged at 15 000g for 20 min at 0°C. The resulting mitochondrial-rich pellet (P$_2$) was stored on ice and the associated supernatant (S$_2$) was re-centrifuged at 135 000g to yield a pellet (P$_3$) which was then resuspended by homogenization in 10 ml of homogenization medium containing 10 mM MgCl$_2$. This was then left to stand on ice for 20 min prior to centrifugation at 10 000g for 15 min to yield pellet P$_4$, the basolateral membrane fraction. The resulting supernatant (S$_3$) was then centrifuged at 55 000g for 30 min. The pellet (P$_5$) resulting from this spin represented the apical-membrane-rich fraction (Fogg et al., 1991). The latter pellet was resuspended by homogenization in a known volume of deionised water, at 4°C.

2.3. Assay of ATPase activities

Each incubation medium, consisting of 100 µl of an appropriate ionic medium (see below) and 50 µl of membrane preparation, was thermoequilibrated for 15 min at 35°C in a water bath prior to starting the reaction by the addition of 50 µl of 12 mM ATP (Tris salt). Incubations were run for 30 min at 35°C and reactions were stopped by adding 400 µl of a 1:1 mixture of 1% Lubrol and 1% ammonium molybdate in 0.9 M sulphuric acid (Atkinson et al., 1973). The tubes were then kept at room temperature for 10 min to allow the yellow colour, which was proportional to the amount of inorganic phosphate released, to develop. Absorbancy was measured at 390 nm. Enzyme activity was measured by determining the amount of inorganic phosphate released.

In determining different ATPase activities, a variety of different ionic media having the following ionic compositions were used (final concentrations):

2.3.1. Na$^+$/K$^+$-ATPase activity

(1) 4 mM MgCl$_2$; (2) 4 mM MgCl$_2$, 100 mM NaCl and 20 mM KCl; (3) 4 mM MgCl$_2$, 100 mM NaCl, 20 mM KCl plus 1 mM ouabain. Each medium contained 20 mM imidazole/HCl (pH 7.2). Na$^+$/K$^+$-ATPase activity was determined as the difference in the amount of inorganic phosphate liberated in the presence of ionic medium 2 and ionic medium 3.

2.3.2. V-type ATPase activity

This was assayed as azide- and orthovanadate-insensitive ATPase activity as described by Schweikl et al. (1989): 1 mM MgCl$_2$, 20 mM KCl, 0.5 mM NaN$_3$, 0.1
mM Na₃VO₄, 0.1 mM EGTA and 0.3 mg BSA ml⁻¹ in 50 mM Tris–MOPS buffer (pH 7.5).

Appropriate controls were run to determine the extent of non-enzymatic hydrolysis of ATP. All ATPase activities are expressed in nmoles Pi liberated mg protein⁻¹ min⁻¹.

2.4. Marker enzyme assays for membrane fraction identification

Basal membrane fractions were identified by enrichment of Na⁺/K⁺-ATPase (EC 3.6.1.3) activity. Succinate dehydrogenase (EC 1.3.99.1) activity was determined according to the method described by King (1967) as a marker for mitochondrial membranes. Alkaline phosphatase (EC 3.1.3.1) activity was determined using the method of Bowers and McComb (1966) to identify apical membrane fractions.

2.5. Determination of protein content

Protein determinations were carried out using the Coomassie Brilliant Blue binding method of Bradford (1976), using bovine serum albumin fraction V as standard.

2.6. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

The method used was essentially that described by Laemmli (1970) for discontinuous gels. Membrane fractions (of ≈ 15 μg protein) in sample buffer were loaded onto a 12.5% (w/v) polyacrylamide gel via a 3.9% (w/v) stacking gel. The gels were run at a constant voltage (40 mV) and then fixed and stained for protein.

2.7. Western blotting

Proteins were blotted from acrylamide gels to nitrocellulose using the semi-dry blotting method of Kyhse-Anderson (1984). Transfer was carried out on a Bio–Rad Trans blot, semi-dry transfer cell at 25 V and 220 mA (5.5 mA.cm⁻²) for 30 min. After transfer, the nitrocellulose sheet was blocked (PBS, 5% Marvel and 1% Tween-20) for 1 h at room temperature. It was then incubated with primary antibody in anti-sera buffer (PBS, 5% Marvel and 0.1% Tween-20) overnight at 4°C, with shaking. Best results were obtained using 1:4000 dilution. Following 3 × 5 min washes with anti-sera buffer, the nitrocellulose sheet was incubated with the secondary antibody, goat anti-mouse IgG (H + L) horseradish peroxidase conjugate (Bio–Rad cat. no. 170-6516), at 1:5000 dilution, for 2 h at room temperature. After thorough washing in 0.1% Tween-20 in PBS and rinsing with distilled water, antibody binding was visualized using an Enhanced Chemiluminescence Detection (ECL) system (Amersham Life Science).

