Triatoma infestans Apyrases Belong to the 5′-Nucleotidase Family*

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Apyrases are nucleoside triphosphate-diphosphohydrolases (EC 3.6.1.3) present in a variety of organisms. The apyrase activity found in the saliva of hematophagous insects is correlated with the prevention of ADP-induced platelet aggregation of the host during blood sucking. Purification of apyrase activity from the saliva of the triatome bug Triatoma infestans was achieved by affinity chromatography on oligo(dT)-cellulose and gel filtration chromatography. The isolated fraction included five N-glycosylated polypeptides of 88, 82, 79, 68 and 67 kDa apparent molecular masses. The isolated apyrase mixture completely inhibited aggregation of human blood platelets. Labeling with the ATP substrate analogue 5′-p-fluorosulfonylbenzoyladenosine showed that the five species have ATP-binding characteristic of functional apyrases. Furthermore, tandem mass spectrometry peptide sequencing showed that the five species share sequence similarities with the apyrase from Aedes aegypti and with 5′-nucleotidases from other species. The complete cDNA of the 79-kDa enzyme was cloned, and its sequence confirmed that it encodes for an apyrase belonging to the 5′-nucleotidase family. The gene multiplication leading to the unusual salivary apyrase diversity in T. infestans could represent an important mechanism amplifying the enzyme expression during the insect evolution to hematophagy, in addition to an escape from the host immune response, thus enhancing acquisition of a meal by this triatomine vector of Chagas’ disease.

Hematophagy in triatomines (Hemiptera: Reduviidae) is associated with the presence of biochemical compounds in the salivary glands that are essential for obtaining blood meals. Indeed, most blood-feeding arthropods have salivary components with vasodilatory, anti-clotting, and anti-platelet aggregation activities that are capable of inhibiting hemostatic reactions of the host (1–4).

Host platelet aggregation is considered to be an important hemostatic barrier against insect feeding, because it can stop the bleeding of small blood vessels regardless of other clotting factors (5, 6). Rhodnius prolixus, a triatome, neutralizes and overrides platelet aggregation induced by collagen, thrombin, thromboxane A2, and ADP (7–10). Similarly, collagen- and thrombin-induced platelet aggregation is inhibited by, respectively, pallidipin and triabin, both of which are present in the saliva of Triatoma pallidipennis (11, 12). However, the importance of ADP as a common mediator of platelet aggregation pathways is evidenced by the presence on the vascular endothelium surface of the CD39 apyrase, which limits platelet aggregation by hydrolyzing ADP, thus preventing thrombus formation (13). Thus, studying insect apyrases may lead to alternative strategies against the diseases they transmit, as well as new pharmaceutical tools for platelet aggregation-associated disorders.

Apyrase removes inorganic phosphate from ATP and ADP, and thus prevents platelet aggregation (6, 8, 14). Apyrase activity has been characterized in the saliva of Anopheles stephensi, Aedes albopictus, and Aedes aegypti as 65-, 61-, and 68-kDa protein, respectively (15–17). Ae. aegypti and Anopheles gambiae apyrases are members of the 5′-nucleotidase family (17, 18), whereas the gene coding for the 37.5-kDa apyrase of Cimex lectularius belongs to a novel protein family showing significant similarity to phlebotomine apyrases (19–22) and to human and rat apyrases (23, 24).

Here, we report the purification and characterization of five salivary apyrases from Triatoma infestans, a vector of Chagas’ disease, the protozoan Trypanosoma cruzi. The sequence of the gene encoding the 79-kDa apyrase confirmed that it belongs to the 5′-nucleotidase family apyrase.

EXPERIMENTAL PROCEDURES

Triatomines and Collection of Saliva—T. infestans were reared in an insectary maintained at 28 ± 2 °C, 70% ± 5 relative humidity, with photoperiods of 12 h. Pipette tips were placed over the triatomines mouthparts and approximately 0.2–1 μl of saliva was collected from each adult triatome. Except for phenotyping, all experiments were performed with pooled saliva obtained from insects at 20 days following a blood meal. The saliva was filtered through an 0.22-μm pore membrane and stored at −80 °C until use.

