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P-glycoprotein ATPase from the resistant pest, *Helicoverpa armigera*: Purification, characterization and effect of various insecticides on its transport function

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ABSTRACT

Helicoverpa armigera is a major pest of agricultural crops and has developed resistance to various insecticides. A P-glycoprotein (Pgp) with ATPase activity likely to be involved in insecticide resistance was purified and characterized from insecticide-resistant *H. armigera*. The purification was 18-fold with 3% yield. The optimum pH and temperature were found to be 7.4 and 30–40 °C, respectively. Kinetic studies indicated that this enzyme had a K_m value of 1.2 mM for ATP. Pgp from *H. armigera* was partially sequenced and found to be homologous to conserved sequences of mammalian Pgps. Pesticides stimulated *H. armigera* Pgp ATPase activity with a maximum stimulation of up to 40%. Quenching of the intrinsic tryptophan fluorescence of purified Pgp was used to quantitate insecticide binding. Using the high-affinity fluorescent substrate, tetramethylrosamine, transport was monitored in real time in proteoliposomes containing *H. armigera* Pgp. The presence of Pgp could be one of the reasons for insecticide resistance in this pest.

1. Introduction

Helicoverpa armigera (Hübner) (Lepidoptera: Noctüidae) is a worldwide agricultural pest affecting more than 182 species of crop plants. In the Indian subcontinent, Australia, China and Africa, *H. armigera* is arguably the most important agricultural pest and has a long history of insecticide resistance [1,2]. It was demonstrated that a population of Australian *H. armigera* has developed resistance (275-fold) to *B. thuringiensis* Cry1Ac toxins in transgenic cotton [3]. In India, crop losses are commonly more than half the yield, and annual losses of cotton and pulses alone have been estimated at US $ 300–500 million. Insects that show resistance to one insecticide generally develop resistance to other classes of insecticides, a phenomenon often referred to as cross-resistance. This phenomenon resembles multidrug resistance (MDR), whereby resistance to one drug is accompanied by simultaneous resistance to a variety of structurally unrelated compounds [4].

MDR in mammalian organisms has been associated with the over-expression of plasma membrane proteins that belong to the ATP-binding cassette (ABC) family [5]. The ABC superfamily of proteins contains a number of membrane-bound, ATP-driven transporters that pump drugs, drug metabolites, and endogenous metabolites out of cells. In general, they comprise two transmembrane domains (TMDs) and two cytoplasmic nucleotide-binding domains (NBDs) that function to hydrolyze ATP. The NBDs are highly conserved throughout this protein family, and contain the Walker A and B motifs commonly found in other proteins that hydrolyze ATP or GTP, and also the Signature C motif that is unique to ABC proteins [6]. Human P-glycoprotein (Pgp; ABCB1; product of the MDR1 gene) is a 170 kDa ABC protein, and its expression confers cellular resistance to a wide spectrum of drugs. Pgp functions in ATP-driven efflux of drugs from the cell, and Pgp-mediated MDR is thought to be an important cause of failure of cancer chemotherapy [7]. Another group of compounds known as Pgp modulators blocks drug efflux by competing with transport substrates in a complex fashion [8]. Pgp displays high levels of constitutive ATPase activity, which may be stimulated or inhibited by the addition of drug substrates [9,10]. Similar ABC proteins have been implicated in the resistance of many organisms to a vast and chemically diverse range of toxic molecules, and this type of resistance has occurred throughout the course of evolution [11,12].

The role of Pgp-like proteins in insects merits study because such transporters may contribute to insecticide resistance. Organochlorine and organophosphorus pesticides such as chlorpyrifos have been reported to bind to Pgp, and exposure to such compounds increases MDR1 gene expression [13,14]. Exposure to the Pgp modulator verapamil increases the toxicity of ivermectin in chironomids [15] and the toxicity of three insecticide classes (cypermethrin, ivermectin, and endosulfan) in mosquitoes [16]. A few studies have examined the

Abbreviations: ABC, ATP-binding cassette; CHAPS, 3[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid; DMPC, dimyristoyl-L-α-phosphatidylcholine; DTT, dithiothreitol; Ha-Pgp, *Helicoverpa armigera* P-glycoprotein; MDR, multidrug resistance/resistant; NBD, nucleotide-binding domain; Pgp, P-glycoprotein; p-NPP, p-nitrophenylphosphate; PMSF, phenylmethanesulfonyl fluoride; TMD, transmembrane domain; TMR, tetramethylrosamine

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possible role of Pgp-like proteins in the malpighian tubules of insects [17–19]. A Pgp homolog was identified as a nicotine pump in the blood brain barrier of tobacco budworm [18] and shown to maintain a decreased level of nicotine in the brain, thereby protecting the tobacco hornworm from central nervous system toxicity. Several mechanisms of insecticide resistance have been identified in the insect pest, *H. armigera* [2], including insecticide insensitivity of acetylcholinesterase and esterase-mediated organophosphorus resistance [2]. The presence of Pgp and its involvement in insecticide resistance have also been reported in tobacco budworm, *Heliothis virescens* [4], and *H. armigera* [2]. Earlier we have shown that various insecticides stimulated the ATPase activity of partially purified *H. armigera* Pgp (Ha-Pgp) which was reconstituted into proteoliposomes [20]. There have been several reports of purification and characterization of Pgp from MDR human and other mammalian cells, but no reports using insects as a source. In the present study, we report the purification and characterization of Ha-Pgp from insecticide-resistant pests, and its interaction with various insecticides as assessed by their effects on its transport function.

2. Materials and methods

2.1. Chemicals

Dimyristoyl-L-α-phosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). ATP, CHAPS, ethylparaoxon and cypermethrin were obtained from Sigma-Aldrich (Oakville, ON, Canada), and tetramethylrosamine (TMR) from Molecular Probes (Eugene, OR, USA). Creatine kinase and creatine phosphate were purchased from Roche Diagnostics (Laval, QC, Canada). Verapamil and doxorubicin were purchased from Sigma-Aldrich. All other chemicals used were of analytical grade. Methylparathion (99.3%) was procured from the Pesticide Analysis Laboratory, Gulbarga, India. Endosulfan (99%) and fenvalerate (99.4%), from Bayer Crop Science and Dow Agroscience, respectively, were obtained from the Department of Environmental Biology, University of Guelph (Guelph, ON, Canada).

2.2. Insects

H. armigera pests which have developed resistance to various insecticides [2] and are broadly referred to as the resistant population were supplied by Dr. S.S. Udikeri (Agriculture Research Station, Gulbarga, India).

