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THE SALIVARY APYRASE OF THE BLOOD-SUCKING SAND FLY *PHLEBOTOMUS PAPATASI* BELONGS TO THE NOVEL *CIMEX* FAMILY OF APYRASES

JESUS G. VALENZUELA[1], YASMINE BELKAID[1], EDGAR ROWTON[2] AND JOSÉ M. C. RIBEIRO[1,*]

[1]*Section of Medical Entomology, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, Building 4, Room 126, 4 Center Drive, MSC 0425, Bethesda, MD 20892-0425, USA* and [2]*Department of Entomology, Walter Reed Army Institute of Research, Washington, DC 20307-5100, USA*

*Author for correspondence (e-mail: Jribeiro@nih.gov)

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Summary

Apyrases are enzymes that hydrolyze nucleotide di- and triphosphates to orthophosphate and mononucleotides. At least two families of enzymes, belonging to the 5′-nucleotidase and to the actin/heat shock 70/sugar kinase superfamily, have evolved independently to serve the apyrase reaction. Both families require either Ca^{2+} or Mg^{2+} for their action. A novel apyrase enzyme sequence, with no homology to any other known protein sequence, was found recently in the salivary glands of the hematophagous bed bug *Cimex lectularius*. This enzyme functions exclusively with Ca^{2+}. Here, we report the finding of a cDNA similar to that of the *C. lectularius* salivary apyrase isolated from a salivary gland cDNA library of *Phlebotomus papatasi*. Transfection of insect cells with the *P. papatasi* salivary gland apyrase cDNA resulted in the secretion of a Ca^{2+}-dependent apyrase whose activity was indistinguishable from that in salivary homogenates of *P. papatasi*. Homologous sequences were found in humans, in another sand fly (*Lutzomyia longipalpis*), in the fruit fly *Drosophila melanogaster*, in the nematode *Caenorhabditis elegans* and in the protozoan *Cryptosporidium parvum*, indicating that this family of enzymes is widespread among animal species.

Key words: apyrase, salivary gland, haematophagy, bed bug, *Cimex lectularius.*

Introduction

Apyrase was a term coined over 50 years ago (Meyerhoff, 1945) to describe those enzymes that could hydrolyze both ADP and ATP to AMP and orthophosphate. Shortly thereafter, the existence of this enzyme was questioned because, in muscle, the activity could be explained by the combined action of myosin and myokinase, although in liver the activity was found without contaminating adenylate kinase (Kalckar, 1945). However, in plants such as potato tubers, the existence of a true apyrase seemed real, although its function remains unknown (Traverso-Cori et al., 1965; Traverso-Cori et al., 1970). Apyrase activity was later described in the salivary glands of hematophagous insects, when Sarkis and others (Sarkis et al., 1986) gave a convincing account of the presence of a true animal apyrase and, in the same paper, postulated that apyrase could serve an extracellular role in purinergic catabolism (such as degrading ADP, which triggers platelet aggregation) and an intracellular role in glycosylation reactions (such as degrading UDP to UMP after it is released during transglycosylation reactions). More recently, apyrases have been found in several vertebrate tissues, and their genes have been cloned (for reviews, see Plesner, 1995; Yoshida and Amano, 1995; Zimmermann and Braun, 1996).

Most apyrases cloned thus far are related to the yeast GTPase/CD39 family, which can be grouped in the actin/heat shock 70/sugar kinase superfamily (Smith and Kirley, 1999). However, the salivary apyrase of the mosquito *Aedes aegypti* belongs to the 5′-nucleotidase gene family (Champagne et al., 1995b), which is not surprising since bacterial 5′-nucleotidases also hydrolyze ADP and ATP (Zimmermann, 1992). In addition, a completely new type of apyrase was found in the blood-sucking bed bug *Cimex lectularius* (Valenzuela et al., 1998). This cDNA has a high level of similarity to the expressed sequence tag (EST) from *Caenorhabditis elegans* and from humans (Jurkat-cell-derived cDNA library), indicating that this family may be widespread. In addition, a cDNA from the sand fly *Lutzomyia longipalpis* also has high similarity to *Cimex lectularius* apyrase (Charlab et al., 1999), indicating that phlebotomine Diptera may have a *Cimex* type of apyrase, rather than the 5′-nucleotidase or CD39 type found in other organisms. However, *Lutzomyia longipalpis* apyrase cDNA was not expressed to confirm its function.