Apyrase Purification—Saliva (800 μg of protein) was applied to an oligo(dT)25 cellulose column (0.7 × 10 cm) pre-equilibrated with 25 mM Tris-Cl, pH 7.5. Bound proteins were eluted with isocratic steps of 10 mM of the same buffer containing 0.1, 0.5, and 1 mM NaCl, respectively, at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected on ice and tested for apyrase activity. The active fractions, which were eluted in 0.5 mM NaCl, were pooled and concentrated to 50 μl by ultrafiltration through a Centricon 30 (Amicon) at 4 °C. The resulting protein solution was loaded on a Superose 12 HR 10/30 gel filtration column (Amersham Biosciences), equilibrated with 25 mM Tris, 500 mM NaCl, pH 7.5, and eluted at a flow rate of 0.4 ml/min. The absorbency at 280
ammonium carbonate were fractionated and concentrated as described above, this time to 0.5 ml, were stored at –80 °C until use.

Deglycosylation—Proteins were deglycosylated with O-glycosidase (0.1 unit) and neuraminidase (0.5 unit) as described by the glycosidase procedures (Bio-Rad). For PNGase F (0.1 unit) treatment, 2 μg of purified saliva were boiled in 1% SDS and 2% 2-mercaptoethanol and diluted 10-fold in 100 mM phosphate containing 0.5% Nonidet P-40 prior to incubation with the enzyme for 12 h at 37 °C. The mix was then subjected to 8% SDS-PAGE analysis.

FSBA Labeling—Purified apyrases were incubated at 37 °C for 30 min with 1 mM FSBA (Sigma) solubilized in Me2SO. To assess labeling specificity, ATP (5 mM) was introduced in the reaction mix before the addition of FSBA. A negative control consisted of deglycosylated and purified apyrases solubilized in Me2SO only (10% final concentration). Then, proteins were submitted to 6% SDS-PAGE under reducing conditions, and immunoblot was performed with serum raised against FSBA in rabbit (a kind gift of Dr. A. Beaudoin). Immunoreactivity was detected by chemiluminescence following the manufacturer’s instructions (Amersham Biosciences).

Platelet Aggregation Assay—From a fasting human donor who had not been exposed to any platelet aggregation-interfering drugs within the preceding 10 days, 5 milliliters of human blood was collected in sodium citrate 0.38%, and 2 ml of the platelet-rich plasma (PRP) was obtained by differential centrifugation (25). The PRP (400 μl) was preincubated under stirring for 10 min at 37 °C, and the aggregation was induced by the addition of ADP (5 μM) to achieve complete platelet aggregation. The effects of pH on the apyrase activity were determined at 37 °C using 50 mM buffering solutions of bis-Tris, pH 7, Tris-HCl, pH 7–8, and borate, pH 9–10. The effect of divalent cations on enzyme activity was determined by substituting the MgCl2 present in the reaction mix with CucI, CuCl2, ZnCl2, MnCl2, or CoSO4 at a final concentration of 1 mM. The effects of inhibitors on the apyrase activity were determined by preincubating purified apyrase at 37 °C for 10 min with putrescine (1 mM), levamisole (1 mM), mycrocistin-LR (0.01 mM), okadaic acid (0.01 mM), sodium azide (20 mM), NaF (1 and 10 mM), vanadate (0.1 an 1 mM), 2-mercaptoethanol (5 mM), iodoacetamide (5 mM), or diadenosine-5’-pentaphosphate (0.01 mM).