2.8. Native microgradient PAGE

Continuous microgradient acrylamide gels were produced by controlled mixing of 3 and 15% buffered acrylamide solutions (Schweikl et al., 1989; Hames, 1990).

Apical membrane-enriched pellets (P₅), produced as described previously, were resuspended and solubilized in a 1% solution of TritonX-100 in 10 mM Tris/HCl (pH 7.5) containing 0.32 mM EDTA and 5% glycerol. This mixture was stirred on ice for 10 min prior to centrifugation at 100 000g for 1 h, using a fixed angle rotor. The supernatant was then mixed with an equal volume of a sample buffer consisting of 0.0625 M Tris/HCl (pH 6.8), 10% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol (modified from Laemmli, 1970). The resulting mixture was loaded onto the stacking gel and electrophoresis carried out at 20 mA constant current for 4–5 h at ~ 4°C.

Following electrophoresis, ATPase activity was identified and localized using the procedure described by Schweikl et al. (1989). Initially, gels were preincubated in a medium consisting of 5 mM MgSO₄ and 100 mM Tris/HCl (pH 7.5) for 15 min at 35°C in a water bath. ATP (5 mM, final concentration) or β-glycerophosphate (control) was then added and the gel incubated for a further 1 h. At the end of this time, the gel was washed in double distilled water and placed in 2.5 mM lead acetate in 80 mM Tris-maleate buffer (pH 7.0) for 30 min, at room temperature. The gel was then thoroughly washed in deionised water and 2% ammonium sulphide added. Sites of ATPase or alkaline phosphatase were revealed as dark brown/black bands on the gel.

2.9. Immunocytochemistry

Malpighian tubules from adult Locusta were dissected out and placed in ice-cold fixative consisting of 2% paraformaldehyde, 0.075% lysine, 0.01 M sodium periodate in 0.1 M sodium phosphate buffer (pH 7.3) for 2 h (McLean and Nakane, 1974). The tissues were washed in phosphate buffer and transferred to 30% sucrose buffer (pH 7.0) for 30 min, at room temperature. The gel was then thoroughly washed in deionised water and 2% ammonium sulphide added. Sites of ATPase or alkaline phosphatase were revealed as dark brown/black bands on the gel.

2.10. Reagents

All solutions were prepared in glass-distilled deionised water. All inorganic salts were AnalAR grade or the purest commercially available. Bafilomycin A₁ was purchased from Professor Dr K. Altendorf, Universität Osnabruck, Germany. The concentration was estimated
photometrically (Bowman et al., 1988) using an absorbance maximum of 245 nm. Bafilomycin A₁ was dissolved in DMSO before adding it to the appropriate solution and the final concentration of DMSO in the experimental solutions was < 1%, the same concentration of the solvent was included in the controls.

Monoclonal antibodies 230-3, 221-67, 224-3 and 90-7, directed to the V-type ATPase from insect plasma membrane (midgut of larval M. sexta), were a gift from Professor Dr. Ulla Klein, Laboratory Wieczorek, Zoological Institute of the University of Munich, Germany.

3. Results

3.1. Rate of fluid secretion

The effect of bafilomycin A₁ on the rate of fluid secretion is shown in Fig. 1(A). Comparison between normalized control and experimental values established that 5 μM bafilomycin A₁ caused a 73% inhibition in the rate of fluid secretion (P < 0.001), the IC₅₀ was calculated to be 0.82 μM. The effect of NEM on the rate of fluid secretion is shown in Fig. 1(B), significant inhibition was observed over the concentration range 10⁻³ to 10⁻⁷ M, with an IC₅₀ of 9.05 μM.