In this paper, we describe a cDNA from the sand fly *Phlebotomus papatasi* which also has a high similarity to the cDNA of the apyrase found in the bed bug *Cimex lectularius*. We present evidence that this cDNA codes for the abundant salivary apyrase of this blood-sucking fly, and argue for the convergent evolution of apyrase enzymes.

Materials and methods

Materials

Organic compounds were obtained from Sigma Chemical Corporation (St Louis, MO, USA). All water used was of 18 MΩ quality and was produced by a MilliQ apparatus from Millipore (Bedford, MA, USA). Sand flies were reared at the Walter Reed Army Medical Research Institute on a fermented mixture of rabbit chow and rabbit feces, as described previously (Modi and Tesh, 1983).

Insects

Adult sand flies *Phlebotomus papatasi* were kept with free access to a 20 % solution of sucrose unless specified otherwise. Salivary glands from 3- to 10-day-old adult flies were dissected and transferred to 10 or 20 μl of 10 mmol l^{-1} Hepes, pH 7.0, 0.15 mol l^{-1} NaCl in 1.5 ml polypropylene vials. Usually groups of 20 pairs of glands were placed in 20 μl of Hepes saline or individual glands were placed in 10 μl of Hepes saline. Salivary glands were kept at −75 °C until needed, when they were disrupted by sonication using a Branson Sonifier 450 homogenizer (Branson Ultrasonics, Danbury, CT, USA). Salivary homogenates were centrifuged at 10 000 ***g*** for 2 min, and the supernatants were used for the experiments.

Polyclonal antibody to C. lectularius *salivary apyrase*

A synthetic peptide (KVLIEETKIDDHKYEGVDFV) based on the predicted C-terminal region of the *Cimex* apyrase clone was produced at the Laboratory of Molecular Structure, National Institute of Allergy and Infectious Diseases, Twinbrook Facility, Rockville, MD, USA. The synthetic peptide (5 mg) was conjugated to 7 mg of keyhole limpet hemocyanin (KLH) at a molar coupling ratio of peptide:carrier of 122:1 and used to immunize rabbits at Spring Valley Laboratories, Woodbine, MD, USA. Pre-immune sample was taken before the first injection, and immune serum samples were taken after two and three injections. Antibody specificity was verified by enzyme-linked immunosorbent assay (ELISA) of crude salivary homogenate and purified *Cimex* apyrase with both immune and pre-immune sera. This antiserum has been used previously (Valenzuela et al., 1998).

Chromatographic experiments

Gel permeation chromatography was performed with Super TSK-2000SW (4 mm×25 cm; TosoHaas, Montgomeryville, PA, USA) isocratically perfused with 10 mmol l^{-1} Hepes, pH 7.0, 1.0 mol l^{-1} NaCl, at a flow rate of 0.2 ml min^{-1}. Fractions were collected at 20 s intervals. Samples of the fractions were used to determine apyrase activity by incubating with 2 mmol l^{-1} ADP or ATP in a medium containing 20 mmol l^{-1} Hepes, pH 7.4, 5 mmol l^{-1} $CaCl_2$ and 100 mmol l^{-1} NaCl. Orthophosphate released from nucleotides was detected by the method of Fiske and Subbarow (Fiske and Subbarow, 1925) adapted to a microtiter plate (Marinotti et al., 1990). All colorimetric assays were performed with a ThermoMax plate reader from Molecular Devices (Menlo Park, CA, USA).