Mass Spectrometry—Following electrophoresis and Coomassie Blue staining, the 88-, 82-, and 79-kDa bands and the 68/67-kDa doublet were excised from the gel, washed, and analyzed in the manner described previously (28). In brief, the gel pieces were washed in 25 mM ammonium hydroxycarbonate (NH4HCO3), pH 8, then in 50% acetonitrile, 25 mM NH4HCO3, and finally with pure water, for 30 min each before centrifuging purified apyrase at 37 °C for 10 min with 30% acetonitrile, 25 mM NH4HCO3, and 10% acetonitrile. The resulting pellets were resuspended with a minimum amount of sequence grade modified porcine trypsin (Promega, Madison, WI) solution containing 0.5 μg of protease (typically 10 μl of an 0.05 μg portion of trypsin/μl solution in 25 mM NH4HCO3 containing 10% acetonitrile). When necessary, NH4HCO3 buffer was added until the gel piece achieved complete resuspension. Digestion occurred at 37 °C for 3–5 h. The tryptic digest was extracted twice with 50% acetonitrile, 25 mM NH4HCO3 solution. The digest solution and the extracts were pooled, dried in a vacuum centrifuge, and desalted with ZipTip C18 (Millipore, Bedford, MA) prior to a nanospray Q-TOF instrument (Micromass, Manchester, UK) was used with a Z-Spray ion source working in the nanospray mode. About 3–5 μl of the desalted sample was introduced into a needle (medium sample needle, Protana Inc., Odense, Denmark) to run MS and MS/MS experiments. The capillary voltage was set to 1000 V and the sample cone to 50 V. Glufibrinopeptide was used to calibrate the instrument in the MS/MS mode. MS/MS spectra were transformed using MaxEnt3 (Masslynx, Micromass Ltd.), and amino acid sequences were analyzed using PepSeq (BioLynx, Micromass Ltd.). Amino acid sequences were used in homology searches with BLAST or FASTA (www.ncbi.nlm.nih.gov/blast/).

PCR Amplification of apy79 cDNA—cDNA inserts of a salivary gland cDNA library constructed in λ ZAP bacteriophage (Stratagene) were PCR-amplified using the M13 reverse and T7 vector primers flanking the inserts as follows. After characterization of the MgCl2 present in SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM MgSO4, 0.01% gelatin) by EDTA used for storage of the library phages, 10 μl of the library boiled for 10 min was used as template in a 35-cycle standard PCR reaction. Amplification products ranging from 0.7 to 2.6 kb were then gel purified using a QiAquick column (Qiagen).

To amplify the apy79 cDNA fragment, primers were derived from the 5′-nucleotidase conserved motif GNHEFD and the VYEEDDDL1 peptide sequence of the 79-kDa apyrase. The degenerated primer set CONSI-1 (GGNAAYCYGARTTYGAY) and MS79-as (ADRTCRTCTCYTCT-RTA) was used. PCR amplification products were obtained from the amplified library with an annealing temperature of 45 °C (8 °C above calculated Tm) to ensure maximum specificity. The single band product of 1.2 kb was cloned into GEM T-easy plasmid (Promega).

Library Screening—The 1.2-kb cDNA fragment was labeled using the Random Primer labeling kit (Invitrogen) and used to screen the salivary gland cDNA library by standard procedures (λ ZAP manual), with washes done at 65 °C in 0.1× SSC, 0.1% SDS. Positive clones were submitted to in vivo excision (λ ZAP manual) to rescue the plasmids from which the inserts were sequenced.

5′-RACE—To get the 5′ missing region of the apy79 cDNA, we used the Stratagene GeneRacer kit that allows selection of complete mRNA by differential ligation of a RNA linker to their capped 5′ end. We used as starting material 2 μg of total RNA TRIzol extracted from the salivary glands of equal numbers of adult insects at 1, 2, 3, 4, 7, and 15 days after feeding. The 79GSP1-as primer (AGGTTGTGATCCTGTA) was used for reverse transcription, and the resulting cDNA was submitted to PCR with the 79GSP2-as primer (GCCGAAGTGTCCACCGGCAATA) and 5′ end linker primer. Specificity of the 1-kb product obtained was confirmed by nested PCR using two other gene-specific primers and Southern blot with the 1.2-kb apy79 cDNA probe. This product was cloned into pCR 4-TOPO and sequenced.

Baculovirus Expression—Recombinant baculovirus was obtained using the Bac-to-Bac system and protocol (Invitrogen). The Apy79 coding region was cloned by standard molecular biology techniques into the pFastBac plasmid with native signal peptide or mellitin signal peptide (kind gift from S. Bibert). In addition, two His-tagged constructions were derived by adding six histidine residues at the C terminus. Sf9 insect cells were infected with the recombinant baculovirus, and supernatant aliquots were taken at 24, 48, 72, and 96 h after infection.

SDS-PAGE and Western Blot—Proteins were separated by 10% SDS-PAGE using the standard Laemmli procedure. After completion of the run, the proteins were transferred to a nitrocellulose membrane in a semidry system. Membranes were blocked by incubation in phosphate-buffered saline containing 5% dry skimmed milk and then probed with anti-apyrase serum raised in rabbit by immunization with purified 79-kDa recombinant apyrase. Secondary antibody was conjugated with horseradish peroxidase, and immunoblot was developed using diaminobenzidine.

