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## SHORT COMMUNICATION

# 5′-*p*-FLUOROSULFONYLBENZOYLADENOSINE BINDS TO THE 28 kDa SUBUNIT OF LARVAL *MANDUCA SEXTA* MIDGUT VACUOLAR-TYPE ATPase

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The larval lepidopteran midgut contains a mechanism for the active transport of potassium ions from blood to lumen (Harvey and Nedergaard, 1964). This insect $K^+$ pump has been localized to the apical membrane of the midgut goblet cells (Dow *et al.* 1984). Recently it has been shown to consist of a proton-motive ATPase of the vacuolar class (V-ATPase) functioning in conjunction with a $K^+/nH^+$ antiporter (Wieczorek *et al.* 1991).

Like other V-ATPases (Forgac, 1989), the midgut goblet cell apical membrane V-ATPase is a multipolypeptide complex. Five major polypeptides of 67, 56, 46, 29 and 16 kDa co-purify with ATPase activity (Schweikl *et al.* 1989). The functions of the various V-ATPase subunits are not well known. The subunits of several V-ATPases have been modified chemically in efforts to elucidate the molecular mechanisms underlying the activity of these enzymes (Arai *et al.* 1987; Bowman, 1983; Moriyama and Nelson, 1987; Percy and Apps, 1986). In the present study the ATP analogue 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA) has been used as a probe for nucleotide binding sites on the subunits of the V-ATPase from the midgut of the tobacco hornworm *Manduca sexta*.

All chemical reagents were from either Sigma (St Louis, MO) or Fisher Scientific (Pittsburg, PA), unless otherwise noted. *Manduca sexta* eggs and larval diet were purchased from Carolina Biological Supply (Burlington, NC). The larvae were raised to experimental size at 27°C under constant light. Fifth-instar larvae, weighing 5.5±0.5 g, were used for the preparation of midgut goblet cell apical membrane V-ATPase. The V-ATPase was prepared as described by Wieczorek *et al.* (1990). Enzyme concentration was determined using the method of Popov *et al.* (1975) as modified and described by Wieczorek *et al.* (1990).

The activity of the V-ATPase was assayed by determining released inorganic phosphate (Schweikl *et al.* 1989; Wieczorek *et al.* 1990). The incubation mixtures for assay of the activity of the purified enzyme contained approximately 0.5 μg of protein, 1 mmol $l^{-1}$ $MgCl_2$, 1 mmol $l^{-1}$ Tris–ATP, 20 mmol $l^{-1}$ KCl, 50 mmol $l^{-1}$ Tris–Mops, 0.1 mmol $l^{-1}$ EDTA, 3 mmol $l^{-1}$ 2-mercaptoethanol and 0.003 %

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$C_{12}E_{10}$ (polyoxyethylene 10 lauryl ether). Incubation mixtures had a total volume of 160 $\mu$l and a pH of 8.1. Each mixture was prepared in triplicate. The mixtures were preincubated in the absence of the substrate for 5–20 min at 30°C. Assays were started by adding ATP. The incubation time was 5 min. Under these conditions, substrate hydrolysis was a linear function of protein concentration and of incubation time. Reactions were stopped by placing the assay tubes in liquid nitrogen.

Reaction mixtures used for studying the inhibition of the ATPase by FSBA were the same as those used in enzyme activity assays except for the addition of various concentrations (0–0.4 mmol $l^{-1}$) of FSBA. FSBA solutions were prepared in dimethylformamide (DMF). Amounts of DMF up to 2 % had no effect on enzyme activity. All reactions to test FSBA inhibition contained less than 2 % DMF.

Labeling of the midgut V-ATPase with [$^{14}$C]FSBA was accomplished by incubating purified ATPase (15 $\mu$g $ml^{-1}$) with 75 $\mu$mol $l^{-1}$ [$^{14}$C]FSBA (40–60 Ci $mol^{-1}$; Dupont-NEN, Boston, MA) in 1 mmol $l^{-1}$ $MgCl_2$, 20 mmol $l^{-1}$ KCl, 50 mmol $l^{-1}$ Tris–Mops (pH 8.1), 0.1 mmol $l^{-1}$ EDTA, 1 mmol $l^{-1}$ 2-mercaptoethanol, 0.003 % $C_{12}E_{10}$, and either 0 or 7.5 mmol $l^{-1}$ ATP for 5–10 min at 30°C. Reactions were stopped by placing the assay tubes in liquid nitrogen.