ELISA assay

Salivary gland proteins eluted from a molecular sieving high-performance liquid chromatography (HPLC) column were assayed with pre-immune and immune serum (anti-apyrase) using conventional ELISA protocols. Dilutions of pre-immune and immune serum were 1:500, and dilutions for the secondary antibody (anti-rabbit IgG peroxidase conjugate; Sigma Chemical Co., St Louis, MO, USA) were 1:2000.

Isoelectrofocusing gel electrophoresis

A Pharmacia Phast System (Uppsala, Sweden), using pH 3–9 gel, was utilized according to the manufacturer's recommendation. Standards provided by Pharmacia were used to calibrate the gels. Two lanes of the gel were run, each with two pairs of homogenized salivary glands containing approximately 2 μg of protein. An additional lane was run with isoelectric pH markers and stained with Coomassie Blue, as recommended by the manufacturer. After the run, cutting the gel and its plastic backing with scissors separated the two lanes containing salivary gland homogenates. These were incubated with either ADP or ATP (5 mmol l^{-1}), 50 mmol l^{-1} Tris-Cl, pH 8.3, 20 mmol l^{-1} $CaCl_2$ and 100 mmol l^{-1} NaCl until bands of calcium phosphate (indicating apyrase activity) were visible (10–20 min of incubation at room temperature). Washing the gel with the same reaction mixture without nucleotide stopped the reaction.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

Tris-glycine gels (10 %), 1 mm thick, were used (Novex, San Diego, CA). To estimate the relative molecular mass (M_r) of apyrase, SeeBlue markers from Novex (myosin, bovine serum albumin, glutamic dehydrogenase, alcohol dehydrogenase, carbonic anhydrase, myoglobin, lysozyme, aprotinin and insulin chain B) were used. Salivary gland homogenates were treated with 8 % SDS in Tris-HCl buffer, 0.5 mol l^{-1}, pH 6.8, 10 % glycerol and 1 % Bromophenol Blue. Five salivary gland pairs per lane (approximately 5 μg of protein) were used for ATPase and ADPase activity, and 10 pairs were used for staining with Coomassie Blue. The gel was run with Tris-glycine buffer according to the manufacturer's instructions. Gels used to measure enzymatic activity were renatured by washing twice with 2.5 % Triton X-100 in water for 20 min, followed by two washes with water. All washes were performed with gentle rocking of the plate containing the gels. Gels were tested for apyrase activity as described above for the isoelectric focusing experiment. For amino-terminal sequencing of the gel band containing apyrase activity, 25 homogenized pairs of glands were electrophoresed and transferred to PVDF membrane using 10 mmol l^{-1} Caps, pH 11, 10 % methanol, as the transfer buffer on a Blot-Module for the XCell II Mini-Cell from Novex. The membrane was then stained with Coomassie Blue in the absence of acetic acid.

Amino-terminal sequencing

The band associated with apyrase activity was cut from the

PVDF membrane and subjected to Edman degradation, using a Procise sequencer (Perkin-Elmer Corp., Norwalk, CT, USA), by Dr Mark Garfield at the Structural Biology Section, Twinbrook II facility of the National Institutes of Allergy and Infectious Diseases.

Construction of a salivary gland cDNA library

Phlebotomus papatasi salivary gland mRNA was isolated from 85 salivary gland pairs from adult females, at days 1, 2 and 3 after emergence. The Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego, CA, USA) was used, yielding a total of 100 ng of poly(A^+) mRNA. The polymerase chain reaction (PCR)-based cDNA library was prepared following the instructions for the SMART cDNA library construction kit (Clontech). The *P. papatasi* salivary gland mRNA (100 ng) was reverse-transcribed to cDNA using SuperscriptII RNAase H^- reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA) and the CDS/3′ primer (Clontech) for 1 h at 42 °C. Second-strand synthesis was performed with a PCR-based protocol using the SMART III primer (Clontech) as the sense primer and the CDS/3′ primer as the anti-sense primer. In addition, these two primers create *Sfi*I A and *Sfi*I B sites, respectively, at the ends of the nascent cDNA. Double-strand cDNA synthesis was performed on a Perkin Elmer 9700 thermal cycler (Perkin Elmer Corp., Foster City, CA, USA) using Advantage Klen-Taq DNA polymerase (Clontech).