Genomic Southern—Genomic DNA was extracted from 50 adult T. infestans insects, and the pooled DNA was probed by Southern blot with 32P-labeled apy79 cDNA (29). Fifty μg of genomic DNA was digested with XhoI, BamHI, or HindIII. This last enzyme promotes two cuts in the 79GSP1-as primer (GCCGAAGTGTCCACCGGCAATA) and 5′ end linker primer. Specificity of the 1-kb product obtained was confirmed by nested PCR using two other gene-specific primers and Southern blot with the 1.2-kb apy79 cDNA probe. This product was cloned into pCR 4-TOPO and sequenced.
the laboratory was used to purify apyrase activity first described by Ribeiro et al. (3). An oligo(dT)12–18 cellulose column was used for a first-step purification of the apyrase because this method has been successfully employed for purification of terminal deoxynucleotidyltransferases (30). A typical experiment showing chromatography of 800 μg of saliva in an oligo(dT)-cellulose column is shown in Fig. 1A. About 10% of the total saliva proteins bound to the matrix and was eluted with 0.5 M NaCl. This fraction, which retained 84% of the salivary apyrase activity, was concentrated and loaded on a Superose 6 column. The apyrase activity was eluted as a large peak with an elution volume corresponding to a 400-kDa globular protein, as shown in Fig. 1B. This two-step chromatographic procedure led to an ~75-fold increase in apyrase specific activity with a yield of 35%.

SDS-PAGE analysis (Fig. 1C) showed that the 400-kDa apyrase fraction contained five proteins with molecular masses of 88, 82, and 79 kDa and a 68–67 kDa doublet, implying that they are associated in a protein complex.2 The presence of the 68–67 kDa doublet is more clearly seen in SDS-PAGE with a lower polyacrylamide concentration (Fig. 2, lane 1). The purified sample was treated with neuraminidase, O-glycosidase, or PNGase F. Only PNGase F changed the migration pattern to 64-, 59-, 57-, and 54-kDa bands in SDS-PAGE (Fig. 2) showing that the various species are N-glycosylated and do not represent different glycosylation levels of one or more proteins.

The five apyrase species were specifically labeled by FSBA (Fig. 3), an ATP analogue that covalently binds its enzyme target and can be detected by anti-FSBA-specific antibodies (31). Antibody specificity was shown by the lack of reactivity against apyrases that were not incubated with FSBA (Fig. 3, lane 1). The intensity of the signal was markedly reduced when the apyrases were preincubated with ATP (Fig. 3, lane 3). Therefore, the five proteins are likely to be functional species with respect to ATP binding.

The T. infestans apyrases inhibited ADP-induced human platelet aggregation (Fig. 4). This inhibition assay was run by incubating increasing concentrations of crude saliva with PRP followed by 5-min incubations with 5 μM ADP. The experiment illustrated in Fig. 4 depicts 27% inhibition of platelet aggregation with 1 μg of crude saliva and complete inhibition with 5 μg and as low as 30 ng of purified apyrases. The apyrase activity of saliva spontaneously ejected by one insect completely abolished aggregation of platelets equivalent to 1 ml of human blood.

Characterization of the Apyrases—Enzymatic characterization was performed to assess the apyrase nature of the purified proteins. The purified mixture displayed the same optimal temperature (37 °C) and optimal pH (pH 8.0) for the hydrolysis of ATP and ADP (maximum specific activities of 1500 and 1200 units/mg, respectively). Moreover, the two activities showed similar dependence upon divalent cations (1 mM final concentration) with a maximal hydrolytic activity against ATP and ADP obtained in the presence of 2 mM of Mg2+, Co2+, or Mn2+, whereas Ca2+, Cu2+, and Zn2+ did not influence enzyme activity.

The enzymatic activity was ranked for nucleoside 5’-triphos-
The presence or absence of 5 mM ATP. Thereafter, proteins were separated by 6% SDS-PAGE under reducing conditions, and Western blot was performed with serum raised against FSBA. Mock control consisted of purified apyrases incubated with Me2SO only. Arrows indicate the positions of the five apyrase, and their respective molecular masses are shown on the left.