2.3. Purification of Ha-Pgp ATPase

Fourth instar larvae were washed in cold 50 mM Tris–HCl buffer (pH 7.4) and dissected to remove the gut contents. The whole bodies (100 g) were then homogenized in 100 ml of 50 mM Tris–HCl (pH 7.4) containing 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM EDTA, and 1 mM phenylthiourea. The homogenate was filtered through four layers of muslin cloth and centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatant was then subjected to ultracentrifugation at 100,000 *g* for 1 h. The pellet was resuspended in the above buffer containing 20 mM 3 [(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid (CHAPS) and solubilized for 2 h at 4 °C. After re-centrifuging the suspension at 100,000 *g* for 30 min, the supernatant was treated with ammonium sulfate to attain 60% saturation and allowed to stand for 2 h at 4 °C. The precipitate was collected by centrifugation at 15,000 *g* for 20 min and dissolved in 50 mM Tris–HCl buffer (pH 7.4) containing 2 mM CHAPS, 1 mM PMSF, and 1 mM EDTA. The above fraction was loaded on to a Concanavalin A Sepharose-4B column (2.5×10 cm) equilibrated with 50 mM Tris–HCl buffer at pH 7.4 containing 2 mM CHAPS, 1 mM dithiothreitol (DTT) and 5 mM $MgCl_2$ and the column was washed with the same buffer. The column was further washed with 20 ml of 10 mM D-methylmannopyranoside containing 0.2 mM CHAPS until no absorbance at 280 nm was detected in the eluate. The adsorbed proteins were then eluted with 20 ml of 0.5 M D-methylmannopyranoside containing 0.2 mM CHAPS and 2 ml fractions were collected. The five fractions containing maximum activity were pooled, concentrated, dialyzed against 50 mM Tris–HCl buffer pH 7.4 containing 0.2 mM CHAPS and loaded onto a hydroxylapatite column (2×10 cm) which was previously equilibrated with the above buffer. After washing with 40 ml of the above buffer, the adsorbed proteins were then eluted in a stepwise manner with 20 ml of increasing concentrations (10, 50, 100 and 200 mM) of phosphate buffer at pH 7.4 containing 0.2 mM CHAPS, and 3 ml fractions were collected. The active fractions were pooled and concentrated using a Viva cell 250 (MWCO 50,000) and dialyzed against 50 mM Tris–HCl buffer of pH 7.4 containing 0.2 mM CHAPS for 18 h by changing the buffer at every 6 h interval. This step removed phosphate completely from the sample.

Gel filtration chromatography was performed using a G-200 (1×60 cm) column equilibrated with 50 mM Tris–HCl buffer 7.4 containing 0.2 mM CHAPS. The above concentrated sample was loaded on to the column followed by elution with the same buffer at a flow rate of 0.1 ml/min with a fraction size of 1 ml. The Ha-Pgp ATPase fractions were pooled and concentrated as above. Protein concentration was determined by the method of Lowry [21]. The presence of a Pgp-like protein in the purified preparation, reconstituted proteoliposomes, cuticle, fat body and midgut preparation was confirmed as described previously [2], using Western blotting with C219 antibodies, which are directed at the C-terminal NBD of mammalian Pgp.

2.4. The reconstitution of purified Ha-Pgp into the proteoliposomes

The reconstitution of purified Ha-Pgp into proteoliposomes was carried out as described in our earlier report [20]. In brief, lipids (25 mg egg yolk L-α-phosphatidylcholine and 2.5 mg egg yolk L-α-phosphatidic acid) were dissolved in chloroform and dried under a stream of nitrogen. A 1.375 ml buffer sample (containing 20 mM Tris–HCl, pH 7.4; 75 mM NaCl; 1 mM DTT; and 0.5 mM EDTA) was added to the lipid film. The mixture was sonicated on ice until the milky suspension become transparent (about 15–20 min). To a 100 µl aliquot of this preparation, 610 µl of buffer and 40 µl of 180 mM CHAPS solution were sequentially added under constant stirring at room temperature. After 15 min, 250 µl of 0.2 mg/ml purified Pgp were slowly added, and the mixture was incubated under constant agitation for another 30 min. To remove the detergent, the whole sample was applied to a Sephadex G-50 column (1 cm×25 cm) that had been preequilibrated with 20 mM Tris–HCl (pH 7.4), 75 mM NaCl, 1 mM DTT, and 0.5 mM EDTA. The proteoliposomes were collected as a single fraction with the same buffer content as that of the elution buffer. They were assayed for ATPase activity.

2.5. ATPase activity

ATPase activity was determined by quantitating the release of inorganic phosphate from ATP [22]. In brief, an aliquot of enzyme was incubated in 1 ml of ATPase assay medium (containing 2.5 mM ATP; 75 mM KCl; 5 mM $MgCl_2$; 0.5 mM EGTA; 2 mM ouabain; 3 mM sodium azide; 50 mM Tris–HCl pH 7.4) for 30 min at 37 °C. The reaction was terminated by the addition of 2 ml ice-cold stopping medium 0.2% (w/v) ammonium molybdate, 0.9% SDS, 2.3% trichloroacetic acid, 1.3% (w/v) sulfuric acid, and freshly prepared 1% (w/v) ascorbic acid. After 30 min incubation at room temperature, the released phosphate was quantitated colorimetrically at 660 nm. To study the effect of various insecticides, drugs, metal ions and inhibitors, different concentrations of these compounds were included in the reaction mixture.

2.6. Electrophoresis

SDS-PAGE was carried out on 7.5% polyacrylamide gels according to Laemmli [23]. Glycoprotein staining was carried out with periodic acid-Schiff reagent using a procedure described previously [24].

2.7. Effect of pesticides on Pgp-ATPase activity

The ATPase activity of Pgp in the purified preparation was estimated by measuring the liberation of inorganic phosphate using an ATP concentration of 2.5 mM and an assay time of 30 min [9]. The purified fraction was used directly with no exogenous phospholipids added. Pesticides were added to Pgp as DMSO solutions 5 min before initiation of the ATPase assay. The final DMSO concentration did not exceed 1% (v/v) and had no effect on Pgp ATPase activity. The insecticide concentrations that gave rise to half-maximal and maximal ATPase stimulation were estimated from the plots of ATPase activity vs. insecticide concentration. The kinetic parameters K_m and V_{max} were determined graphically by Lineweaver-Burk plot [25].