PCR conditions were the following: 94 °C for 2 min; 19 cycles of 94 °C for 10 s and 68 °C for 6 min. Double-strand cDNA was immediately treated with proteinase K (0.8 $\mu g\,\mu l^{-1}$) for 20 min at 45 °C and washed three times with water using Amicon filters with a 100 kDa cut-off (Millipore Corp.). Double-strand cDNA was then digested with *Sfi*I for 2 h at 50 °C (the *Sfi*I sites were inserted into the cDNA during the second-strand synthesis using SMART III and the CDS/3′ primer). The cDNA was then fractionated using columns provided by the manufacturer (Clontech). Fractions containing cDNA of more than 400 bp were pooled, concentrated and washed three times with water using an Amicon filter with a 100 kDa cut-off. The cDNA was concentrated to a volume of 7 µl and then ligated into a lambda triplex2 vector (Clontech). The resulting ligation reaction was packed using the Gigapack gold III from Stratagene/Biocrest (Cedar Creek, TN, USA) following the manufacturer's instructions. The library thus obtained was plated by infecting log-phase XL1-blue cells (Clontech), and the number of recombinants was determined by PCR using vector primers flanking the inserted cDNA and visualized on a 1.1 % agarose gel with ethidium bromide (1.5 $\mu g\,ml^{-1}$).

Sequencing of P. papatasi *apyrase*

The *P. papatasi* salivary gland cDNA library was plated to approximately 200 plaques per plate (150 mm diameter Petri dish). The plaques were randomly picked and transferred to a 96-well polypropylene plate containing 100 µl of water per well. The plate was covered and placed on a gyrator/shaker for 1 h at room temperature (24–27 °C). The phage sample (5 µl) was used as a template for a PCR reaction to amplify random cDNAs. The primers used for this reaction, sequences from the triplex2 vector, were: PT2F1 (5′-AAGTACTCTAGCAA-TTGTGAGC-3′), which is positioned upstream of the cDNA of interest (5′-end), and PT2R1 (5′-CTCTTCGCTATT-ACGCCAGCG-3′), which is positioned downstream of the cDNA of interest (3′-end). High-fidelity platinum Taq polymerase (Gibco-BRL) was used for these reactions. Amplification conditions were: one hold of 75 °C for 3 min, one hold of 94 °C for 3 min, 34 cycles of 94 °C for 30 s, 49 °C for 30 s and 72 °C for 80 s. Amplified products were visualized on a 1.1 % agarose gel with ethidium bromide. The concentration of double-strand cDNA was measured with Hoechst dye 33258 on a Flurolite 1000 plate fluorimeter (Dynatech Laboratories, Chantilly, VA, USA).

The PCR product (3–4 µl) containing 100–200 ng of DNA was then treated with ExonucleaseI (0.5 units μl^{-1}) and shrimp alkaline phosphatase (0.1 units μl^{-1}) for 15 min at 37 °C and 15 min at 80 °C on a 96-well PCR plate. This mixture was used as a template for a cycle sequencing reaction using the DTCS labeling kit (Beckman Coulter, Inc., Fullerton, CA, USA). The primer used for sequencing (PT2F3) is upstream of the inserted cDNA and downstream of the primer PT2F1. The sequencing reaction was performed on a Perkin Elmer 9700 Thermalcycler. Conditions were 75 °C for 2 min, 94 °C for 4 min and 30 cycles of 96 °C for 20 s, 50 °C for 20 s and 60 °C for 4 min. After cycle sequencing, a cleaning step was performed using the multiscreen 96-well plate cleaning system from Millipore. The 96-well multiscreening plate was prepared by adding a fixed amount (according to the manufacturer's specification) of Sephadex-50 (Amersham/Pharmacia Biotech, Piscataway, NJ, USA) and 300 µl of deionized water. After a 1 h incubation at room temperature, the water was removed from the multiscreen plate by centrifugation at 750 ***g*** for 5 min. When the Sephadex in the multiscreen plate was partially dried, the whole cycle sequencing reaction was added to the center of each well and centrifuged at 750 ***g*** for 5 min, and the clean sample collected on a sequencing microtiter plate (Beckman Coulter, Inc.). The plate was then dried on a Speed-Vac SC (model 110) with a microtiter plate holder (Savant Instruments, Inc., Holbrook, NY, USA). Dried samples were immediately resuspended with 25 µl of deionized ultrapure formamide (J. T. Baker, Phillipsburg, NJ, USA), and one drop of mineral oil was added to the top of each sample. Samples were sequenced immediately on a CEQ 2000 DNA sequencing instrument (Beckman Coulter, Inc.) or stored at −30 °C.

