Partial Purification and Immunohistochemical Localization of ATP Diphosphohydrolase from *Schistosoma mansoni*

IMMUNOLOGICAL CROSS-REACTIVITIES WITH POTATO APYRASE AND *TOXOPLASMA GONDII* NUCLEOSIDE TRIPHOSPHATE HYDROLASE*

Eveline G. Vasconcelos‡, Sérgio T. Ferreira§, Técia M. U. de Carvalho¶, Wanderley de Souza¶, Ana M. Kettlun‖, Marta Mancilla‖, M. Antonieta Valenzuela‖, and Sergio Verjovski-Almeida**

From the Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Caixa Postal 26077, 05599-970 São Paulo, SP, Brazil, the §Departamento de Bioquímica Médica, Universidade Federal do Rio de Janeiro, 21941-590 RJ, Brazil, the ¶Laboratório de Biologia Celular e Tecidual, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, 28015-620 RJ, Brazil, and the ‖Departamento de Bioquímica y Biologia Molecular, Universidad de Chile, Casilla 233 Santiago, Chile

ATP diphosphohydrolase from tegumental membranes of *Schistosoma mansoni* was solubilized with Triton X-100 plus deoxycholate and separated by preparative nondenaturing polyacrylamide gel electrophoresis. Two isoforms with ATP-hydrolytic activity were identified and excised from nondenaturing gels. For each of the active bands, two protein bands (63 and 55 kDa) were detected with Coomassie Blue staining, following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blots developed with polyclonal anti-potato apyrase antibody revealed a single protein of 63 kDa, either with samples excised from active bands or with total *S. mansoni* tegument. Anti-potato apyrase antibody immobilized on Sepharose-Protein A depleted over 95% of ATPase and ADPase activities from detergent-solubilized tegument. Confocal laser scanning microscopy showed anti-potato apyrase antibody on the outer surface of *S. mansoni* tegument. A different antibody against a fusion protein derived from recently cloned *Toxoplasma gondii* nucleoside triphosphate hydrolase (Bermudes, D., Peck, K. R., Afifi, M. A., Beckers, C. J. M., and Joiner, K. A. (1994) *J. Biol. Chem.* 269, 29252–29260) revealed the same 63-kDa band in Western blots of *S. mansoni* tegument. Since anti-potato apyrase antibodies exhibited cross-reactivity with *S. mansoni* ATP diphosphohydrolase, we decided to gain further information on the primary structure of potato apyrase by sequencing the protein. Three novel peptides were obtained: amino-terminal sequence and two internal sequences from tryptic fragments. Eight sequences recently deposited in the data bank, including that of *T. gondii* nucleoside triphosphate hydrolase, have considerable homologies to potato apyrase suggesting a new family of nucleoside triphosphatases which contains a conserved motif (I/V)(V/M/I)(I/L/F/C)DAGS(S/T) near the amino-terminal. Antibody cross-reactivities in the present work suggest that conserved epitopes from *S. mansoni* ATP diphosphohydrolase are present in this family of nucleotide-splitting enzymes.

A novel ATP diphosphohydrolase (apyrase, EC 3.6.1.5) has been described on the external surface of *Schistosoma mansoni* tegument by ultrastructural histochemical techniques, which identified formation of lead phosphate precipitates on the surface of the parasite upon splitting of external ATP or ADP (1). It was the first demonstration of an ATP diphosphohydrolase on the surface of a blood-dwelling endoparasite. ATP diphosphohydrolases or apyrases are capable of splitting ATP or ADP to AMP and are present on the surface of a number of different cells (for reviews, see Refs. 2 and 3), including blood cells and endothelial cells of blood vessels. We have proposed that in *S. mansoni* this enzyme may participate in escape mechanisms of the parasite by splitting ATP or ADP which could be released on its surface by activated platelets (1) or activated cytotoxic T lymphocytes.

ATP diphosphohydrolases share several kinetic features, namely a broad nucleoside di- and triphosphate specificity, activation of ATP hydrolysis by micromolar concentrations of either Mg^{2+} or Ca^{2+}, and inhibition of Ca^{2+}-activated ATP hydrolysis by millimolar Mg^{2+} (2, 3). One of the first ATP diphosphohydrolases to be purified was potato apyrase (4), a 49-kDa enzyme from potato tuber (4–6).

In the original report of ATP diphosphohydrolase from *S. mansoni* tegument (1), the apparent molecular weight of the enzyme was not determined. In the present work, *S. mansoni* ATP diphosphohydrolase was partially purified. Western blot analysis using either an antibody against a fusion protein derived from recently cloned nucleoside triphosphate hydrolase of *Toxoplasma gondii* protozoan (7) or an anti-potato apyrase antibody have identified *S. mansoni* ATP diphosphohydrolase as a 63-kDa protein. Since anti-potato apyrase antibody exhibited cross-reactivity with *S. mansoni* ATP diphosphohydrolase, we decided to gain further information on the primary structure of potato apyrase. Amino acid sequences of three tryptic fragments of potato apyrase were obtained in the present work, and a search against the data bank revealed considerable homology with *T. gondii* nucleoside triphosphate hydrolase (7, 8) and with a recently cloned nucleoside triphosphatase of *Pisum sativum* (garden pea) (unpublished sequence, PIR S48859).

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The peptide sequence data reported in this manuscript appear in the Swiss-Prot Sequence Data Base under the accession number P80595.

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** To whom correspondence should be addressed. Tel.: 55-11-818-7973; Fax: 55-11-818-7986; E-mail: verjo@quim.iq.usp.br.

Further homology searches in the data bank showed that the above sequences and the sequences of yeast *Saccharomyces cerevisiae* GDPase (guanosine-diphosphatase, EC 3.6.1.42) (9) and hypothetical 71.9-kDa protein in PMI40-PAC2 intergenic region of *S. cerevisiae* chromosome V (unpublished sequence, Swiss-Prot P40009), of human and mice CD39 lymphoid cell activation antigen (10) and of unpublished conceptual translations of *Caenorhabditis elegans* cosmids C33H5 (NCBI sequence ID 1086594) and R07E4 (NCBI sequence ID 1049394) have significant homologies which point to a novel family of nucleotide-splitting enzymes. Cross-reactivities detected in the present work seem to indicate that conserved epitopes from *S. mansoni* ATP diphosphohydrolase are present in this family.

EXPERIMENTAL PROCEDURES

Materials—Potato apyrase (49 kDa) was purified from an homogeneous strain of *Solanum tuberosum* cv Desirée. Purified apyrase was obtained in the native conformation by the Cibacron Blue column procedure (5) and was used for mice immunization. Purified apyrase was denatured by running on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the 49-kDa band was used for microsequencing as described below and for rabbit immunization. Antibody against Sigma potato apyrase was developed in rabbits and was a gift from Dr. J. Sarkis, Universidade Federal do Rio Grande do Sul. ATP, ADP, ouabain, $C_{12}E_8$, and protease inhibitors were obtained from Sigma. All other reagents were of the highest analytical grade available.