2.8. Effect of pH and temperature

To determine optimal pH and temperature, the purified protein was incubated with different pH Tris–HCl buffer (pH 7.0 to 9.0), glycine–NaOH buffer (pH 9.5 to 11.5), and temperature 10–80 °C, for 10 min and the enzyme activity was assayed as described above.

2.9. Effect of metal ions and inhibitors

To study the effect of metal ions, chelators, detergents and inhibitors, purified Ha-Pgp was incubated at various concentrations for 10 min at 37 °C and then assayed for enzyme activity as above.

2.10. MALDI-TOF LC–MS of Ha-Pgp

A Coomassie Blue-stained spot was cored from a 1.5-mm-thick 1-D SDS-PAGE gel digested with trypsin. The gel piece was collected in a sterile, siliconized microcentrifuge tube and submitted to the Center for Genomic Application, New Delhi, India, for LC–MS/MS analysis. The MALDI-MS mass spectra of the tryptic peptide mixture were searched for among all entries in the database. The partial peptide sequences were compared with those of the proteins from a MASCOT or BLAST similarity search (http://www.ncbi.nlm.nih.gov/BLAST) to obtain homology with higher confidence.

2.11. Measurement of pesticide and drug binding affinity by tryptophan fluorescence quenching

The affinity of binding of doxorubicin, verapamil and different insecticides, such as cypermethrin, endosulfan, parathion and paraoxon, to purified Ha-Pgp was determined using Trp fluorescence quenching titrations as described previously [26]. Briefly, Ha-Pgp (50 μg in 1 ml) was titrated in 2 mM CHAPS buffer with increasing concentrations of insecticides, while quenching of Trp fluorescence was monitored at 322 nm following excitation at 280 nm (slit width for both, 5 nm). K_d and ΔF_{max} values were extracted following fitting of the data to an equation describing binding to a single affinity site,

$$\Delta F / F_0 \times 100 = \frac{\Delta F / F_{max} \times (100) \times [S]}{K_d + [S]}$$

Where $(\Delta F/F_0 \times 100)$ represents the percent change in fluorescence intensity relative to the initial value after addition of pesticide at a concentration [S], and $(\Delta F_{max}/F_0 \times 100)$ is the maximum percent quenching of the fluorescence intensity that occurs upon saturation of the substrate-binding site.

2.12. Effect of pesticides on TMR transport by Ha-Pgp in reconstituted proteoliposomes

The effect of paraoxon, parathion, endosulfan and cypermethrin on TMR transport was assayed by fluorescence measurements as described previously [27], using a Cary Varian Eclipse fluorescence spectrophotometer. Excitation was carried out at 550 nm (slit width, 5 nm) and fluorescence emission was monitored continuously at 575 nm (slit width, 5 nm). A 450-μl aliquot of proteoliposomes containing 10–20 μg of reconstituted Ha-Pgp was preincubated with the appropriate concentration of TMR in transport buffer at room temperature. The sample was transferred to a quartz cuvette, and allowed to equilibrate for about 300 s to stabilize the fluorescence intensity. Transport of TMR was initiated by the addition of a 25-μl aliquot of buffer containing ATP (final concentration 1 mM) and an ATP-regenerating system (30 μg/ml creatine kinase, 3.5 mM creatine phosphate) followed by mixing for 5–10 s. Fluorescence intensity data were collected for a further 150–200 s. Fluorescence intensity vs. time was normalized to the intensity measured immediately before addition of ATP, which was taken as 100%. The relative rate of TMR transport was estimated from the slope of the fluorescence traces for the first 20 s after the addition of ATP. Different concentrations of pesticides were added at various time points during the transport process, either before addition of ATP, or after the establishment of a new steady-state fluorescence value. In the latter case, fluorescence intensity data were collected for a further 150–200 s. Pesticides were prepared as stock solutions in DMSO, and the final concentration never exceeded 0.1% (v/v).

3. Results

3.1. Enzyme purification

The results of the purification procedure are summarized in Table 1. The highest ATPase activity was found in the 60% ammonium sulfate fraction. Most of the enzyme activity, along with other glycoproteins, was eluted by 0.5 M D-methylmannopyranoside from the Concanavalin A column. This preparation showed low specific activity as D-methylmannopyranoside suppresses the ATPase activity at higher concentration (0.5 M) (date not shown). Upon dialysis, purification was increased to 15-fold. Following the hydroxylapatite column, pure enzyme with high specific activity was eluted with 100 mM phosphate buffer. The recovery of Ha-Pgp-ATPase was 3% and 18-fold purification was achieved. The purified enzyme was homogeneous as judged by SDS-PAGE (Fig. 1A) with a molecular mass of 150 kDa when compared with authentic standards. The presence of Ha-Pgp in the purified preparation, reconstituted proteoliposomes, cuticle, fat body and midgut preparation was further confirmed by western immunoblotting using C219 antibodies (Fig. 1C and E). This protein gave a violet band when stained with periodic acid-Schiff reagent, indicating that it is glycosylated (Fig. 1D). The ATPase activity of the reconstituted Ha-Pag ATPase was found to be 400 ± 4.5 nmol/min/mg.

3.2. Effects of pH, temperature and different substrates

The optimum pH and temperature of Ha-Pgp ATPase were found to be 7.5 and 35–40 °C, respectively. The substrate specificity of the

Table 1
Purification steps of Pgp ATPase from *Helicoverpa armigera*.

Purification steps	Total protein (mg)	Total ATPase activity (nmol/min)	Specific ATPase activity (nmol/min/mg)	Yield (%)	Fold-purity
Crude	1200	20,000	16.6	100	1
CHAPS	400	17,000	42.5	85	2.5
Ammonium sulfate precipitation	20	4000	200	20	12
Concanavalin A followed by dialysis	8	2000	250	10	15
Hydroxylapatite followed by dialysis	4	1200	300	6	18
Sephadex G-200	2	600	300	3	18