3.2. Marker enzyme activities of cell fractions

Table 1 shows the subcellular distribution of Na⁺/K⁺-ATPase (basolateral marker), succinate dehydrogenase (mitochondrial marker) and alkaline phosphatase (an apical membrane marker) activities in the various cell fractions studied. Pellets P₃ and especially P₄ exhibited the highest specific levels of Na⁺/K⁺-ATPase while alkaline phosphatase activity was largely associated with P₅. Previous cytochemical studies (Fogg et al., 1991) have shown that alkaline phosphatase is confined to the apical cell surface in Malpighian tubules of Locusta. These differential distributions of Na⁺/K⁺-ATPase and alkaline phosphatase are consistent with P₄ and P₅ representing basal and apical membrane enriched fractions, respectively. Table 1 also shows that SDH activity was maximal in pellet P₂ with minimal levels of activity being observed in P₃ suggesting low mitochondrial contamination of the apical membrane enriched fraction.

3.3. Effect of NEM and bafilomycin A₁ on azide- and orthovanadate-insensitive ATPase (V-type ATPase) activity

Pellet P₃ also contained significant levels of ATPase activity that was insensitive to both azide and orthovanadate, these reagents inhibit F-type and P-type ATPases, respectively. This ATPase activity, in the apical membrane-enriched fraction, was very sensitive to bafilomycin A₁; all concentrations used effecting a significant inhibition (Fig. 1(C)) with an IC₅₀ of 0.44 nM. NEM also inhibited this ATPase activity of the apical membrane fraction over the concentration range 0.5 to 100 μM, with an IC₅₀ of 2.15 μM (Fig. 1(D)). These results are consistent with this ATPase activity being a V-type in its properties (Al-Awqati, 1986; Bowman et al., 1988; Mattsson et al., 1991).

3.4. Effects of pH and the requirements for ATP and Mg²⁺ for azide and orthovanadate-insensitive ATPase activity (V-type ATPase)

Fig. 2(A) shows the effect of pH on the V-type ATPase activity, maximal activity was determined at pH 7.5. Fig. 2(B and C) show that maximal activity of the enzyme was also obtained at equimolar (4 mM) concentrations of Mg²⁺ and ATP.

3.5. Effect of various ions on azide- and orthovanadate-insensitive ATPase (V-type ATPase) activity

The effect of various ions on V-type ATPase activity were determined in the presence of 1 mM MgCl₂, 5 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 0.5 mM Na₃VO₄ and 0.05% Triton X-100 (Schweikl et al., 1989). The effect of altering the concentrations of a number of monovalent (K⁺, Na⁺, Li⁺, Rb⁺, choline and Tris) chlorides on V-type ATPase activity was determined. Maximal activation occurred in all cases between 30 and 40 mM, higher concentrations tended to be inhibitory. The maximal activation achieved varied between 251% for Tris to 141% for Rb⁺, the stimulation obtained with K⁺ was 191%. Cations were stimulatory in the following sequence Tris > K = Na > choline > Li = Rb. The effect of different anion salts of K⁺ (SO₄²⁻, F⁻, Br⁻, gluconate and NO₃⁻) on V-type ATPase activity was also determined. Once again activation was maximal between 30 and 40 mM. Gluconate and SO₄²⁻ gave about 50% stimulation whereas Br⁻ and F⁻ produced a smaller stimulation of activity of about 25–30%. It is significant that NO₃⁻ caused progressive inhibition with an IC₅₀ of about 9 mM; 10 mM NaSCN also strongly inhibited the V-type ATPase reducing its activity to 24.2 ± 5.7% of control values. What is clear is that both the anion and cation can affect enzyme activity, with HCO₃⁻ being the most effective stimulating anion (252.4 ± 8.4%), with bromide, fluoride and sulphate being less effective than chloride.

3.6. SDS–PAGE

The different membrane fractions isolated from Malpighian tubules of Locusta and a mixture of standard
molecular weight markers were subjected to analysis by SDS–PAGE, under denaturing conditions (Fig. 3(i) lanes A–D). Western blots of these protein bands were probed with monoclonal antibodies 230-3, 221-67, 224-3 and 90-7, which recognized various subunits of V-type ATPase in crude homogenates of midgut of M. sexta (Klein et al., 1991). Only monoclonal antibody 230-3 showed immunoreactivity with preparations from Locusta and then only to one of the protein bands in the apical membrane-rich fraction (Fig. 3(ii), lanes A and B). This corresponded to the protein band with an estimated mass of 28 kDa.
Table 1
Distribution of enzyme activities in the various fractions derived from the differential centrifugation of a homogenate made from Malpighian tubules of *Locusta*