FIG. 3. FSBA labeling of *T. infestans* apyrases. Purified apyrases from *T. infestans* saliva were incubated with 1 mM FSBA for 30 min in the presence or absence of 5 mM ATP. Thereafter, proteins were separated by 6% SDS-PAGE under reducing conditions, and Western blot was performed with serum raised against FSBA. Mock control consisted of purified apyrases incubated with Me2SO only. Arrows indicate the positions of the five apyrase, and their respective molecular masses are shown on the left.

FIG. 4. *T. infestans* apyrase anti-platelet aggregating activity. Complete inhibition of platelet aggregation was obtained with 5 µg of crude saliva and 30 ng of purified apyrases in triplicate repeats. 400 µl of PRP was incubated at 37 °C under stirring in the presence of either saliva or purified apyrase for 4 min. Then, 5 µM ADP (final) was added, and platelet aggregation was measured in an aggregometer.

Phosphates (ATP > CTP > GTP > UTP > ITP) and nucleoside 5'-diphosphates (ADP > IDP > CDP > GDP > UDP), which corresponds to a typical apyrase pattern. Consistent with these observations, the purified fraction did not hydrolyze nucleoside 5'-monophosphates, glycerol phosphate, glycose 6-phosphate, and UDP-galactose. A series of ATPases, ADPases, alkaline phosphatase, protein phosphatase 1 and 2A, and adenylate cyclase inhibitors were tested as described under “Experimental Procedures.” Both ATPase and ADPase activities were inhibited to similar levels by 1 mM vanadate and 1 mM NaF, indicating the two enzymatic activities are unlikely to be held by different active sites. This was confirmed by a mixed substrate experiment (32). The apyrase activity against a mixture of ATP and ADP was close to the mean of activities determined for hydrolysis of each of the two substrates used separately (data not shown). Taken together, all of these observations are consistent with a single site for nucleoside 5'-diphosphates and -triphosphates in each protein and exclude the possibility that the purified molecular species could correspond to a mixture of ATPases and ADPases.

**Analysis of the Saliva Apyrases by Mass Spectrometry**—The masses of peptides obtained by in-gel digestion of the 88- and 79-kDa proteins were compared with the *in silico* digestion of Swiss Protein and TrEMBL data base entries (33, 34). The peptide mass maps from the 88- and 79-kDa proteins presented striking similarities with the *Ae. aegypti* apyrase map, and partial sequences were also conserved. To confirm these results, MS/MS peptide sequences were obtained from the 88-, 82-, 79-, and 68-67-kDa proteins of the saliva of *T. infestans*. Peptide sequences were aligned with sequences from members of the 5'-nucleotidase family: *Aedes aegypti* (Swiss Protein Database accession no. (Swiss-Prot) P50635), *Anopheles gambiae* (TrEMBL Q9TW03), *Lutzomyia longipalpis* (Swiss-Protein Q9XX43), *Drosophila melanogaster* (Swiss-Protein Q9V825, and Q9V824), and *Discopoge omnata* (Swiss-Protein P29240). Boxed letters indicate conserved regions among *T. infestans* and the preceding species.

**DNA Sequence of the 79-kDa Apyrase**—We decided to clone the gene of the 79-kDa apyrase because this polypeptide is abundant in all individuals in the insectary. Degenerated oligonucleotides were derived from a peptide sequence obtained by mass spectrometry sequencing and a sequence motif conserved among the 5'-nucleotidase family. These oligonucleotides were used to PCR-amplify a unique cDNA fragment from a salivary gland cDNA library. This 1.2-kb fragment was used as a probe to screen the library, leading to the isolation of five clones. These clones presented cDNA inserts of different lengths, which were sequenced. Blotted letters indicate conserved regions among *T. infestans* and the preceding species.