Isolation of P. papatasi *apyrase cDNA*

After identifying a cDNA with high similarity to *Cimex lectularius* apyrase, a sample (approximately 100 ng) of *P. papatasi* apyrase PCR sample was re-amplified using the PT2F1 and PT2R1 primers (same conditions as described above but only 25 PCR cycles), and the entire cDNA was fully sequenced using custom-designed primers. *P. papatasi* apyrase cDNA was then cloned into a TOPO TA cloning vector (Invitrogen) and stored at −70 °C.

Expression of P. papatasi *apyrase*

To express the *P. papatasi* apyrase, the full-length cDNA was used as a template to amplify only the cDNA that begins at the initial methionine and ends at the first stop codon. The single amplified product obtained was immediately cloned into the vector pIB/V5-His TOPO (Invitrogen) following the manufacturer's specifications. The ligation mixture was used to transform TOP10 cells (Invitrogen), and the cells were incubated overnight at 37 °C. Eight colonies were picked and mixed with 10 μl of sterile water. From each sample, 5 μl was transferred to Luria broth with ampicillin (100 μg ml^{-1}) and grown at 37 °C. The other 5 μl was used as a template for a PCR reaction using two vector-specific primers from the pIB/V5-His TOPO vector to confirm the presence of the insert and for sequencing analysis. After visualization of the PCR product on a 1.1 % agarose gel, we sequenced the eight PCR products described above using a CEQ2000 DNA sequencing instrument (Beckman Coulter, Inc.).

We chose two samples, one containing the complete sequence (from methionine to the stop codon) in the correct orientation of *P. papatasi* apyrase for expression on this vector and a second sample, which became our control sample, containing the *P. papatasi* apyrase complete sequence in reverse orientation for the expression in this cell vector. Cells containing the sample and control were grown overnight at 37 °C in Luria broth with ampicillin (100 μg ml^{-1}), and plasmid isolation was performed using the Wizard miniprep kit (Promega Corporation, Madison, WI). After plasmid isolation, sample and control plasmids were washed three times with ultrapure water using an Amicon-100 (Millipore Corp.), the concentration of sample was measured, and the samples were stored at −30 °C before High Five cell transformation.

Expression of P. papatasi *apyrase in insect cell line BTI-TN-5B1-4 (High Five)*

High Five insect cells (Invitrogen, Carlsbad, CA, USA) were cultivated in High Five serum-free medium (Invitrogen) supplemented with 10 μg ml^{-1} gentamycin. A cell density of 2.0×10^6 cells ml^{-1} and 60–70 % confluency were used for transfection of *P. papatasi* apyrase cDNA and the *P. papatasi* apyrase anti-sense construct (control). Transfections were performed using 70 μl of Insectin-Plus liposomes (Invitrogen) and added to a mixture of 5 ml of Ultimate Insect serum-free medium (Invitrogen) containing 1–25 μg ml^{-1} plasmid DNA in a 15 ml sterile tube. After vortexing for 10 s, the mixture was incubated at room temperature for 15 min. The mixture was then added very slowly to a monolayer of High Five cells (in a 25 cm^3 flask) immediately following removal of culture medium. The flasks were then transferred to a rocking platform at a speed of two side-to-side movements per minute and left for 4 h at room temperature. Finally, the cells were incubated at 27 °C for up to 4 days. After 3 days of incubation, the supernatant (5 ml) was collected and centrifuged on a Centricon Plus-20 (5 kDa cut-off) (Millipore Corp.) at 1800 ***g*** for 40 min. The retentate of the Centricon Plus-20 (200 μl) was collected and stored for further analysis.