<table>
<thead>
<tr>
<th>Membrane fraction</th>
<th>Mg(^{2+}) -ATPase (nmoles Pi liberated mg protein(^{-1}) min(^{-1}))</th>
<th>Na(^{+})K(^{-}) -ATPase (nmoles Pi liberated mg protein(^{-1}) min(^{-1}))</th>
<th>Alkaline phosphatase (nmoles p-nitrophenol liberated mg protein(^{-1}) min(^{-1}))</th>
<th>Succinic dehydrogenase (nmoles succinate oxidized mg protein(^{-1}) min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>163.4 ± 8.0</td>
<td>66.8 ± 4.6</td>
<td>325.4 ± 31.0</td>
<td>123.0 ± 6.0</td>
</tr>
<tr>
<td>P1</td>
<td>196.7 ± 12.2</td>
<td>55.4 ± 9.0</td>
<td>181.6 ± 12.1</td>
<td>115.4 ± 12.3</td>
</tr>
<tr>
<td>P2</td>
<td>279.4 ± 21.1</td>
<td>71.4 ± 6.9</td>
<td>466.3 ± 19.4</td>
<td>287.2 ± 29.6</td>
</tr>
<tr>
<td>P3</td>
<td>440.7 ± 27.9</td>
<td>131.9 ± 11.2</td>
<td>1017.7 ± 38.5</td>
<td>103.2 ± 15.0</td>
</tr>
<tr>
<td>P4</td>
<td>446.5 ± 11.7</td>
<td>331.9 ± 21.9</td>
<td>530.1 ± 45.5</td>
<td>133.1 ± 23.4</td>
</tr>
<tr>
<td>P5</td>
<td>597.4 ± 38.1</td>
<td>26.7 ± 5.6</td>
<td>3603.3 ± 145.5</td>
<td>10.0 ± 1.5</td>
</tr>
<tr>
<td>S1</td>
<td>192.3 ± 8.6</td>
<td>65.1 ± 7.7</td>
<td>467.4 ± 30.7</td>
<td>115.8 ± 13.2</td>
</tr>
<tr>
<td>S2</td>
<td>169.6 ± 8.7</td>
<td>48.2 ± 5.5</td>
<td>402.4 ± 93.2</td>
<td>69.4 ± 15.3</td>
</tr>
<tr>
<td>S3</td>
<td>21.8 ± 1.8</td>
<td>1.1 ± 0.5</td>
<td>36.4 ± 4.1</td>
<td>16.5 ± 2.9</td>
</tr>
<tr>
<td>S5</td>
<td>37.5 ± 6.2</td>
<td>2.4 ± 0.6</td>
<td>182.0 ± 10.8</td>
<td>0</td>
</tr>
</tbody>
</table>

ATPase activities are expressed as nmoles Pi liberated mg protein\(^{-1}\) min\(^{-1}\). Alkaline phosphatase activity is expressed as nmoles p-nitrophenol liberated mg protein\(^{-1}\) min\(^{-1}\). Succinic dehydrogenase activity is expressed as nmoles succinate oxidized mg protein\(^{-1}\) min\(^{-1}\). Mean value ± SEM for three independent experiments.

3.7. Native microgradient PAGE

Apical membrane enriched fractions of tubules of *Locusta*, were subjected to native microgradient PAGE. Two protein bands showing ATPase activity, as revealed by hydrolysis of ATP, were identified (Fig. 3(iii)). Band 1 was specific for ATP whereas band 2 also effected hydrolysis of β-glycerophosphate (lane F), suggesting that the latter was a non-specific alkaline phosphatase. The ATPase activity was particularly sensitive to inhibition by bafilomycin (lane B) and NEM (lane C) but was not sensitive to the presence of either azide or orthovanadate (lanes D and E). In a parallel study, unstained protein bands were transferred to nitrocellulose and probed with antibody 230-3. Fig. 3(iv) (lane B) shows that only the protein corresponding to band 1 (i.e. that showing ATPase activity) was recognized by the V-type ATPase specific antibody from *M. sexta*. In a control experiment a sample of a crude homogenate of *Locusta* Malpighian tubules and a mitochondrial fraction were also probed with antibody 230-3 and neither preparation showed cross-reactivity with the antibody.