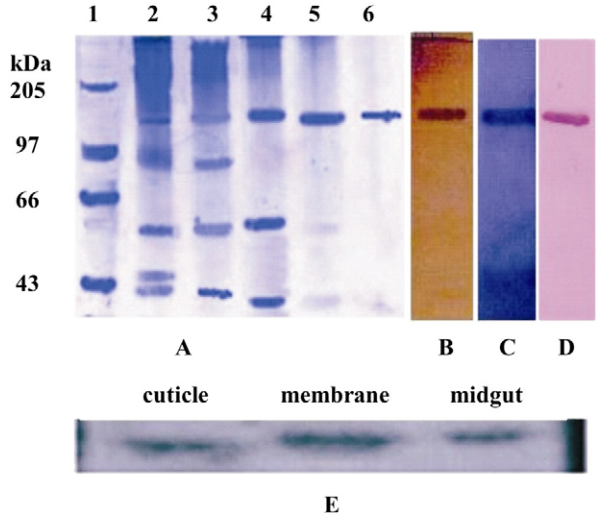

Fig. 1. A. SDS-PAGE (7.5%) profile at different stages of purification of Ha-Pgp ATPase. Lane1, molecular weight markers myosin (205 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa); lane 2, CHAPS extract (100 μg); lane 3, ammonium sulfate precipitation (100 μg); lane 4, Concanavalin A fraction (40 μg); lane 5, hydroxylapatite fraction (20 μg); lane 6, gel filtration fraction (10 μg). The gel was stained with Coomassie blue. (B) Silver stained gel and (C) detection of Ha-Pgp in the purified sample, and (E) cuticle, fat body and mid gut by C219 antibodies. (D) Detection of glycosylation of Ha-Pgp. In brief, the purified protein was resolved by 7.5% SDS-PAGE and the gel was dried at 100 °C followed by treatment with ethanol and periodic acid-Schiff reagent for 20 min.

purified Ha-Pgp ATPase was investigated using various phosphorylated compounds. The enzyme could cleave phosphoester bonds effectively from a broad range of substrates such as ATP, AMP, p-nitrophenylphosphate (p-NPP) and β-glycerophosphate. However, at higher concentrations, ADP acts as an inhibitor. The K_m for ATP was found to be 1.2 mM and the maximum ATPase activity was 250 nmol/min/mg. For ADP, AMP, and p-NPP the maximum ATPase activity was 4–20 nmol/min/mg.

3.3. The effect of ATP concentration on Ha-Pgp ATPase

The effect of ATP concentration on the Ha-Pgp ATPase in the presence or absence of different stimulators is shown in Fig. 2. Ethylparaoxon and fenvalerate at 30 μM enhanced ATPase activity. For basal ATPase the K_m is 1.2 mM, whereas in the presence of paraoxon and fenvalerate the K_m values are 0.5 mM and 0.5 mM, respectively.

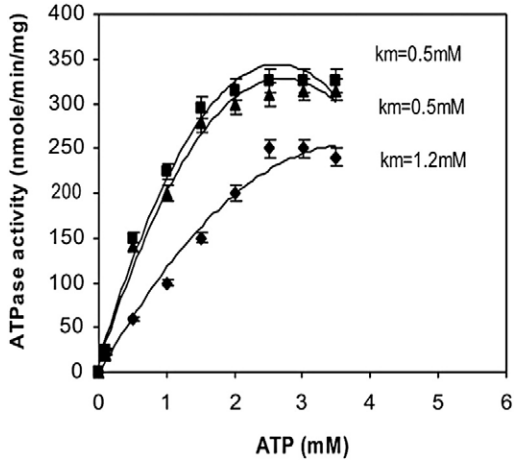

Fig. 2. The effect of paraoxon and fenvalerate on K_m and V_{max} of purified Ha-Pgp ATPase. The specific ATPase activity of purified Ha-Pgp was measured at different concentrations of ATP, in the (♦) absence and presence of (■) ethylparaoxon (30 μM), and (▲) fenvalerate (30 μM).

3.4. Effect of vanadate on stimulated ATPase activity

Verapamil and fenvalerate stimulated the basal ATPase activity by 2-fold and 0.15-fold, respectively. Verapamil-stimulated ATPase activity was enhanced by fenvalerate, indicating that verapamil and fenvalerate may bind to different sites on Ha-Pgp. The basal and fenvalerate or verapamil-stimulated ATPase activities were inhibited by vanadate at 10 μM, but were not affected by agents that inhibit other ATPases and phosphatases (Table 2).

3.5. Effect of insecticides on purified Ha-Pgp ATPase activity

In the previous study we reported the effect of insecticides on reconstituted proteoliposomes containing partially purified Ha-Pgp [20]. In this study, we examined the effect of various insecticides at different concentrations on purified Ha-Pgp ATPase activity (Fig. 3). ATPase activity was stimulated by all the tested insecticides, ranging from 20% (for ethylparaoxon) to 40% (for cypermethrin) at an insecticide concentration of 100 μM. Endosulfan and monocrotophos at a concentration of 100 μM stimulated ATPase activity by 30% and 25%, respectively. Methylparathion at a concentration of 50 μM stimulated ATPase activity by 30% and inhibited at higher concentrations.

3.6. Inhibition

We examined the effect of metal ions, chelators and inhibitors on purified Ha-Pgp ATPase (Table 2). NaCl and $MnCl_2$ were found to increase the activity by 20 and 10%, respectively. Ba^{2+}, Pb^{2+}, sodium vanadate, probenecid, and N-ethylmaleimide strongly inhibited ATPase activity, whereas SDS, Triton X-100, urea, EGTA, EDTA, DTT and 1,10-phenanthroline had no significant effect.

3.7. Amino-acid sequence analysis

The pure protein was subjected to digestion with trypsin and the peptides were separated and sequenced by LC–MS. Some of the fragments showed high homology to human Pgp, which is involved in

Table 2
Effect of metal ions, chelators, detergents, inhibitors and modulators on purified Ha-Pgp ATPase activity.

Compounds	Concentrations	ATPase activity (%)
Control	0	100
Nacl	5 mM	120
Mncl	1 mM	110
Ba	1 mM	10
Pb	3 mM	23
EGTA	1 mM	90
EDTA	1 mM	90
SDS	1%	50
Triton X-100	1%	90
Urea	5 mM	100
DTT	1 mM	100
1,10-Phenanthroline	1 mM	100
Sodium vanadate	10 μM	10
Probenicid	100 μM	20
N-ethylmaleimide	100 μM	30
Verapamil	30 μM	200
Verapamil + vanadate	30 + 10 μM	50
Fenvalerate	30 μM	115
Fenvalerate + vanadate	30 + 10 μM	30
Verapamil + fenvalerate	30 + 30 μM	210
Verapamil + ouabain	30 + 1000 μM	200

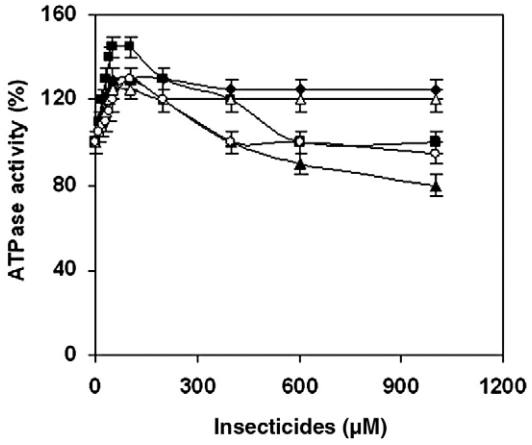

Fig. 3. Effect of various insecticides on purified Ha-Pgp ATPase activity. The graph shows the percent stimulation compared with a control (100%, in the absence of pesticides). (♦) Paraoxon; (■) cypermethrin; (▲) endosulfan; (Δ) monocrotophos; (○) parathion. All insecticides were stimulated at lower concentrations and inhibited at higher concentrations. Data points represent the mean of at least three determinations.