[$^{14}$C]FSBA-treated ATPase was precipitated by trichloroacetic acid (final concentration 10 %) and washed twice with 100 % acetone. The pellets were air-dried and prepared for SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) by resuspension in sample buffer (0.125 mol $l^{-1}$ Tris–HCl, pH 6.8; 4 % SDS; 20 % glycerol; 10 % 2-mercaptoethanol) with 2 min of heating in boiling water. 0.75 mm thick, 10 % gels were run at 25 mA constant current until the solvent front was near the bottom of the gel. Gels were incubated in transfer buffer (25 mmol $l^{-1}$ Tris, 192 mmol $l^{-1}$ glycine, 15 % methanol, pH 8.2) for 20–30 min. The gel and a wet Immobilon membrane (Millipore, Bedford, MA) were sandwiched between sheets of filter paper wetted with transfer buffer. The sandwich was placed in the transferring cassette of a Bio-Rad (Richmond, CA) transblot apparatus and transferred at 40 V and 4°C for 5–15 h. The membranes were dried in air overnight. The dried membranes were exposed to Kodak X-OMAT film for 2–4 weeks at −70°C.

The effects of FSBA on midgut V-ATPase activity are shown in Fig. 1. In the presence of 1 mmol $l^{-1}$ ATP, 0.22 mmol $l^{-1}$ FSBA inhibits ATPase activity by approximately 50 %. At an FSBA concentration of 0.37 mmol $l^{-1}$, ATPase activity is inhibited by more than 80 %. The natural substrate, Mg–ATP, can protect the enzyme against inhibition by FSBA. At a molar ratio of ATP to FSBA of 10:1, approximately 75 % of the enzyme activity is restored (data not shown).

In the absence of ATP, predominantly the 28 kDa subunit of midgut V-ATPase is labeled by 75 $\mu$mol $l^{-1}$ [$^{14}$C]FSBA (Fig. 2, lane B). In the presence of a 100-fold excess of ATP, no subunits are labeled (Fig. 2, lane C).

FSBA is a highly specific ligand for adenine nucleotide binding sites (Wyatt and Colman, 1977). It has been used to identify the active sites as well as regulatory

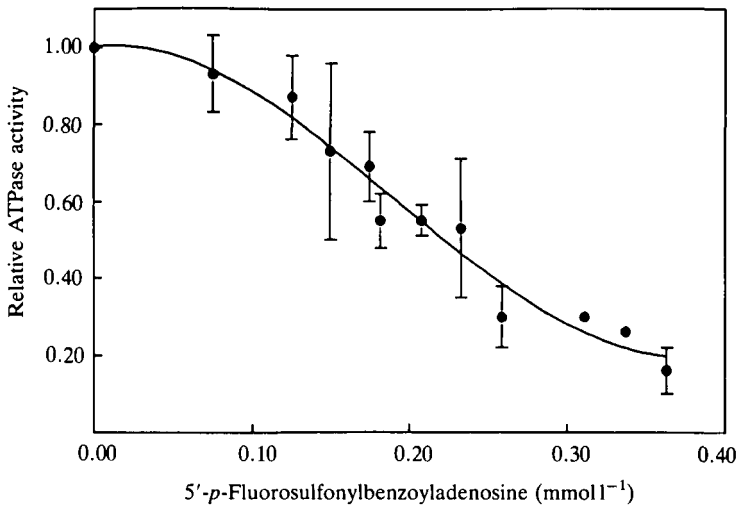


Fig. 1. Activity of solubilized midgut V-ATPase *versus* 5'-*p*-fluorosulfonylbenzoyladenosine concentration. Assay conditions are described in the text. Mean values ±S.E.M. are from three independent assays (except for 0.31 mmol $l^{-1}$ FSBA, which was tested only once). A smooth curve was fitted to the data using Slide Write Plus (Advanced Graphics Software, Inc., Sunnyvale, CA).

sites of both P-ATPases and F-ATPases (DiPietro *et al.* 1979; Cooper and Winter, 1980; Verburg and Allison, 1990). In the present study FSBA was used both as an inhibitor of catalytic activity and as a probe to identify which subunits of the V-ATPase from the apical membrane of goblet cells of larval *M. sexta* midgut contain putative nucleotide binding sites.