Parasites and Isolation of Tegument—Adult *S. mansoni* worms were perfused either from Syrian hamsters or from mice infected 6 weeks previously with approximately 350 cercariae each (11). Isolated tegument was obtained from live parasites as described previously (1) by incubation for 10 min at 37 °C in phosphate-buffered saline containing 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and protease inhibitors: leupeptin (0.5 μg/ml), pepstatin (0.07 μg/ml), soybean trypsin inhibitor (50 μg/liter), and phenylmethylsulfonyl fluoride (2 μg/ml). Isolated tegument was stored in liquid nitrogen in the presence of 5 mM Tris-HCl, pH 7.4, 8% sucrose plus the protease inhibitors described above. Protein concentration was determined according to Lowry *et al.* (12).

Activity Measurements—Activity measurements were performed as described previously (1) in standard reaction medium containing 50 mM MOPS[1] buffer, pH 7.4, 5 mM $CaCl_2$, 2 mM $MgCl_2$, 150 mM NaCl, 5 mM KCl, 1 mM ouabain, 10 mM NaN_3, 0.01–0.05 mg of protein/ml, and 3 mM concentration of either ADP or ATP. P_i liberated was determined spectrophotometrically according to Taussky and Shorr (13).

Partial Purification with Nondenaturing Polyacrylamide Gel Electrophoresis—This was performed as described by Laemmli (14) using a 6% polyacrylamide gel and 0.1% (v/v) Triton X-100 plus 0.1% (w/v) sodium deoxycholate instead of sodium dodecyl sulfate in the gel and running buffer. For each gel lane, an aliquot of 100 μg of tegumental protein was solubilized in standard reaction medium without nucleotides supplemented with 0.2% Triton X-100 plus 0.4% sodium deoxycholate and subjected to electrophoresis for approximately 80 min at 150 V in the cold room. The gel was washed for 40 min in standard reaction medium without nucleotides. Fresh standard reaction medium containing 10 mM $CaCl_2$ (higher calcium was used here to induce calcium phosphate precipitation) and 5 mM ADP or ATP were added. After incubation at 37 °C for approximately 40 min, white calcium phosphate precipitates appeared and were photographed against a dark background. Bands containing ATPase or ADPase activity were cut out and electroeluted in a Gel Eluter (Hoefer Scientific Instruments, San Francisco, CA) for 3–4 h at approximately 50 V in 300 μl of 4 × Laemmli buffer (0.1 M Tris and 0.768 M glycine, pH 8.3, plus 0.4% SDS) according to manufacturer instructions. Eluted samples were precipitated with 10% trichloroacetic acid, washed by centrifugation with water, and resuspended in gel loading buffer. Two samples were combined (eluted protein originated from 200 μg of total tegumental protein) and submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gels (14). Gels were either stained with Coomassie Blue or electroblotted onto nitrocellulose membrane.

Western Blotting—Proteins were electroblotted onto nitrocellulose filters, followed by blocking with non-fat milk using standard procedures (15). Primary antibodies were used at a dilution of 1:5,000 (antibody anti-apyrase from Sigma), 1:10,000 (antibodies anti-native or denatured apyrase from *S. tuberosum* cv Desirée), or 1:2,000 (*T. gondii* antibody) and were incubated overnight. Assays were developed by chemiluminescence with secondary antibody coupled to horseradish peroxidase and Luminol as substrate using the ECL kit (Amersham) and exposed to x-ray film.

Immunoprecipitation—Nonionic detergent octaoxyethylene lauryl ether ($C_{12}E_8$) (Sigma) at a concentration of 1 mg/ml was added to a reaction medium containing 50 mM MOPS buffer, pH 7.4, 2 mM $CaCl_2$, 1 mM EGTA, 0.3 mM $MgCl_2$, 150 mM NaCl, 5 mM KCl, 1 mM ouabain, 10 mM NaN_3, 1 mM vanadate, 0.01 mg of protein/ml in the absence of nucleotides, followed by ultracentrifugation at 14,000 × *g* for 20 min at 4 °C. Immune serum at a final dilution of 1:200 (Sigma apyrase) or 1:50 (Desirée apyrase) was added to the soluble supernatant and incubated for 3 h at room temperature. Protein A-Sepharose (Pharmacia Biotech Inc.) was added and incubated for an additional 2 h. Control assays with preimmune serum were run in parallel. The resin was sedimented by centrifugation in an Eppendorf centrifuge for 5 min. Supernatants were used for determination of hydrolytic activities by addition of either ATP or ADP as described above, and organic phosphate liberated was determined by the method of Lin and Morales (16).

Indirect Immunofluorescence—Adult worms were fixed in a solution containing freshly prepared 3.7% formadehyde, 0.1% glutaraldehyde, 0.1 M sodium cacodylate buffer, pH 7.2, and 2 mM calcium chloride for 1 h at 4 °C. Worms were then washed with 1% phosphate-buffered saline-albumin (PBS-A), incubated in 50 mM NH_4Cl for 30 min to block free aldehyde groups, washed with PBS-A, and incubated with a 1:100 dilution of antibody against the denatured conformation of purified apyrase for 2 h. Adult worms were washed with PBS-A and incubated with a 1:50 dilution of tetramethylrhodamine isothiocyanate (TRITC)-labeled goat anti-rabbit IgG (Sigma, T5268) for 2 h, washed again with PBS-A, and mounted with *n*-propyl gallate. Control was done by incubating adult worms with TRITC-labeled goat anti-rabbit IgG for 2 h, washing with PBS-A, and mounting with *n*-propyl gallate. Observations were done in an inverted confocal laser scanning microscope (LSM 410-Zeiss).

Protein Microsequencing—Potato apyrase eluted as a single peak of active enzyme (950 units/ml of ADPase activity) in the last step of Cibacron Blue affinity chromatography (5). Protein from the active peak was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by electroblotting onto PVDF membranes (Millipore). The membrane was stained with Ponceau S, and a single band of 49 kDa was observed. The center of the stained band was cut and submitted to sequencing at Yale University W. M. Keck Foundation Biotechnology Resource Laboratory. Quantitation of blotted protein was done by amino acid analysis using a Beckman 6300 analyzer following a 16-h acid hydrolysis at 115 °C of a sample corresponding to 10% of the membrane. Sequencing was performed on an Applied Biosystems Model 470A instrument. Amino-terminal sequence was done on 66 pmol of apyrase on PVDF with sequencing initial yield of 38%. A sample of 400 pmol of apyrase on PVDF was digested with trypsin, the resulting peptides were separated by reverse phase HPLC, and 3-μl samples from several peptide peaks were subjected to laser desorption mass spectrometry on a VG/FISONS TofSpec spectrometer. Peaks were selected for their suitability for microsequencing based on sample homogeneity as revealed by the presence of a single species in mass spectra.