MDR (Fig. 4A). Conserved motifs such as the Walker A and B motifs, the Signature C motif, and the Q, D and H loops were present, and homologous to other Pgps (Fig. 4B). These results indicate that the isolated enzyme from *H. armigera* is likely to be a Pgp homolog, as it shows many similarities with known sequences of other Pgps.

3.8. Affinity of binding of insecticides and drugs to Ha-Pgp

Tryptophan quenching experiments were carried out in the presence and absence of ATP using four different pesticides and two drugs, and the K_d values for binding were estimated by fitting the fluorescence quenching data to an equation describing a single binding site (Fig. 5A–C). Addition of ATP to Ha-Pgp resulted in a large quenching of the fluorescence (Fig. 5D), suggesting interaction of ATP to Ha-Pgp. Ha-Pgp in the presence of insecticides also show a large quenching but with a shift on the λ_{max} of Trp residues fluorescence emission. A small 3-nm red shift on emission maximum was observed with parathion, while 8-nm red shift was found for monocrotophos. These results suggest the formation of an association between the macromolecule and the insecticides with a change of Trp environment. All the pesticides and drugs displayed substantial levels of Trp quenching. Fitting of the experimental data showed that all the pesticides and drugs interacted with Ha-Pgp with relatively high affinity with K_d values for binding in the 0.8–50 μM range. Doxorubicin and verapamil K_d values were 0.8 and 1.5 μM respectively, whereas for cypermethrin, endosulfan, parathion and paraoxon, K_d values were 9 μM, 25 μM, 25 μM and 50 μM, respectively (Fig. 5A–C).

3.9. Ability of pesticides to compete for drug transport by Ha-Pgp in proteoliposomes

In previous studies, TMR was chosen as a substrate for measuring Pgp-mediated transport into reconstituted proteoliposomes in vitro, and the kinetics of the transport process were characterized [27]. The rhodamine derivative TMR was found to be a much superior substrate to rhodamine 123, which has been widely used in dye exclusion studies in intact cells [28]. We have employed TMR to measure the rates of transport by reconstituted Ha-Pgp in real time. The addition of 1 mM ATP and an ATP-regenerating system to reconstituted DMPC proteoliposomes containing Ha-Pgp in the presence of 1 μM TMR led to a rapid drop in TMR fluorescence until a new lower steady-state level was established (Fig. 6, trace c). The decrease in fluorescence intensity arises from transport of TMR into the proteoliposomes, where its fluorescence is lower, likely as a result of self-quenching as it accumulates in the lumen. Intensity measurements were made (after mixing) every 1 s for about 20 s to allow for estimation of the rate of fluorescence decrease, which is proportional to the initial rate of TMR transport. No decrease in TMR fluorescence was observed in the presence of non-hydrolysable ATP analogs, and heat denaturation of Pgp abolished the change in TMR fluorescence (data not shown). Thus the process of TMR transport into proteoliposomes is dependent on ATP hydrolysis and requires functionally active Ha-Pgp. Pesticides at concentrations that completely block transport of TMR into the proteoliposome interior would be predicted to prevent the decrease of rapid fluorescence when added prior to ATP. This was indeed observed for all four insecticides and is shown for paraoxon in Fig. 6, trace a. Addition of pesticide to the proteoliposomes after prior establishment of the TMR concentration gradient resulted in immediate collapse of the gradient, as indicated by a rapid increase in TMR fluorescence. Experimental results obtained with paraoxon are shown in Fig. 6, trace c, and are representative of similar data obtained for the other three insecticides. When TMR transport was measured in the presence of different insecticide concentrations, both the rate of the fluorescence decrease and the magnitude of the total decrease seen at a steady state were changed in a concentration-dependent fashion. For example, Fig. 6, trace b, shows the rate of TMR transport in the presence of 10 μM paraoxon. The initial rate of TMR transport declined in a saturable fashion with increasing concentrations of insecticide (Fig. 7), with 50–70% inhibition observed at 60 μM for all the compounds. Thus, insecticides inhibited the net rate of transport of TMR, and prevented the generation of a substrate concentration gradient at high concentrations. All the insecticides were able to compete effectively with TMR for transport via Pgp.

4. Discussion

This study is the first to purify a Pgp homolog from the pest *H. armigera* and investigate the interaction of insecticides with the purified protein in detergent solution and proteoliposomes, rather than intact cell systems. This approach allows the application of several novel fluorescence-based tools, so that the interaction of these chemicals with the transporter can be characterized at the molecular level. There are several reports of the isolation of highly purified Pgp from mammalian cells, insect cells or a yeast expression system. Pgp has been purified from Chinese hamster ovary cells by several groups [9,29]. The expression of Pgp in *Saccharomyces cerevisiae* [30], and insect cells [31], also led to high yields of catalytically active protein.

Several independent lines of evidence indicate that the ATPase activity of our purified preparation arises from Ha-Pgp itself and not a contaminating ATPase. Fig. 3 shows that Ha-Pgp ATPase activity is stimulated by the addition of various insecticides. The maximal levels of ATPase stimulation observed were high (20–40%) for all the insecticides, compared to those observed for mammalian Pgp with other drug substrates [32]. Several pesticides were shown to stimulate the ATPase activity of Pgp from the MDR Chinese hamster ovary cell line, CH^RB30 [33]. The stimulation of ATP hydrolysis by the substrate vinblastine was due to an increase in the maximal velocity of ATP hydrolytic activity without affecting the apparent K_m for ATP, whereas several drugs and modulators behaved as mixed activators, producing changes in both the V_{max} of the ATPase and the K_m for ATP [9]. Several studies have shown that proteoliposomes containing reconstituted Pgp can carry out ATP-dependent transport of drugs and peptides [27,32,34].