FSBA inhibition of midgut V-ATPase activity is consistent with it modifying catalytic or regulatory nucleotide binding sites. Loss of enzyme activity is low at FSBA concentrations below 0.1 mmol $l^{-1}$, it increases greatly with FSBA concentrations between approximately 0.15 and 0.25 mmol $l^{-1}$, but it increases much less with higher FSBA concentrations (Fig. 1). A complete elimination of enzyme activity by FSBA would not be expected, since ATP is likely to be a better substrate for the enzyme than FSBA (DiPietro *et al.* 1979). FSBA not only inhibits midgut V-ATPase but also binds preferentially to its 28 kDa subunit (Fig. 2). This binding is specific; it is prevented by the natural substrate Mg–ATP.

This communication is the first report of studies of the interactions of FSBA with a V-ATPase. V-ATPases are related much more closely to F-ATPases than to P-ATPases (Nelson and Taiz, 1989). Careful studies of inactivation and labeling of F-ATPases by FSBA and related nucleotide analogs have shown that, depending upon conditions, inactivation is accompanied by labeling of different residues associated with either catalytic or noncatalytic nucleotide binding sites (Verburg and Allison, 1990). For example, inactivation of bovine heart mitochondrial $F_1$-ATPase with FSBA at pH 6.0 is correlated predominantly with modification of $\beta$-

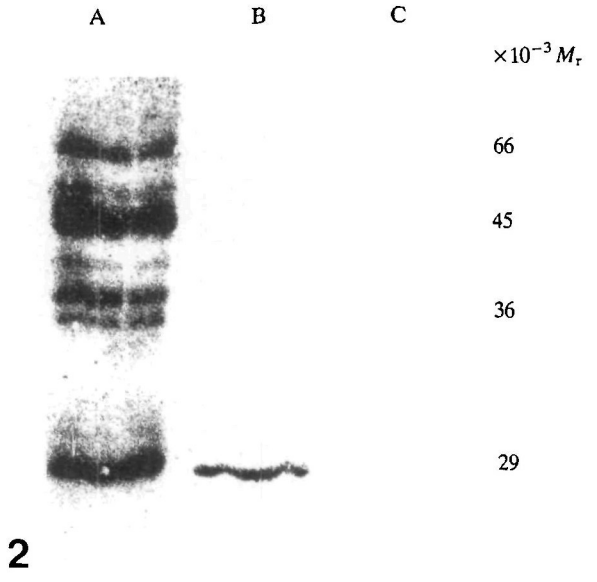


Fig. 2. Labeling of midgut V-ATPase by [$^{14}$C]FSBA. Reaction mixtures and experimental conditions are described in the text. Lane A, blot of midgut V-ATPase separated by SDS–PAGE stained for protein. Lanes B and C, autoradiogram of a blot of midgut V-ATPase incubated with 75 $\mu$mol l$^{-1}$ [$^{14}$C]FSBA (lane B) or 75 $\mu$mol l$^{-1}$ [$^{14}$C]FSBA plus 7.5 mmol l$^{-1}$ ATP (lane C) before denaturation, separation by SDS–PAGE and electroblotting. The positions of standard proteins are indicated.

tyrosine-368, but at pH 8.0 modification of $\beta$-histidine-427 predominates (Bullough and Allison, 1986). Both of these residues are associated with a noncatalytic ATP binding site on the beta subunit of this mitochondrial enzyme. Therefore, although specific FSBA labeling may be a reliable method for identifying nucleotide binding sites, identification of the function of a site requires additional information. Studies with radioactive ATP, *N*-ethylmaleimide and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole have shown that the 70 kDa subunit of several V-type ATPases possesses a nucleotide binding site that is believed to be the catalytic site (Randall and Sze, 1987; Arai *et al.* 1987). However, these reagents also label other subunits of V-ATPases, such as the 39 kDa subunit of the chromaffin granule V-ATPase (Moriyama and Nelson, 1987). These results combined with the results reported here suggest that V-ATPases have nucleotide binding sites on more than one of their subunits. The site on at least one subunit, perhaps the 67 kDa subunit of midgut V-ATPase, will be a catalytic site. The sites on other subunits, such as the 28 kDa subunit of midgut V-ATPase, will probably be shown to have noncatalytic functions.

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