![Figure 4](http://www.jbc.org/)

**FIG. 5.** MS/MS peptide sequences from the 88, 82-, 79-, and 68-67-kDa proteins of the saliva of *T. infestans*. Peptide sequences are aligned with sequences from members of the 5'-nucleotidase family: *Aedes aegypti* (Swiss Protein Database accession no. (Swiss-Prot) P50635), *Anopheles gambiae* (TrEMBL Q9TW03), *Lutzomyia longipalpis* (Swiss-Protein Q9XX43), *Drosophila melanogaster* (Swiss-Protein Q9V825, and Q9V824), and *Discopoge omnata* (Swiss-Protein P29240). Boxed letters indicate conserved regions among *T. infestans* and the preceding species.
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Fig. 6. Protein sequence of Apy79 deduced from apy79 cDNA. The arrow indicates the cleavage site of signal peptide predicted by the SignalP program. Peptides sequences obtained by MS/MS are underlined. Alignment with the periplasmic 5'-nucleotidase from E. coli, for which the structure is known (Protein Data Bank code 1hpu), revealed that the most important residues in catalytic pocket are conserved; the residues involved in coordination of metal ions are in bold, and residues involved in substrate recognition are boxed. Two residues for which alignments are ambiguous are shaded.

Fig. 7. Recombinant Apy79 kDa of T. infestans. Sf9 insect cells were infected with recombinant baculovirus containing the apy79 open reading frame or an unrelated open reading frame. Supernatants and salivary apyrases were submitted to 6% SDS-PAGE followed by Western blot with antibodies specific to 79-kDa apyrase (lanes 2–4). In parallel, salivary apyrases were submitted to electrophoresis, transferred to nitrocellulose membrane, and stained by Amido Black. Lane 1, Amido Black staining of salivary apyrases; lane 2, mock control; lane 3, recombinant Apy79; lane 4, salivary apyrases.

Fig. 8. Genomic Southern blot. Southern hybridization of restriction digests of T. infestans genomic DNA probed with the apy79 cDNA. T. cruzi DNA was used as negative control. DNA and restriction enzymes combinations are listed above the corresponding lanes. Migration of the 1 kb-plus DNA ladder from Invitrogen is shown on the right.

25% identity with this 5'-nucleotidase, for which the structure is known (42), and the most important residues involved in metal coordination and substrate recognition were aligned (Fig. 6). These findings confirm that the 79-kDa apyrase from T. infestans belongs to the 5'-nucleotidase family. The genomic fragment corresponding to the coding sequence was amplified by PCR, and sequencing showed it to be intron-free.

Mapping the apy79 Gene in the Genome of T. infestans—Southern blot hybridizations were carried out on genomic DNA pooled from 50 adult T. infestans insects and hybridized with the 32P-labeled apy79 cDNA. Genomic DNA digested with XhoI or BamHI, two sites that are absent within the apy79 cDNA sequence, produces two high molecular weight DNA fragments hybridizing with the probe (Fig. 8). Digestion with HindIII restriction enzyme, which is expected to cut within the apy79 gene, led to five bands: four high molecular weight species and the expected 468-bp fragment resulting from internal diges-
tion. These results are consistent with the presence of two copies of the apy79 gene.

**DISCUSSION**

Triatoma vectors of Chagas’ disease were shown to overcome host platelet aggregation by secreting apyrase activity in their saliva during feeding (3). In the present report, we show that pools of saliva from laboratory populations of *T. infestans* contain five glycosylated proteins of 88, 82, 79, 68 and 67 kDa associated with apyrase activity. The apyrase nature of these proteins is supported by peptide sequences similar to those of the 5′-nucleotidases. Moreover, they all specifically bound an ATP analogue, and the sequence of the gene encoding the 79-kDa apyrase displayed unambiguous similarities with the 5′-nucleotidase family. Furthermore, we showed that the purified apyrase mixture is a very potent inhibitor of human platelet aggregation.

The presence of five apyrases in *T. infestans* saliva is likely explained by gene multiplication and subsequent divergence. We considered that these features of the *T. infestans* salivary apyrases are unusual, as other arthropod saliva described thus far display a unique apyrase. Recently, two apyrase clusters were identified (15). Moreover, the recent finding of two clusters showing similarities with 5′/H11032 were identified (15). Additionally, the recent finding of two clusters showing similarities with 5′/H11032 (18) by functional genomics, but a conclusion as to whether the already characterized apyrase gene (17). However, an apyrase and an apyrase-like gene were identified in *An. gambiae* (18) by functional genomics, but a conclusion as to whether they represent two different apyrases could not be drawn. In *An. stephensi*, three apyrase activities with distinguishable pls were identified (15). Moreover, the recent finding of two clusters showing similarities with 5′-nucleotidases supports the hypothesis that different apyrase genes exist in this species (44). In this paper we present evidence that the apyrase activity found in *T. infestans* saliva is associated with five different proteins.