RESULTS

Partial Purification of ATP Diphosphohydrolase by Nondenaturing Gel Electrophoresis—It was previously shown that *S. mansoni* tegument could be submitted to nondenaturing gel electrophoresis in the presence of 0.2% nonionic detergent Triton X-100 plus 0.4% sodium deoxycholate (1) with preservation of ATP diphosphohydrolase activity. Solubilized enzyme was able to catalyze hydrolysis of either ATP or ADP, as observed by formation of a white calcium phosphate precipitate at the active band upon incubation of the gel slab with substrate (1). Under those detergent concentrations, phosphohydrolytic activity gave rise to the appearance of a single calcium phosphate precipitate band displaying identical electrophoretic mobilities when either ATP or ADP was used (1). Excision of active bands from nondenaturing 0.2% Triton X-100 plus 0.4% sodium de-

[1] The abbreviations used are: MOPS, 3-(*N*-morpholino)propanesulfonic acid; TRITC, tetramethylrhodamine isothiocyanate; PVDF, polyvinylidene difluoride; HPLC, high performance liquid chromatography; NTPase, nucleoside triphosphatase or nucleoside triphosphate hydrolase.

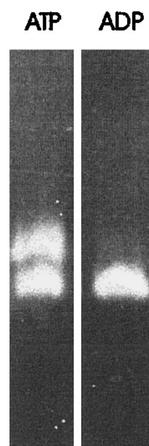

FIG. 1. **Two isoforms of active ATP diphosphohydrolase are separated by nondenaturing gels.** *S. mansoni* tegument was solubilized in nonionic detergent Triton X-100 plus deoxycholate and separated by polyacrylamide gel electrophoresis in buffer containing the same detergents. Two lanes run in parallel were immersed in standard medium for activity measurements (see "Experimental Procedures") containing either ATP (*left*) or ADP (*right*) as substrate. After 30–40 min of incubation at 37 °C, white deposits of calcium phosphate appeared as a result of nucleotide hydrolysis catalyzed by the enzyme. Gels were photographed against a dark background.

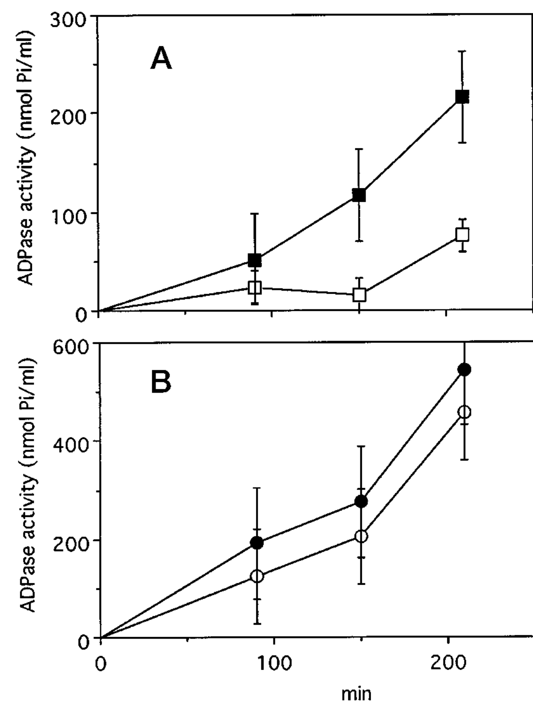

FIG. 2. **ADPase activity of both isoforms of ATP diphosphohydrolase in the absence or presence of $C_{12}E_8$.** A nondenaturing gel similar to that shown in Fig. 1 was run, and one lane was developed with ATP. Both the upper (□, ■) and lower (○, ●) bands were cut out from adjacent lanes which were not developed. Each gel piece was incubated for the indicated times in standard assay medium with 3 mM ADP in the absence (○, ■) or the presence (●, ■) of 1 mg/ml nonionic detergent $C_{12}E_8$. Inorganic phosphate produced was determined colorimetrically.

oxycholate gels followed by separation of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that a number of distinct bands ranging from 65 to 30 kDa were present (not shown). We have therefore tested different concentrations of detergents in the nondenaturing gel as an attempt to further separate active ATP diphosphohydrolase from other contaminants. Fig. 1 shows a nondenaturing polyacrylamide gel electrophoretic pattern of tegumental membranes solubilized with 0.1% Triton X-100 plus 0.1% sodium deoxycholate. Gels were developed with either ATP (Fig. 1, *left lane*) or ADP (Fig. 1, *right lane*) as substrate. When ATP was used, two distinct calcium phosphate precipitate bands were formed corresponding to two enzymatic forms with different electrophoretic mobilities (Fig. 1, *left lane*). A single band matching one of the bands developed with ATP was obtained when ADP was the substrate (Fig. 1, *right lane*). The results suggest that two isoforms of ATP diphosphohydrolase with different isoelectric points exist in *S. mansoni* tegument.

While the mixture of Triton X-100 and deoxycholate at the concentrations described in Fig. 1 gave the best resolution of isoforms and the sharpest bands among a number of nonionic detergent combinations tested, we found that removal of detergents by washing as described under "Experimental Procedures" was essential for developing ATPase and ADPase activities on the gels. In addition, high calcium (10 mM) is required in this assay for visualization of precipitates. Lack of calcium phosphate precipitate in the upper band of Fig. 1 in the presence of ADP could eventually be due to a greater sensitivity of one of the isoforms to incomplete removal of detergents and/or to inhibition due to high calcium. The two separate isoforms were then assayed in test tubes by cutting out the upper or lower band from additional nondenaturing gel lanes run in parallel to those shown in Fig. 1 and exposing the gel slices to standard reaction medium and substrate as described under "Experimental Procedures." Fig. 2*A* (*open squares*) shows that for the upper band ADPase activity was 0.31 nmol of P_i ml^{-1} min^{-1} when assayed in the standard medium in the absence of detergent. Nonionic detergent $C_{12}E_8$ is known to preserve sarcoplasmic reticulum Ca^{2+}-ATPase activity (17) and was found to solubilize and preserve the activity of schistosomal ATP diphosphohydrolase. When $C_{12}E_8$ was added, the ADPase activity of the upper band increased to 1.0 nmol of P_i ml^{-1} min^{-1} (Fig. 2*A*, *closed squares*). ADPase activity of the lower band (Fig. 2*B*) was 2.0 to 2.4 nmol of P_i ml^{-1} min^{-1} and was relatively insensitive to the presence of $C_{12}E_8$. The two isoforms have ADPase activity (Fig. 2, *A* and *B*) and lack of optimal conditions is probably the reason for absence of calcium phosphate precipitate in the upper band when the nondenaturing gel was developed *in situ* with ADP (Fig. 1). ATPase activity assayed in the test tubes in the presence of $C_{12}E_8$ showed that the upper band isoform had an activity of 0.39 nmol of P_i ml^{-1} min^{-1} and the lower band 0.87 nmol of P_i ml^{-1} min^{-1}.