The ATPase activity of purified Ha-Pgp was characterized with respect to its ionic requirements and inhibitor sensitivity. The presence of Na^+, K^+, and Cl^- at 150 mM had no effect on basal activity. Various agents that are known to inhibit F, P, and V-type ATPases and phosphatases were tested; except for vanadate, all other agents were found to be without any effect [20]. It is clear that common ATPases (e.g., Na^+K^+, Ca^{2+} and F_0F_1-ATPases) and

phosphatases do not contribute significantly to the activity. ATP protects the Pgp ATPase from inactivation by these compounds at two sites per molecule [35]. A conserved Cys residue is found in the Walker A motif of all Pgp gene classes, and it is known that this residue is the target of attack by sulfhydryl reagents [36]. The inhibitor profile of Ha-Pgp is thus very similar to that of the mammalian Pgps.

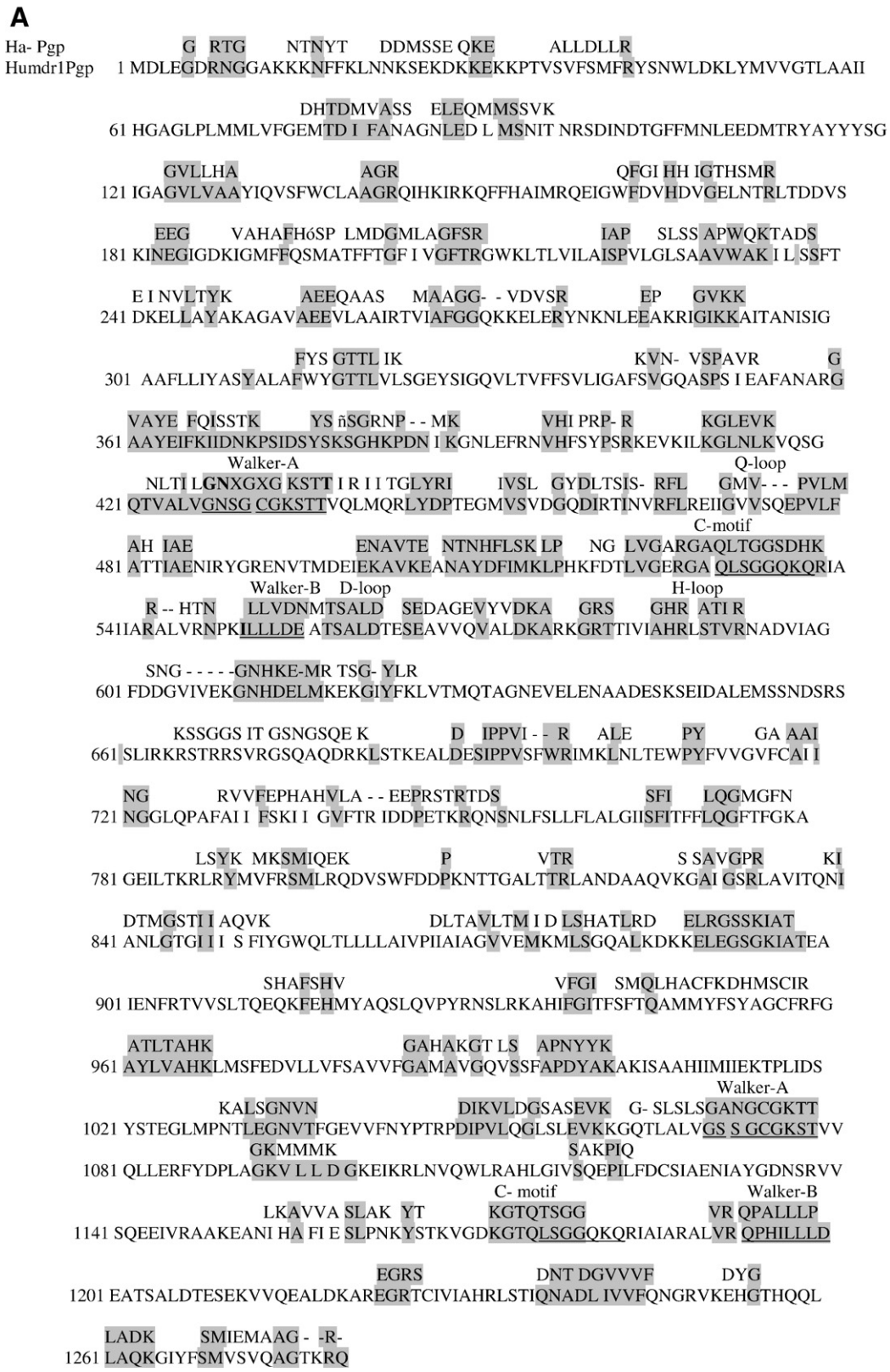

Fig. 4. (A) Partial amino-acid sequence alignment of Ha-Pgp with human Pgp (MDR1/ABCB1). The ATP-binding sites are underlined and the shaded boxes represent identical amino acids in two of the NBD sequences. The conserved motifs, Walker A, Walker B and Signature C, are labeled and underlined. The Q, D, and H loops are labeled and shaded. (B) Alignment of the amino acid sequence of Ha-Pgp with the ATP-binding cassette of *Anopheles gambiae* protein (XM 315658), *Drosophila melanogaster* proteins mdr49 (M59076), and human MDR1/ABCB1 Pgp (GI:238054374). The Walker A, Walker B and Signature C motifs are labeled and underlined.