3.8. Immunocytochemistry

Incubations with antibodies 221-67, 224-3 and 90-7 showed no significant binding to the cells of Malpighian tubule cells of *Locusta*. In contrast, examination Fig. 4, shows that antibody 230-3 bound specifically to the apical microvillar surface of the tubule cells indicating the
Fig. 3. Characterization and separation of proteins present in membrane fractions of *Locusta* Malipghian tubules. (i) Microgradient SDS–PAGE gel. Lane A, mitochondrial fraction (P$_2$); lane B basal membrane fraction (P$_4$); lane C apical membrane fraction (P$_5$); lane D molecular weight markers. Coomassie blue staining. (ii) Western blot of an SDS–PAGE microgradient gel prepared identically to (i) above. The blot was probed with a monoclonal antibody 230-3 to the 28 kDa subunit of the midgut V-type ATPase from *Manduca sexta* (lane A). Molecular weight markers are shown in lane B. (iii) Native microgradient PAGE of the apical membrane fraction of *Locusta* Malipghian tubules. The gels were stained for ATPase activity (lanes A–E) or β-glycerophosphatase activity (lane F). The ATPase activity was demonstrated in control media (lane A); in the presence of 5 µM bafiloycin A$_1$ (lane B); 10 mM NEM (lane C); 100 mM NaN$_3$ (lane D); or 1 mM Na$_3$VO$_4$ (lane E). Phosphate release was visualized by lead acetate plus ammonium sulphite precipitation. (iv) Microgradient gels prepared as described in (i) above. Lane A is stained for ATPase activity and lane B was probed with antibody 230-3.
4. Discussion

As described previously by Fogg et al. (1991), differential separation of the apical cell membrane marker enzyme alkaline phosphatase (Ernst and Mills, 1980) and the basolateral membrane marker, Na\(^+\)/K\(^+\)-ATPase, was observed at the 10 000g spin. Pellet P\(_4\) contained relatively high specific Na\(^+\)/K\(^+\)-ATPase activity with little apical membrane contamination, reflected by the relatively low alkaline phosphatase activity. The high specific activity reported of 331 nmoles Pi liberated mg protein\(^{-1}\) min\(^{-1}\) was greater than that reported for the same tissue using a deoxycholate/NaI extraction procedure, which gave specific activities of 220–290 (Anstee and Bowler, 1984); however the latter preparations contained appreciably less Mg\(^{2+}\) -ATPase activity (Table 1). The high specific activity for Na\(^+\)/K\(^+\)-ATPase confirms that P\(_4\) is basolateral-membrane rich. The specific activity of alkaline phosphatase was greatest in pellet P\(_5\), which, in turn, exhibited relatively low Na\(^+\)/K\(^+\)-ATPase activity. Mitochondrial contamination of P\(_5\) fraction, as indicated by succinate dehydrogenase activity, was extremely low.

Subsequent studies on the apical membrane-rich fraction (P\(_3\)) revealed the presence of azide- and orthovanadate insensitive ATPase activity similar to that reported in lepidopteran midgut goblet cells (Schweikl et al., 1989; Wieczorek et al., 1989). This enzyme activity was substantially inhibited by NEM (IC\(_{50}\) ~ 2 \(\mu\)M) in agreement with the reported NEM sensitivity for the enzyme of \textit{M. sexta} (Schweikl et al., 1989). This, together with the high bafilomycin sensitivity of the enzyme activity, suggested that a substantial proportion of this azide- and orthovanadate-insensitive activity present was due to a V-type ATPase (Schweikl et al., 1989); however, neither bafilomycin or NEM totally eradicated apical ATPase activity.

Monoclonal antibodies which had been raised to different subunits of the V-type ATPase from the gut of \textit{M. sexta} were used to probe different enriched membrane fractions of the Malpighian tubules of \textit{L. migratoria}. Of these antibodies, only one, antibody 230-3 (specific for the 28 kDa subunit in \textit{M. sexta}), recognized a protein present in the Malpighian tubules of \textit{Locusta} and this protein was confined to the apical membrane rich fraction. Support for the apical localization of a V-type ATPase was provided by the immunocytochemical study, where once again, antibody 230-3 bound specifically to the cell’s apical surface. This is supported by the fact that ATPase activity was confined to one protein band separated by native electrophoresis, and this band reacted with antibody 230-3 rather than the band which showed alkaline phosphatase activity. Furthermore, this ATPase activity was sensitive to bafilomycin A\(_1\) and NEM. The fact that only one of the \textit{Manduca} monoclonal antibodies cross-reacted with a subunit of the \textit{Locusta} V-type ATPase suggests that this antibody recognized a conserved epitope between the species. There is therefore clear evidence that a V-type ATPase is present in Malpighian tubule cells of \textit{Locusta}, and that this activity is associated with their apical surface. Vacuolar-type ATPases were originally considered to be localized in the endomembrane systems of eukaryotic cells, e.g. lysosomes and the Golgi complex, although, more recently, they have also been reported in the plasma membranes of vertebrate urinary epithelia (Gluck and Caldwell, 1987; see also Forgac, 1989).