Extraction of either the upper or lower activity bands from nondenaturing gels followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie Blue staining revealed in both lanes two major bands of 55 and 63 kDa (Fig. 3*A*, *lanes U* and *L*). Comparison with a total tegumental extract (Fig. 3*A*, *lane T*) showed that considerable purification was attained by separation of tegumental proteins by nondenaturing electrophoresis and excision of the active band (compare Fig. 3*A*, *lane T* with *lanes U–L*).

In order to identify the ATP diphosphohydrolase, three different polyclonal antibodies raised against native or denatured potato apyrase of different origins were used to develop a Western blot (Fig. 3, *B–D*). The three anti-potato apyrase antibodies reacted with a band of 63 kDa (Fig. 3, *B–D*, *lanes U* and *L*) in samples excised from the upper and lower active bands of nondenaturing gels. The antibody against apyrase from Sigma further recognized two diffuse bands of higher molecular masses (132 and >175 kDa) which could be glycosylated forms. In total *S. mansoni* tegument, anti-potato apyrase antibody recognized a single band of 63 kDa (Fig. 3*B*, *lane T*). The 63-kDa protein is not a major tegumental protein as seen by Coomassie Blue staining (Fig. 3*A*, *lane T*).

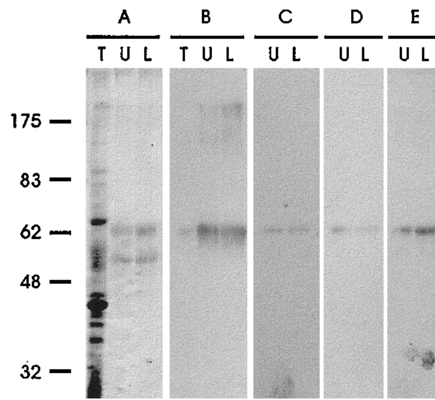

FIG. 3. **Separation by SDS-PAGE of proteins extracted from active bands of nondenaturing gels.** Nondenaturing gels similar to that shown in Fig. 1 were run and developed with ATP. Both the upper (*U*) and lower (*L*) bands were extracted and samples were separated by SDS-PAGE. On *A*, the gel was stained with Coomassie Blue and, on *B* to *E*, proteins were electroblotted onto nitrocellulose and developed with anti-apyrase antibodies against Sigma apyrase (*B*), against native (*C*) or denatured (*D*) apyrase highly purified from potato variety Desirée, or antibody against *T. gondii* NTPase (*E*). *Lanes T* show a control of 80 μg of total *S. mansoni* tegument.

S. mansoni ATP Diphosphohydrolase Was Recognized by Anti-T. gondii NTPase Antibody—Western blots were developed with a polyclonal antibody anti-fusion protein obtained from translation of *T. gondii* nucleoside triphosphate hydrolase cDNA fused to glutathione *S*-transferase (7). Fig. 3*E* shows that anti-toxoplasma NTPase antibody reacted with the same 63-kDa band in schistosomal samples excised from upper (Fig. 3, *lane U*) and lower (Fig. 3, *lane L*) active bands of nondenaturing gels similar to that of Fig. 1.

Anti-apyrase Antibody Immunoprecipitated ATPase and ADPase Activities—Anti-potato-apyrase antibodies were tested for their ability to immunoprecipitate ATP diphosphohydrolase from detergent-solubilized *S. mansoni* tegument. Table I shows that antibody against Sigma apyrase (Experiment 1) immobilized on Sepharose-Protein A completely removed ATPase and ADPase activities from the solution. Antibody against the single isoform of native potato apyrase cv Desirée (Experiment 2) was able to deplete 90% of the ADPase activity while the ATPase activity remaining in solution was not affected.

ATP Diphosphohydrolase Was Detected on the Tegumental Surface by Apyrase Antibody—In order to localize ATP diphosphohydrolase in *S. mansoni* tegument, we observed adult worms by indirect immunofluorescence using confocal laser scanning microscopy, after initial incubation with anti-potato-apyrase antibody followed by TRITC-labeled goat anti-rabbit IgG. As shown in Fig. 4 (*D*, *arrows*), a very strong labeling was seen on the external surface of *S. mansoni* tegument. No labeling was observed when worms were incubated with TRITC-labeled antibody alone (Fig. 4*B*).

Partial Primary Sequence of Potato Apyrase Revealed a Possible New Family of Nucleotidases—There is little information in the literature about primary structure and possible conserved sequences among nucleoside triphosphate hydrolases, ecto-ATPases, or apyrases. In view of the immunological cross-reactivities between *S. mansoni* ATP diphosphohydrolase and potato apyrase (Fig. 3, *B–D*) and due to the relatively large amounts available of highly purified potato apyrase, when compared to small amounts of *S. mansoni* ATP diphosphohydrolase obtainable by preparative electrophoretic purification, we have opted to obtain the primary sequence of potato apyrase. Purified potato apyrase was electroblotted onto PVDF membranes and submitted to microsequencing. Three novel peptide sequences were obtained. The amino-terminal sequence obtained was **ESEHYAVIFDAGS**. Apyrase blotted onto PVDF membrane (400 pmol) was submitted to *in situ* tryptic digestion followed by reverse phase HPLC separation of the resulting peptides. Two of the eluted peaks were selected for their suitability for direct microsequencing based on laser desorption mass spectrometry, which showed that each sample contained a single peptide with masses 3044.3 and 2149.5 Da, respectively. Complete sequencing of the first tryptic digest peptide showed a 28-amino acid peptide with a predicted mass of 3041.5 Da (0.09% deviation from the observed spectrometric mass) with the sequence **LGLLPIGNNIEYFMATEPG-LSSYAEDPK**. Complete sequencing of the second peptide gave an 18-amino acid peptide with a predicted mass of 2147.4 (0.1% deviation from the observed) with the sequence **DYNLYVHSYLNYGQLAGR**.

TABLE I
Depletion of detergent-solubilized ATP diphosphohydrolase activity from the medium by anti-potato apyrase antibodies immobilized on Sepharose-Protein A

In Experiment 1, antibodies against the two isoforms of native potato apyrase from Sigma were raised in a rabbit. Preimmune and immune sera were used at a dilution of 1:200. In Experiment 2, antibodies against the single isoform of native potato apyrase cv Desirée purified by the method of Kettlun *et al.* (6) were raised in mice. Preimmune and immune sera were used at a dilution of 1:50. The experiments shown are typical results; the experiments were repeated three times with similar results.