B

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                                                    Walker-A
Ang21663    GSMQGNIKFSNVFFRYPARNDVQVLQGLNLEIKTGQTVALVGPSGCGKSTCLQLIQRLYD 479
Dmdr65      YGLRGDVEFQDVFFRYPSRPEVIVHRGLNIRIRAGQTVALVGSSGCGKSTCVQLLQRFYD 458
Hmdr1       DNIKGNLEFRNVHFSYPSRKEVKILKGLNLKVQSGQTVALVGNSGCGKSTTVQLMQRLYD 445
HaPgp       -----------------------------------NLTILGNXGXGKSTTIRIITGLYR 24
                                                .::::*  * **** :::: :*
                                 Q-loop
Ang21663    PLSGSVTIDGTKVSELNIGWLRSFIGLVGQEPVLFATTIAENIRYGNPDASQSEIERAAK 539
Dmdr65      PVFGSVLLDDLDIRKYNIQWLRSNIAVVGQEPVLFLGTIAQNISYGKPGATQKEIEAAAT 518
Hmdr1       PTEGMVSVDGQDIRTINVRFLREIIGVVSQEPVLFATTIAENIRYGRENVTMDEIEKAVK 505
HaPgp       ----IIVSLGYDLTSISR--------FLGMVPVLMAHIAEEN---------------AV 56
                 :   . .:   .          .:.  ***:     :*                .
                                  C-motif             Walker-B  D-loop
Ang21663    IANCHSFITKLPNGYATMIGERGAQLSGGQKQRIAIARALVRNPKILLLDEATSALDPNS 599
Dmdr65      QAGAHEFITNLPESYRSMIGERGSQLSGGQKQRIAIARALIQNPKILLLDEATSALDYQS 578
Hmdr1       EANAYDFIMKLPHKFDTLVGERGAQLSGGQKQRIAIARALVRNPKILLLDEATSALDTES 565
HaPgp       TENTNHFLSKLPN---GLVGARGAQLTGGSDHKRHTN---------LLVDNMTSALDSED 104
              .   *: :**.    ::* **:**:**..::              **:*: ***** :.

Ang21663    EKRVQDALERASKGRTTLVVSHRLSTITNADKIVYIDKGLVMEQGTHEELMAARGLYYDL 659
Dmdr65      EKQVQQALDLASKGRTTIVVSHRLSAIRGADKIVFIHDGKVLEEGSHDDLMALEGAYYNM 638
Hmdr1       EAVVQVALDKARKGRTTIVIAHRLSTVRNADVIAGFDDGVIVEKGNHDELMKEKGIYFKL 625
HaPgp       AGEVYVDKAGRSGHRATIRSNG----------------------GNHKEMRTSG--YLR- 139
              *          *:*:                           *.*.::      *
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Fig. 4 (*continued*).

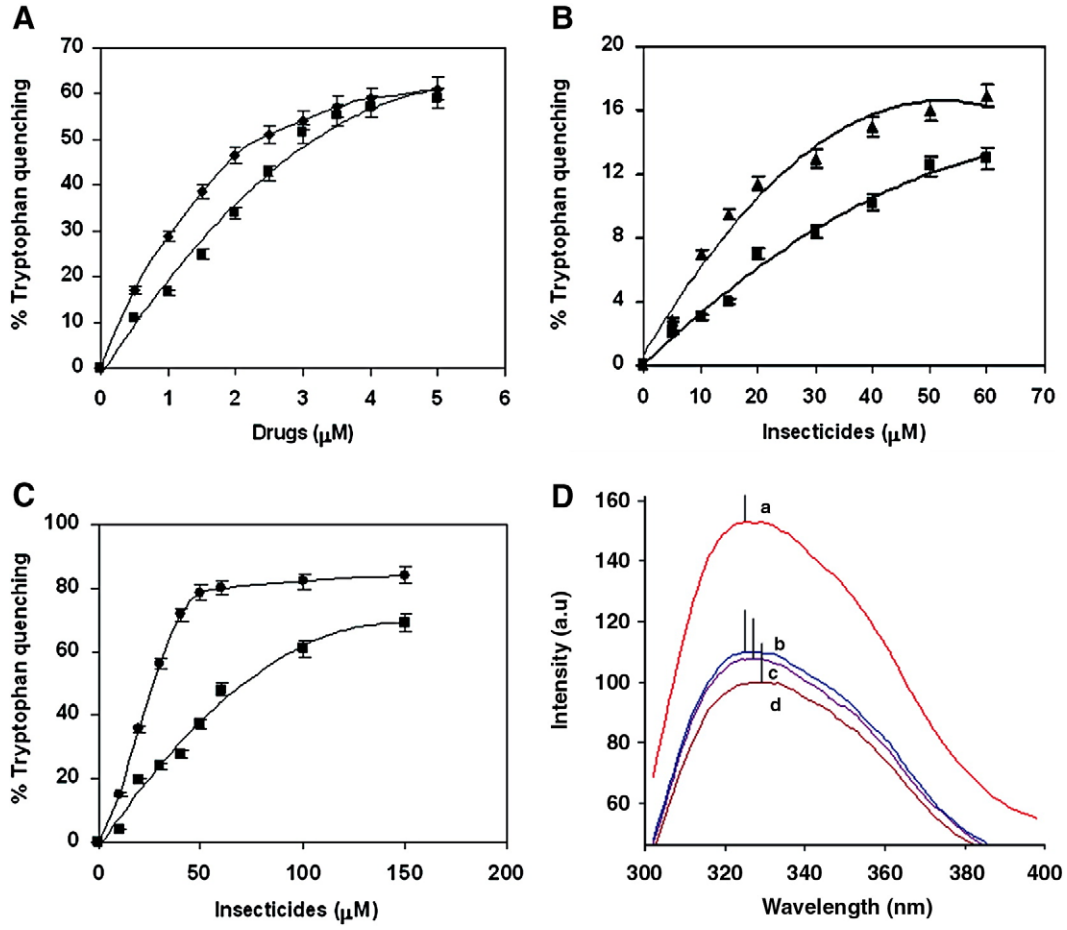

Fig. 5. Binding of insecticides and drugs to purified Ha-Pgp as assessed by Trp fluorescence quenching at increasing concentrations of insecticides and drugs. (A) (♦) Doxorubicin, (■) Verapamil; (B), (▲) cypermethrin, (■) endosulfan; (C), (●) parathion, and (■) paraoxon. (D) Fluorescence emission spectra of Ha-Pgp Trp residues in the absence (a) and presence of 1 mM ATP (b); 10 μM parathion (c); 10 μM monocrotophos (d), titrated with 50 μg/ml purified Ha-Pgp at room temperature (23 °C). Trp fluorescence was monitored at 322 nm following excitation at 280 nm. The percent quenching of fluorescence ($\Delta F/F_0 \times 100$) was calculated relative to Ha-Pgp in the absence of insecticide. The continuous line represents the best computer-generated fit of the data points (shown by symbols) to an equation describing interaction of the pesticide with a single type of binding site (see Materials and methods). K_d and ΔF_{max} values were estimated by the fitting process. Data points represent the mean ± standard error for triplicate determinations.