Apyrase activity

Apyrase activity was determined by orthophosphate release from nucleotides using the method of Fiske and Subbarow (Fiske and Subbarow, 1925) with modifications (Marinotti et al., 1990).

Sequence analysis

Sequence similarity searches were performed using the Blast (Altschul et al., 1997) program. Cleavage site predictions of the mature proteins used the SignalP (Nielsen et al., 1997) program. Alignments of protein sequences were performed with the ClustalW program version 1.7 (Thompson et al., 1994).

Results

While sequencing a salivary gland cDNA library from the sand fly *P. papatasi*, a cDNA clone with high similarity to the

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   1    gccggggattctgttgtgggaaatctcttgaacattttcaaa

  43 atgtttctcaaattttgtattgttgcttttgccatttgcctctca
     M  F  L  K  F  C  I  V  A  F  A  I  C  L  S
  88 ataaatctctcagaaggagctccaagaagtggaacaatctataat
     I  N  L  S  E  G  A  P  R  S  G  T  I  Y  N
 133 tttgccattatagctgatttggataaaaaatctatcagcccaaag
     F  A  I  I  A  D  L  D  K  K  S  I  S  P  K
 178 aatgataataattacaaaagtatcgtgaaagtgggtgaattgatt
     N  D  N  N  Y  K  S  I  V  K  V  G  E  L  I
 223 gaagtaggagataagtacagtgtcaaaatgaagaaagaagatcat
     E  V  G  D  K  Y  S  V  K  M  K  K  E  D  H
 268 gaaatattcactaaatatgcatacaaaggacgaggagctgaatta
     E  I  F  T  K  Y  A  Y  K  G  R  G  A  E  L
 313 tctgaattcttaatttataaatggaaactttacacttttgatgac
     S  E  F  L  I  Y  K  W  K  L  Y  T  F  D  D
 358 aaaagtggaattgtctttagactgaaaaccaatgcagacctcatt
     K  S  G  I  V  F  R  L  K  T  N  A  D  L  I
 403 ccttgggtaactctcgcaaatggcaatggagatcaaactgatggc
     P  W  V  T  L  A  N  G  N  G  D  Q  T  D  G
 448 tttaaggcagaatgggcaacaactaaaggtgacaaaatgtacgtt
     F  K  A  E  W  A  T  T  K  G  D  K  M  Y  V
 493 ggatcaactggaatttcttttactgacaaaacaggcaaattaaat
     G  S  T  G  I  S  F  T  D  K  T  G  K  L  N
 538 agcaactccctctggatcaaagaaatcgatcaagacggaaaggtt
     S  N  S  L  W  I  K  E  I  D  Q  D  G  K  V
 583 cagagtttagattggaaagaacaatacgacaaaataaaaagtgct
     Q  S  L  D  W  K  E  Q  Y  D  K  I  K  S  A
 628 atgaaaatccctaatggatttatttggcacgaagctgttaattgg
     M  K  I  P  N  G  F  I  W  H  E  A  V  N  W
 673 tcaaaactcaaaaaccaatgggtctttctaccaagaaaatgctca
     S  K  L  K  N  Q  W  V  F  L  P  R  K  C  S
 718 gaacgtccatttgataccaaaactgaagagactattggatgtaac
     E  R  P  F  D  T  K  T  E  E  T  I  G  C  N
 763 aagataatcattgccagtgaaaatttcgaaataatcaaatctatt
     K  I  I  I  A  S  E  N  F  E  I  I  K  S  I
 808 cagatcaaaggaaaatctattaatcgtgccgcaggatttcttca
     Q  I  K  G  K  S  I  N  R  A  A  G  F  S  S
 853 ttcaaattcctccccgatagtgatgaccaaatccttcttgcattg
     F  K  F  L  P  D  S  D  D  Q  I  L  L  A  L
 898 aagactatcgaaaaggacgacaaaactgctacatacattacagta
     K  T  I  E  K  D  D  K  T  A  T  Y  I  T  V
 943 attgatatcactggaagagttttaatgccagaaatgcagatcaat
     I  D  I  T  G  R  V  L  M  P  E  M  Q  I  N
 988 agcgataaatacgaaggaattgtgcttttgaaaagcaccgaagga
     S  D  K  Y  E  G  I  V  L  L  K  S  T  E  G
1033 tttttgaaacgcagtcaataataaaaaaaaaaaaaaaaaaaa  1075
     F  L  K  R  S  Q  *
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Fig. 1. cDNA and translation product of *Phlebotomus papatasi* salivary apyrase cDNA (PpApy). The underlined amino acid sequence represents amino acids found by Edman degradation (see Fig. 4).