		Activity	
		Preimmune	Immune
		nmolP$_i$mg^{-1}min^{-1}	
Experiment 1	ATPase	64	0
	ADPase	64	0
Experiment 2	ATPase	117	130
	ADPase	198	20

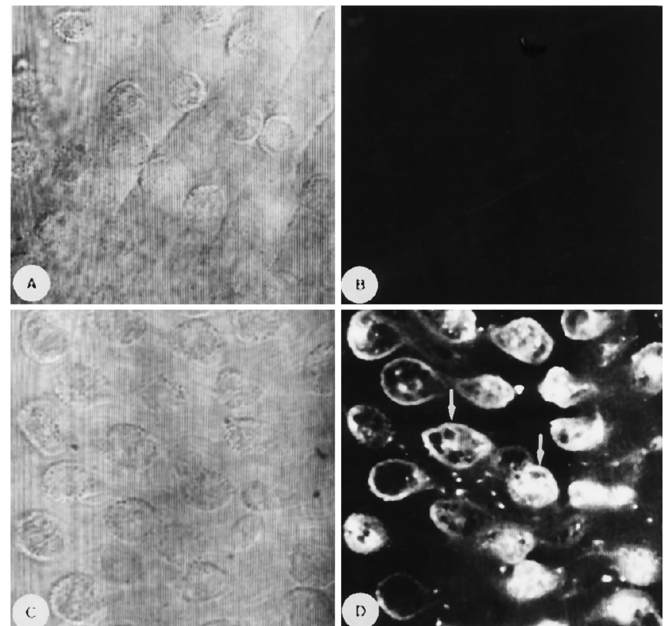

FIG. 4. **Immunolocalization of ATP diphosphohydrolase by indirect immunofluorescence using confocal laser scanning microscopy.** *S. mansoni* adult intact worms were fixed and incubated either in the absence (*A* and *B*) or the presence (*C* and *D*) of anti-potato apyrase antibody followed by rhodamine-labeled secondary antibody (*A-D*). The same microscopic field was observed by interferential contrast (*A* and *C*) or by immunofluorescence (*B* and *D*) microscopy. No fluorescence was observed in control preparation (*B*). Intense fluorescence was observed on the external surface (*arrows*) of *S. mansoni* tegument (*D*). *A* and *B*, × 241; *C* and *D*, × 337.