We hypothesized that the different apyrases could derive from a single gene by alternative splicing as it occurs for other apyrases (45). However, this possibility was discarded following comparison of the 79-kDa apyrase cDNA and genomic DNA showing the absence of an intron. These observations lead to the conclusion that the apyrases are encoded by at least two independent genes. Moreover, because peptides sequences obtained from different protein bands differed in few amino acids residues, it is likely that the various apyrases do not represent different post-translational modifications of a single protein core.

Because the 79- and 68–67-kDa proteins are present in the saliva of each triatomine in our colony, whereas the 88- and 82-kDa apyrases are absent in some individuals,3 the 88- and 82-kDa apyrases may correspond to a gene different from the 79- and 68–67-kDa apyrases. As the 79- and 68–67-kDa species are present in all individuals but display differences in amino acid sequences, they should be encoded by different loci. Combining these elements leads to the conclusion that at least three apyrase loci exist in *T. infestans*, whereas it is not possible to determine the relation between the 68- and 67-kDa apyrases. However, it might be that these two latter proteins originate from the same gene product with different post-translational modifications including limited proteolysis. Southern hybridization with the apy79 cDNA as a probe showing two bands with the *T. infestans* genomic DNA digests of XhoI and BamHI and five bands with the HindIII is consistent with the presence of two copies of the apy79 gene. This observed gene duplication supports the hypothesis that the different apyrases arose from gene duplication and subsequent divergence.

Differences in the sequence of the vasodilator maxadilan among populations of the sandfly are thought to be related to antigenic variations (46). We propose that the gene multiplication shown by *T. infestans* could be an important mechanism of enzyme amplification, in addition to a mechanism of escape from the host immune response. Therefore, the apparent redundancy of multiple apyrases may represent a *T. infestans* mechanism to enhance meal acquisition, developed during evolution to homotrophy. Cloning of the other apyrase genes would provide stimulating evolutionary data by comparing the different genes and assessing the gene duplication hypothesis. Furthermore, it would be interesting to examine whether these genetic features are also present in the field populations. If different apyrase genetic patterns were found in different geographical locations, salivary apyrases could be used, together with other polymorphic loci, as a molecular marker for studying the settlement history of *T. infestans* in the American continent.

Convergent evolution is the prevailing model for adaptation to blood feeding, because species that diverged before emergence of hematophagy independently developed the same molecular tools. Indeed, peptide and cDNA sequencing show that *T. infestans* apyrases belong to the same family as those of the mosquitoes *Ae. aegypti*, *An. gambiae*, and *An. stephensi* (17, 18, 44) but differ from the apyrase from the bedbug *C. lectularius* (20) to which it is phylogenetically closer. Enzymatic characterization showed that *T. infestans* apyrases hydrolyze nucleosides 5′-triphosphates and 5′-diphosphates with preference for ATP and ADP, characteristic of this enzyme family. No phosphohydrolase activity on AMP or other nucleoside 5′-monophosphates was observed, in contrast to ancestral 5′-nucleotidases, which are able to hydrolyze nucleoside 5′-monophosphates (39). Thus ADP degradation by apyrases leads to AMP production and not adenosine. Both AMP and adenosine are thought to benefit blood feeding (47), but the evolution of apyrase from 5′-nucleotidase to favor AMP production may reflect a more important anti-hemostatic effect of AMP.

Bacterial 5′-nucleotidases hydrolyze nucleoside 5′-tri-, di-, and monophosphates and can be membrane-anchored or soluble. On the other hand, vertebrate 5′-nucleotidases only hydrolyze nucleoside 5′-monophosphates and are often bound to membrane by a glycosylphosphatidylinositol anchor (39). In contrast, *T. infestans* 79 kDa apyrase is a truly soluble protein, as it lacks the C-terminal hydrophilic region and the conserved Ser residue present in the vertebrate glycosylphosphatidylinositol-anchored 5′-nucleotidase. This situation is also found in salivary 5′-nucleotidases from mosquito, sandfly, and tick (17, 21, 48).

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