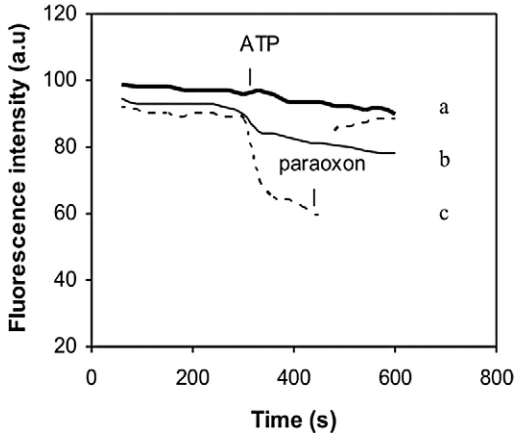

Fig. 6. Real-time fluorescence measurements of TMR transport into reconstituted proteoliposomes containing Ha-Pgp. (a) 100 μM paraoxon was added to the proteoliposomes before the addition of 1 mM ATP. (b) 10 μM paraoxon was added to the proteoliposome before the addition of ATP. (c) ATP was added to the proteoliposomes to initiate TMR transport and lower fluorescence intensity was reached after 250 s as a result of inward pumping of TMR. 100 μM paraoxon was then added, collapsing the inward flow and restoring the fluorescence to close to its original value.

Sequence alignment shows that some of the fragments of Ha-Pgp are aligned with human MDR1 (ABCB1). Characteristic NBD motifs such as the Walker A, Walker B, and Signature C motifs, and the Q, D and H loops, were found in the sequenced fragments (Fig. 4A and B) indicating that the Pgp homolog isolated from *H. armigera* was similar to other known Pgps. ABC family members share 30–50% sequence similarity. The regions of highest homology include the two NBDs that are located on the cytoplasmic side of the membrane [37]. The TMDs are thought to contain the substrate-binding site, and it has been suggested that differences in substrate specificities are a consequence of structurally divergent TMDs [38,39]. The NBDs are the sites of binding and hydrolysis of cytoplasmic ATP. All ABC transporters contain within each NBD at least three highly conserved sequence motifs; the Signature C sequence, and the Walker A and B motifs. All three motifs are directly involved in binding of nucleotide [37]. Specific amino acids are important in the Walker A and B motifs. Indeed, mutation of the lysine and aspartate residues in the Walker A and B motifs, respectively, in either NBD, resulted in loss of the ATP hydrolysis activity of Pgp [40–42]. Sequence comparisons of cloned mammalian Pgp genes divided the family into three classes, I, II and III. Only class I (human mdr1/ABCB1, rodent pgp1, mouse mdr3) and class II (rodent pgp2, mouse mdr1) genes confer MDR [43].

Trp fluorescence quenching confirmed that four insecticides and two drugs interacted directly with purified Ha-Pgp, and allowed estimation of their binding affinity. All of them bound to the transporter with relatively high affinity. Binding of various drugs and modulators to the substrate-binding site of mammalian Pgp results in saturable concentration-dependent quenching of the intrinsic Trp fluorescence of the protein [26]. This approach can be used to quantitate the affinity of binding of many different Pgp substrates and modulators [44]. As Trp residues are quite sensitive to the surrounding medium, changes in accessibility to solvent can be used to detect conformational changes upon binding of compounds to the protein and in the presence of all insecticides an association conformational change on Ha-Pgp seems to occur detected by red shift on the maximum wavelength on the emission spectrum and showing a change of the Trp to a more polar environment [26]. To determine whether the isolated Ha-Pgp is involved in insecticide transport, it was necessary to first demonstrate that it was functional. To do this, Ha-Pgp was reconstituted into proteoliposomes and the transport of a fluorescent compound was examined. Transport was abolished, and the TMR gradient collapsed, by the addition of high concentrations of pesticides. The binding affinities of the pesticides cover a narrow range, perhaps reflecting the fact that binding represents only the first step in the ATP-driven transport process, whereas both ATPase stimulation and transport inhibition involve additional steps of the catalytic cycle. Taken together, these results indicate that the four insecticides used in this study interact with Ha-Pgp with high affinity, and suggest that they may be transported by the protein. Similar studies were carried out on mammalian Pgp and it was found that different insecticides inhibited Pgp-mediated transport of TMR [33]. The data showing that the pesticides bind to Pgp and block Pgp-mediated transport suggests that they may be Pgp inhibitors (also known as modulators or chemosensitizers). Modulators interact with Pgp and are often transported, but a concentration gradient is not built up across the membrane, probably because these compounds repartition rapidly into the bilayer, cross to the inner leaflet, and interact with Pgp once more [10]. Thus, Pgp may operate in a futile cycle in the presence of a modulator. A recent study of 14 structurally diverse pesticides also suggested that several of them were Pgp modulators [45].

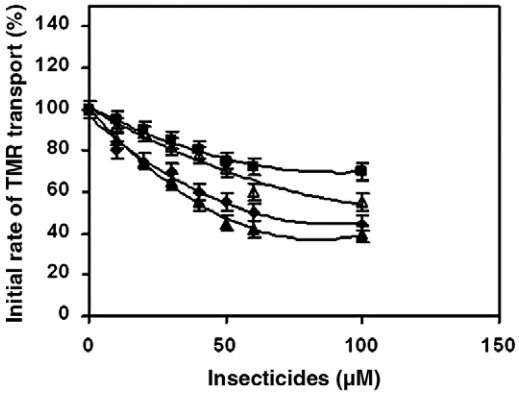

Fig. 7. Rate of Ha-Pgp-mediated TMR transport into reconstituted proteoliposomes and its inhibition by increasing concentrations of insecticides; (■) fenvalerate; (◊) cypermethrin; (♦) ethylparaoxon; and (▲) methylparathion.

ABC drug efflux pumps are widely recognized to play a central role in tissue defence [5]. Expression of Pgp-like transporters has been correlated with resistance to xenobiotics and environmental pollutants in many different species of insects and marine organisms, and Pgp appears to play an important role in environmental toxicology [46–48]. The distribution of Pgp in the different tissues suggests that the accumulation of insecticides could be prevented at every level, thereby achieving the decreased intracellular level of insecticides which is the hallmark of MDR. From the above studies and an earlier report [2], we conclude that the Pgp ATPase of *H. armigera* parallels many mammalian Pgps in terms of its functions, and could participate in insecticide resistance by actively excreting these compounds from the membrane, thus preventing their accumulation in target cells. The details of kinetics, structure and function of Ha-Pgp ATPase will be important in order to develop new strategies for overcoming insecticide resistance in these pests.

5. Dedication

This paper is dedicated to Prof. V. H. Mulimani, Department of Biochemistry, Gulbarga University, Gulbarga, on the occasion of his 60th birth anniversary.

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