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                    1                                                                                                   90
      yeast GDPase  mapifrnyrf aigafavImL iLLiktssig pPsiartvtp nasIpktPeD iSIlpvndep gylqdskTeq nypeladAVk sqtsqtcsEe
        pea NTPase  .......... melliklItF lLFsmpaits sqylgnnLLt srkIFlkqe. .......... .......... .......... ........Ei
      mouse lymph   .......... .......... .......... .......... ....MEDikD SkVkRFCSkN IliIlgFTsI laVIALiAVG lTQnkplPEN
      human lymph   .......... .......... .......... .......... ....MEDtkE SnVktFCSkN IlAIlgFssI iaVIALlAVG lTQnkAlPEN
  C. elegans R07E4  .......... .......... .......... .......... .........m rvslRFtila VsAmifFpvI vfIyvveAht spkviAddqe
  C. elegans C33H5  .......... .......... .......... .......... .......... .......... msggsserv. .......qrs vrsvvetknN
yeast GDA1 homolog  .......... .......... .......... .......... .......... .......... .......... .......... ...mlientN
 Toxoplasma NTPase  .......... .......... ........mw lPvhvplLLv fgvsLslPhg SlgtdssSlr gvdadtekrI nvgkthlqtl rnletrchDs
    potato apyrase  .......... .......... .......... .......... .......... .......... .......... .......... ........Es
         Consensus  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
                    91                                                                                                  180
      yeast GDPase  hKYvImiDAG StGsRvhIYK Fd........ vctSPptLLd .....ekFD. ...mlEPGLS SFdtd..... .......... ..svgAAnS.
        pea NTPase  ssYaVVFDAG StGsRihVYh Fn........ ...qnlDLLh IgkgVeyYn. ...KitPGLS SYAhn..... .......... ..PEqAAkS.
      mouse lymph   VKYGIVLDAG SShTnLYIYK W......... ....PaEkeN dtgVVqQLEE CqVK.gPGiS kYAqK..... .......... ..tDei.gaY
      human lymph   VKYGIVLDAG SShTsLYIYK W......... ....PaEkeN dtgVVHQvEE CRVK.gPGiS kFvqK..... .......... ..vnei.giY
  C. elegans R07E4  rsYGVIcDAG StGTRLFVYn WisT...... ...SdsELiq IepVIydnkp vmkKisPGLS tFgtK..... .......... ..PaqA.aeY
  C. elegans C33H5  IKYGVIcDAG SSGTRLFVYt Lkpl...... ...Sgg.LtN IdtlIHesEp vvkKvtPGLS SFgdK..... .......... ..PEqv.veY
yeast GDA1 homolog  drFGIViDAG SSGsRihVFK WqdTeSlLha tnqdsqsiLq svphIHQekD WtfKlnPGLS SFekK..... .......... ..PqdAykSh
 Toxoplasma NTPase  lqalVViDAG SSsTRtnVF. LakTrScpnr grsidpDsir lirdgkrFtg lRVvlEewLd tYAgKdwesr pvdarilfqy vpqmhegakk
    potato apyrase  ehYaVIFDAG S......... .......... .........l gllpIgnniE YfmatPGLS SYAedpk... .......... ..........
         Consensus  -------DAG S--------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
                    181                                                                                                 270
      yeast GDPase  LdPLLkvAmn yVPikaRsc. .........T PVavkATAGL RLLgD..... AksskILsav RdhLekdypF pVVEg.DG.. .VsImgGdEE
        pea NTPase  LiPLLEqAED VVPDdlqpk. .........T PVrlGATAGL RLLng..... dasEkILqsv RdmLsNrstF n.Vqp.Da.. .VsIIDGtqE
      mouse lymph   LaecMELstE lIPtSkhhq. .........T PVYlGATAGM RLLrmEsEqs Ade..VLaav stsLKsypf. ....DFqG.. .akIItGqEE
      human lymph   LTdcMErArE VIPrSQhqE. .........T PVYlGATAGM RLLrmEsEel Adr..VLdvv ersLsNypf. ....DFqG.. .aRIItGqEE
  C. elegans R07E4  LrPLMELAEr hIPEekRpy. .........T PVFifATAGM RLipDEyvli gqkEAVLknl RnkLpkitsM qVlk..Eh.. .IRIIEGkwE
  C. elegans C33H5  LTPLLrFAEE hIPyeQlgE. .........T dlLifATAGM RLLPE..... AqkDAIiknl qngLKsvtaL rVsD..sn.. .IRIIDGawE
yeast GDA1 homolog  ikPLLDFAkn IIPEShWss. .........c PVFidATAGM RLLPqDi... ..qssILdgl cqgLKhpaeF lVeDcsaq.. .IqVIDGetE
 Toxoplasma NTPase  LmqLLEedtv aIlDSQlnEe qkvqvkalgi PVmlqsTAGv RdFhEwy... ..rDAlFvll RhlinNpspa hgykfFtnpf wtRpItGaEE
    potato apyrase  .......... .......... .......... .......... .......... .......... .......... .......... ..........
         Consensus  ---------- ---------- ---------- -----TAG- R--------- ---------- ---------- ---------- ------G--E
                    271                                                                                                 360
      yeast GDPase  GVFaWITtNY LLGniGan.. .......... gpKlpTaaVf DLGGgSTQIv F...Ep.tfP iNekmvdGEh kFdLk..fgD En....YtlY
        pea NTPase  GsYlWVTVNY aLGnLGKk.. .......... ytK..TvGVi DLGGgSvQmA YAvskk.tak napkVadGDd pYikkvvlKg ip....YLlY
      mouse lymph   GaYGWITINY LLGRFtqeqs W..LSliS.D sqKQETfGal DLGGASTQIt F.......VP qNstIeSpEN sLqFrLygeD ......YtVY
      human lymph   GaYGWITINY LLGkFsqktr W..FSivpyE tnnQETfGal DLGGASTQVt F.......VP qNqtIeSpDN aLqFrLygKD ......YnVY
  C. elegans R07E4  GIYsWIaVNY aLGkFnKtat l.dFpgtSPa HarQkTvGmi DMGGASaQIA F......ElP .dtDsfSsiN veniNLgcrE DdsLFKYklF
  C. elegans C33H5  GIYsWIaVNY iLGRFdK... .......... .endskvGmi DMGGASvQIA F......EIa nekEsynGgN vYeiNLgsiE tnEdYKYkIY
yeast GDA1 homolog  GlYGWlglNY LyGhFn.... .....DynPE vsdhfTfGfm DMGGASTQIA FAphDsgEIa rhrDdiatif lrsvN..... .gDLqKWDVF
 Toxoplasma NTPase  GlFaFITlNh LsrRLGedpa RcmidEygvk HcrnDlaGVv EvGGASaQIv FplqEgtvlP ......Ssvr avnLqrerll perypsaDVv
    potato apyrase  .......... .......... .......... .......... .......... .......... .......... .......... .....dYrlY
         Consensus  G-------N- ---------- ---------- ---------- --GG-S-Q-- ---------- ---------- ---------- ----------
                    361                                                                                                 450
      yeast GDPase  qfShLGYGlk eGRnKvnsvL Venalkdgki lkgDntkThq LssPcLppkv naTnekvtLE skEtYtidFi gpd....... ..........
        pea NTPase  VHSYInFGre asR....... .......aei lk....LTPr spnPcL...L aGfngiytYs gEE.....FK ata....... ..........
      mouse lymph   tHSFLcYGkd qalW...QKL akdiQvsSGG VlkDPCFnPG YeKvvnvSEL yGTPcTKrFE ....kkLPFd QFr....... ..........
      human lymph   tHSFLcYGkd qalW...QKL akdiQvaSne IlrDPCFhPG YkKvvnvSDL ykTPcTKrFE ....mtLPFq QFe....... ..........
  C. elegans R07E4  VttFLGYGvn eGiRKYehmL lsklkdqnGt ViqDdCMpln LhKtvtleng en........ .......... .Fv....... ..........
  C. elegans C33H5  sttFLGYGan eGlkKYensL V......ksG nsNDsCsprG LNrli..... ge........ .......... .Ft....... ..........
yeast GDA1 homolog  VstWLGFGan qaRRrYlaqL Intlpentnd yeNDdfsTrn LNdPcMprgs st.......D ....FeFkdt iFh....... ..........
 Toxoplasma NTPase  svSFMqLGma ssaglFlkeL csndeflqGG IcsnPCLfkG FqqscsagEv evrPdgsasv nEDvrknrLK pLatycsvhn peisfkvtne
    potato apyrase  VHSYLnYGql aGR....... .......... .......... .......... .......... .......... .......... ..........
         Consensus  -------G-- ---------L ---------- ---------- ---------- ---------- ---------- ---------- ----------
                    451                                                                                                 540
      yeast GDPase  .......... .......... .....epsga ...QCrfltd EiLNKdaqcq .....sppCs FNGVhqPSLv rtfkesnDiY iFSyFYdrTr
        pea NTPase  .......... .......... .....ytsga NFnkCkntIr kaLklnypcp .....YqnCt FgGIWngg.. .ggngqknLF asSsFFYlpE
      mouse lymph   .......... .......... .....IQGTG dYEQCHqSIl ELFNnshcP. .....YsqCa FNGVFlPpLh ......gsFg aFSaFYFvmD
      human lymph   .......... .......... .....IQGiG NYqQCHqSIl ELFNtsycP. .....YsqCa FNGIFlPpLq ......gDFg aFSaFYFvmk