Biochemical studies on the V-type ATPase revealed that maximal ATP hydrolysis was obtained at pH 7.5, a value similar to that reported for the enzyme from mammalian brush border (Ait-Mohamed et al., 1986; Wang and Gluck, 1990, but lower than that reported for the enzyme from \textit{Manduca} midgut (Wieczorek et al., 1986). In common with many other ATPases the enzyme was maximally stimulated at equimolar (4 mM) concen-
trations of Mg$^{2+}$ and ATP, which is consistent with a MgATP complex forming the substrate for the enzyme. V-type ATPases are reported to be activated by a variety of both anions and monovalent cations. The fact that this activation occurred in the range 30–40 mM for a variety of salts, suggested that it may be non-specific and probably a response to the change in ionic strength. The enzyme seemed more specifically sensitive to anions than cations. It was strongly inhibited by nitrate, a feature of V-type ATPases from other sources, (O’Neill et al., 1983; Lichko and Okorokov, 1984) and by thiocyanate, both features of the anion-activated ATPase described in this tissue (Fogg et al., 1991; Fathpour and Dahlman, 1994). Fogg et al. (1991) suggested that the anion-stimulated activity present in the apical membranes of Malpighian tubules may be due to a V-type ATPase. It is of interest that recently Dschida and Bowman (1995) have questioned the mechanism of enzyme inhibition by NO$_3^-$ and SCN$^-$, whether it be by a chaotropic action or by an influence of the redox state of the enzyme. The low concentration of the reagents used in our study would argue against a chaotropic action.

The physiological significance of this V-type ATPase in tubule function is suggested from the studies on tubule secretion in vitro. NEM and bafilomycin A$_1$ both inhibited fluid secretion. The concentrations of bafilomycin A$_1$ needed to produce a significant inhibition of fluid secretion in the present study were higher than those reported to inhibit the V-type ATPase activity in the apical membrane rich fractions. Nevertheless they were lower than those that inhibit P- and F-type ATPases (Forgac, 1989). However, it is likely that the intracellular concentration of bafilomycin in these physiological experiments is much lower than that applied in the medium bathing tubules (0.5–5 μM). Other workers report similar findings e.g. bafilomycin A$_1$ (1 μM) caused significant inhibition of fluid production by tubules of *Drosophila hydei* and 10 μM completely arrested it within 15–30 min (Bertram et al., 1991); fluid secretion was also significantly inhibited in the tubules of *Formica polyctena* using 5 μM bafilomycin A$_1$ (Weltens et al., 1992).

A higher concentration of NEM (100 μM) was also needed to produce significant inhibition of fluid secretion than was required to inhibit the partially purified ATPase. The concentrations of NEM compared with that reported by other workers. Weltens et al. (1992) working on the tubules of *F. polyctena* found that a concentration of 500 μM was required to inhibit in this species and Bertram et al. (1991) found significant effects on secretion of fluid by *D. hydei* at concentrations of NEM greater than 10 μM with complete inhibition at 1 mM NEM being found. The significance of this inhibition of fluid production by NEM is complicated because 100 μM NEM is also at the lower end of the sensitivity of P-type ATPases, and even though it is unlikely that the intracellular concentrations would be that high, the possibility that the inhibitory effect on fluid secretion is not due to V-type ATPase inhibition cannot be excluded.

The biochemical properties of the enzyme reported here, together with the immunocytochemical evidence, strongly support the existence of a classical V-type ATPase on the apical surface of the Malpighian tubules of *Locusta*. It is proposed that, in common with other insect secretory epithelia, this enzyme creates a proton motive force across the apical membrane, and acts in parallel with a K$^+$/H$^+$ antiporter to effect the active transport of K$^+$ across the apical membrane which in turn is the driving force for fluid secretion. *Locusta* Malpighian tubules also secrete appreciable quantities of Na$^+$ and it is possible that Na$^+$ and K$^+$ share a common antiporter, however Pivovarova et al. (1994) have provided evidence in support of separate exit ports for these two ions, both of which may depend on the proton motive force generated by the V-type ATPase.

References


lomycin A$_3$ is a potent inhibitor of urine formation by Malpighian tubules of *Drosophila hydei*; is a vacuolar-type ATPase involved in fluid secretion [1]. J. Insect Physiol. 37, 201–209.