  C. elegans R07E4  .......... .......... .....rrGTG NWntCsneVk kLLNpesssE vckaEaakCY FgaVpAPSip Ls...NiEMY gFSEYWYsTh
  C. elegans C33H5  .......... .......... .....VnGTG eWDvClaqVs sLigdkaqPs cpN...ptCF LrnViAPSvn Ls...tvqLY gFSEYWYtTs
yeast GDA1 homolog  .......... .......... .....IaGsG NYEQCtkSIy pLLlKnmpcD .....depCL FNGVhAPrid Fa...NdkFi gtSEYWYtan
 Toxoplasma NTPase  mqcrensidp tkplaermki encsiIeGTG NFDkCvsqVe siLvapklPl paNiEaassg FesV.dqvFr FasstapmFi tgrEmLasiD
    potato apyrase  .......... .......... .......... .......... .......... .......... .......... .......... ..........
         Consensus  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
                    541                                                                                                 630
      yeast GDPase  pLg...MplS ftlnELndLa rIvcK.geet WnsVFsGiag s...ldelEs dShFCLdlsF qvSLLHtGY. dip..lqrel rtGkki....
        pea NTPase  dtg...Mvda stpnfilrpv DIetK.akea calnFEdaKs Typfldkknv aSyvCMdliY qyvLLvDGF. gLD..plQki tsGkeieYQd
      mouse lymph   FFk...kvak nsvisqEKMt EItknFCSKs WeEt.....K TsYPsVkEkY LSEYCFsgAY ILS.LlqGY. nFtd...... ..ssWeqihf
      human lymph   FLn...Ltse k..vsqEKvt EmmkKFCaqp WeEI.....K TsYagVkEkY LSEYCFsgtY ILSLLlqGY. hFta...... ..dsWehihf
  C. elegans R07E4  ..d...vLGl GGqYDaEnia kktqqYCSKr WstIqaeSKK qlYPradEEr LrtqCFksAW ItSvLHDGF. svDk...... ...thnkFQS
  C. elegans C33H5  ..n....FGS GGEYhYqKFt DevRKYCqKD WnDIqDGfKr neFPnadiEr LgtnCFkaAW VtSvLHDGF. nvDk...... ...tkhLFQS
yeast GDA1 homolog  ..d...vFkl GGEYnFDKFs kslReFCnsn WtqILanSdK gvYnsIpEnF LkDaCFkgnW VLniLHEGF. dMpriDvdae nVndRpLFQS
 Toxoplasma NTPase  tLkdhrLLrS dfsgDvEeLa EaaReFCSsE viirtDGpvi qlpnargEqk LnsLnFdlck tMaLtvslLr hMaagEnQps fIkweksiag
    potato apyrase  .......... .......... .......... .......... .......... .......... .......... .......... ..........
         Consensus  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
                    631                                                                                                 720
      yeast GDPase  ....AnkEiG WcLGAsLpLl KadnWkckiq sa........ .......... .......... .......... .......... ..........
        pea NTPase  aiv....EAa WpLGhaveai salPkferlm YfV....... .......... .......... .......... .......... ..........
      mouse lymph   mgKIkdsnAG WtLGyMLnLt nmiPaeqpLs pplpHSTYIg LMVLFS.... .......... .......... ...LLLVAVA ItglFIYsKP
      human lymph   IgKIqGsDAG WtLGyMLnLt nmiPaeqpLs tplsHSTYVf LMVLFS.... .......... .......... ...LvLftVA IiglLIFHKP
  C. elegans R07E4  VstIAGqEvq WaLGAMiyhm rFfPLrdssr nlIvkeT... .......... .......... ..hSSSeSLw apLFFLsAVf clfvLVCaKe
  C. elegans C33H5  VlKIAGeEmq WaLGAMLyhs KdlkFnlleq LeVaqST... .......... .......... ..QqiSnfFs ...FFvIlIi VlavalYrql
yeast GDA1 homolog  VeKVeenEls WtLGriLlya sgsiLagndd FmVGiapser rtkLtgkkfi pgkllesdql rkQSSSlSnk gfLMWFaiIc cifyLIFHrs
 Toxoplasma NTPase  pdgkplaDlG WqvGviLhhv lFteewgrta YeaGyShnl. .......... .......... .......... .......... ..........
    potato apyrase  .......... .......... .......... .......... .......... .......... .......... .......... ..........
         Consensus  ---------- W--G------ ---------- ---------- ---------- ---------- ---------- ---------- ----------
                    721                                                                                                 810
      yeast GDPase  .......... .......... .......... .......... .......... .......... .......... .......... ..........
        pea NTPase  .......... .......... .......... .......... .......... .......... .......... .......... ..........
      mouse lymph   syfWKEaV.. .......... .......... .......... .......... .......... .......... .......... ..........
      human lymph   syfWKDmV.. .......... .......... .......... .......... .......... .......... .......... ..........
  C. elegans R07E4  qsv....... ....lcFddk rRssFgmsRs qysYkmLkEn rtsss...FL EnFa...... .......... .......... ..........
  C. elegans C33H5  qse....... ....stYKk. .......... ...YnfLrtd .skpD...FL nV........ .......... .......... ..........
yeast GDA1 homolog  hiiRrrfsgl ynitkdFKtg iRrrLkflRr sdpFsrLeEg elgtDvdgFk DVYrmksssm fdlgkssatm qrehepqrta sqsanlapsn
 Toxoplasma NTPase  .......... .......... .......... .......... .......... .......... .......... .......... ..........
    potato apyrase  .......... .......... .......... .......... .......... .......... .......... .......... ..........
         Consensus  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
                    811       830
       yeast GDPas  .......... ..........
        pea NTPase  .......... ..........
      mouse lymph   .......... ..........
      human lymph   .......... ..........
  C. elegans R07E4  .......... ..........
  C. elegans C33H5  .......... ..........
yeast GDA1 homolog  lrpafsmadf skfkdsrlyd
 Toxoplasma NTPase  .......... ..........
    potato apyrase  .......... ..........
         Consensus  ---------- ----------
```

FIG. 5. **Alignment of data bank sequences presenting homology with the three potato apyrase peptides obtained by microsequencing.** Alignment was obtained with the PILEUP tool of the Wisconsin package. *Dashed boxes* indicate regions showing four to six conserved amino acids. *Solid boxes* show sequences which are identical in potato and pea apyrases. *potato apyrase* corresponds to the three peptide sequences obtained in the present work (accession number P80595). Other sequences and their accession numbers are: *yeast GDPase*, *Saccharomyces cerevisiae GDA1* guanosine diphosphatase (Ref. 9), P32621; *pea NTPase*, *P. sativum* (garden pea) nucleoside triphosphatase (unpublished), S48859; *mouse lymph*, mouse lymphoid cell activation antigen CD39 (Ref. 10), gi765257; *human lymph*, human lymphoid cell activation antigen CD39 (Ref. 10), S73813; *C. elegans R07E4*, *C. elegans* cosmid R07E4 (unpublished), sequence ID gi1049394; *C. elegans C33H5*, *C. elegans* cosmid C33H5 (unpublished), sequence ID gi1086594; *yeast GDA1 homolog*, hypothetical 71.9-kDa protein in PMI40-PAC2 intergenic region of *S. cerevisiae* chromosome V (unpublished), P40009; *Toxoplasma NTPase*, *T. gondii* nucleoside triphosphatase (Refs. 7 and 8), L39077.

Comparison of the above sequences against the National Center for Biotechnology Information (NCBI) protein data bank using the BLAST routine (18) showed that all three sequences obtained were novel and had significant homology with garden pea nucleoside triphosphatase, an unpublished sequence recently deposited (accession number S48859). Searching the data bank for neighbors with the ENTREZ tool of NCBI revealed seven additional sequences with considerable similarity to garden pea nucleoside triphosphatase. Fig. 5 shows alignment of the three potato apyrase novel peptides with these data base sequences. Potato apyrase has a **VIFD-AGS** sequence near its amino-terminal end, and the alignment of Fig. 5 showed a conserved motif (I/V)(V/M/I)(I/L/F/C)D-AGS(S/T) among all nine proteins (Fig. 5, *first dashed box*). Two additional sequences with strong homology were observed between potato apyrase and garden pea NTPase (Fig. 5, *solid boxes*): sequence **PGLSSYA** in the second peptide and sequence **LYVHSYL** in the third peptide obtained in the present work were identical to the pea sequence (Fig. 5, *solid boxes*). We note that there is less conservation in these segments when all

nine proteins are considered. Potato apyrase and garden pea NTPase showed 59% identity and 76% similarity when the three potato peptide sequences were aligned to the complete pea NTPase sequence using the local homology algorithm (19) with the BESTFIT program of the Wisconsin package.

DISCUSSION

This work reports immunological cross-reactivity of *S. mansoni* tegumental ATP diphosphohydrolase with antibodies against potato apyrase and *T. gondii* nucleoside triphosphate hydrolase. Immunoblotting of *S. mansoni* tegument revealed ATP diphosphohydrolase as a single band of molecular weight 63,000 (Fig. 3, *B–E*). The most striking finding in the experiments of Figs. 1 to 3 was that under nondenaturing conditions, isoforms of active ATP diphosphohydrolase could be separated by their different electrophoretic mobilities as two species possessing different net charges (Fig. 1) yet showing identical molecular weights of 63,000 (Fig. 3, *B–E*). The species with lower net negative charge (lower mobility) hydrolyzed ATP and ADP in the presence of nonionic detergent $C_{12}E_8$ at half the hydrolytic rate of the species with higher net negative charge (higher mobility). ADPase activity of the species with lower net charge was sensitive to the presence of detergent (Fig. 2, *upper panel*). This suggests that the two isoforms of *S. mansoni* ATP diphosphohydrolase possess different catalytic efficiencies. Two isoforms of nucleoside triphosphate hydrolase are expressed in *T. gondii*, namely NTP1 and NTP3 (7). The isoforms have identical molecular weights of 63,000 (7) and isoelectric points calculated from their deduced complete amino acid sequences are pI = 5.93 for NTP1 and pI = 6.39 for NTP3. Potato apyrase from *S. tuberosum* variety Ultimus has two isoforms of molecular weights 48,000 and 46,500 (6) and ATPase/ADPase ratios of 1 and 10, respectively. Apyrase from the *S. tuberosum* variety Desirée which we have used for obtaining partial primary sequence has a single molecular weight of 49,000 and an ATPase/ADPase ratio of 1 (5). More information about primary structures of apyrase isoforms from different sources will be required in order to identify possible domains responsible for different catalytic efficiencies toward nucleoside di- and triphosphates.

It is noteworthy that different antibodies raised against potato apyrase were apparently able to distinguish between isoforms of *S. mansoni* ATP diphosphohydrolase. Thus, antibody raised against highly purified apyrase isolated in its native state from *S. tuberosum* clonal variety Desirée which has a low ATPase/ADPase ratio (5) was able to immunoprecipitate 90% of ADPase activity with no effect on ATPase activity (Table I, Experiment 2). We suggest that this antibody recognized an isoform with very low ATPase/ADPase ratio leaving in the soluble fraction an isoform with ATPase activity of 130 nmol of P_i mg^{-1} min^{-1} and ADPase activity of 20 nmol of P_i mg^{-1} min^{-1} (Table I, Experiment 2) which corresponds to an ATPase/ADPase ratio of 6. In addition, antibody raised against Sigma apyrase which has a mixture of two isoforms did immunoprecipitate all ATPase and ADPase activities from solubilized *S. mansoni* tegument (Table I, Experiment 1).

A new family of nucleoside triphosphatases and apyrases so far unnoticed seems to emerge from the alignment shown in Fig. 5 of the three novel potato apyrase peptides determined in the present work with eight sequences deposited in GenBank™. A well-defined motif near the amino-terminal appears among the nine aligned sequences, which is (I/V)(V/M/I)(I/L/F/C)DAGS(S/T) (Fig. 5, *first dashed box*). This sequence is similar to β-phosphate binding site motif (I/L/V)V(I/L/V/C)DXG(T/S/G)(T/S/G)XX(R/K/C) in the ATP binding domain of the actin-hsp70-hexokinase family (20). The other four conserved motifs of the actin-hsp70-hexokinase family are not present in the family of nucleoside triphosphatases. Three additional regions showing four to six conserved amino acids are indicated in Fig. 5 (*dashed boxes*). These sequences do not match any previously assigned motifs as compiled in the Genetics Computer Group Wisconsin package. Immunological cross-reactivities shown in the present work (Fig. 3, *B–E*) seem to indicate that *S. mansoni* tegumental ATP diphosphohydrolase belongs to the family of nucleotide-splitting enzymes shown in Fig. 5. Elucidation of the primary sequence of parasite enzyme will be required to positively identify this enzyme as a member of the family.

It is noteworthy that the sequences shown in Fig. 5 do not have homology to the recently reported mosquito salivary gland apyrase (21), an enzyme that was described as a member of the 5′-nucleotidase family.

T. gondii is an obligate intracellular protozoan that can invade and replicate in essentially all nucleated mammalian cells. This parasite produces a nucleoside triphosphate hydrolase (NTPase) which has apyrase activity toward all nucleoside and deoxynucleoside triphosphates, sequentially degrading these substrates to the monophosphate form (7, 8). In *T. gondii*, the enzyme is located in dense granules and secretory vesicles of the parasite and is secreted into the vacuolar space of infected cells (22). It has been postulated that *T. gondii* NTPase may participate in the pathway of salvage of purine nucleotides. The function of *S. mansoni* ATP diphosphohydrolase is not clear at present. *Schistosoma* adult worms may live for several years in the mesenteric vasculature of infected human host individuals (23) effectively evading cytotoxic responses of the host. Host responses to *Schistosoma* infection involve platelet aggregation and cytotoxicity (24, 25) and cytolytic T lymphocyte reactivity (26). For unknown reasons, cytolytic T lymphocytes recognize and bind to schistosomula but cause no lysis (26). Recently, ATP released on the external surface of cytotoxic T lymphocytes has been implicated in the mechanism of lysis of target cells (27). Ectolocalization of *S. mansoni* ATP diphosphohydrolase has already been shown by electron microscopy which identified electron-dense lead phosphate deposits on the outer surface of *S. mansoni* tegument upon hydrolysis of ATP or ADP and production of inorganic phosphate (1). The ectolocalization is confirmed by confocal immunofluorescence microscopy (Fig. 4). Thus, we hypothesize that ATPase and ADPase activities on the surface of *S. mansoni* might preclude activation of cytotoxic responses of the host involving external ATP or ADP and contribute to evasion of the parasite from host defense mechanisms (1, 3). Identification of possible specific inhibitors of *S. mansoni* ATP diphosphohydrolase is the present subject of study of our laboratory and may contribute to characterization of the role of the enzyme.

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