Cimex apyrase cDNA (accession number gi:4185746) (Valenzuela et al., 1998) was found (Fig. 1). The cDNA for the putative apyrase of *P. papatasi* (PpApy) indicates the possible presence of a secretory signal sequence (Nielsen et al., 1997) predicting a mature peptide starting at position 22 (APRSGTI...), an estimated M_r of 35 856.06 and a pI of 8.93.

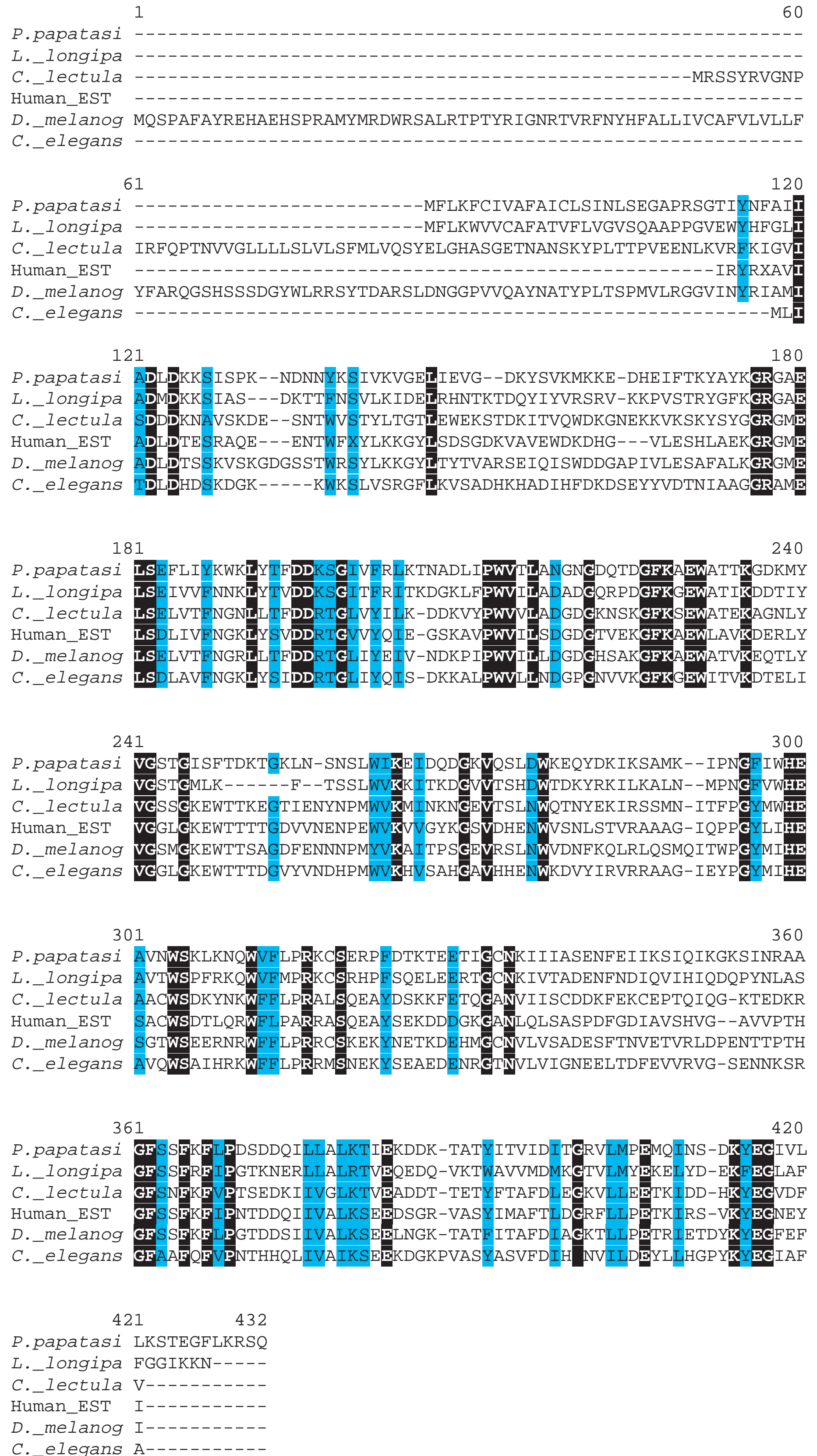

Fig. 2. Alignment of selected cDNA sequences translated from apyrases of *Phlebotomus papatasi* (AF261768), *Lutzomyia longipalpis* (AF131933), *Cimex lectularius* (AF085499), human expressed sequence tags (AA632390, AA337541, AA311735, AA075972, AA356674, AA337180, AA348005, AA112425 and AA074378), *Drosophila melanogaster* (*D. melanog.*) (AAF54638) and *Caenorhabditis elegans* (U29378). Identical amino acids are printed in white on a black background, and highly conserved amino acids are shown on a blue background.

This cDNA was also similar to the putative apyrase cDNA from the fly *Lutzomyia longipalpis* (accession number gi:4928274) (Charlab et al., 1999), an expressed sequence tag (EST) from *Caenorhabditis elegans* (accession number gi:7498612) and an EST from both mouse and human (Valenzuela et al., 1998; Fig. 1). More recently, a *Cryptosporidium parvum* genomic DNA (accession number gi:6205157) coding for a protein similar to *Cimex lectularius* apyrase was found during a genomic survey, as well as a putative gene product from *Drosophila melanogaster* (accession number gi:7299449). Alignments of the predicted protein sequences, not including *Cryptosporidium parvum,* are shown in Fig. 2.

There is marked sequence similarity between *Cimex lectularius* and *Phlebotomus papatasi* apyrase cDNA, so we investigated whether an antiserum raised against the carboxyterminal region of *Cimex lectularius* (Valenzuela et al., 1998) would recognize *Phlebotomus papatasi* apyrase. To this end, we submitted 10 pairs of glands from *P. papatasi* to gel filtration chromatography, assayed the fractions for apyrase activity, and used the fractions to coat an ELISA plate. We found co-elution of the salivary apyrase with material recognized by the antiserum used (Fig. 3), indicating that *Phlebotomus papatasi* apyrase shows sequence similarity with the carboxy-terminal region of *Cimex lectularius* apyrase.

To determine the M_r of the salivary apyrase of *P. papatasi*, we submitted five pairs of salivary glands to SDS–PAGE using a 10 % gel. The gel was renatured with Triton X-100 and washed with water before addition of the reaction mixture to visualize the apyrase activity by calcium phosphate precipitation. The results indicate (Fig. 4) an apparent M_r of 38×10^3 when compared with the standards run in the same gel. We also observed that the apyrase activity appeared in a region of the chromatogram gel containing a band strongly stained by Coomassie Blue. Transfer of this band (from another gel

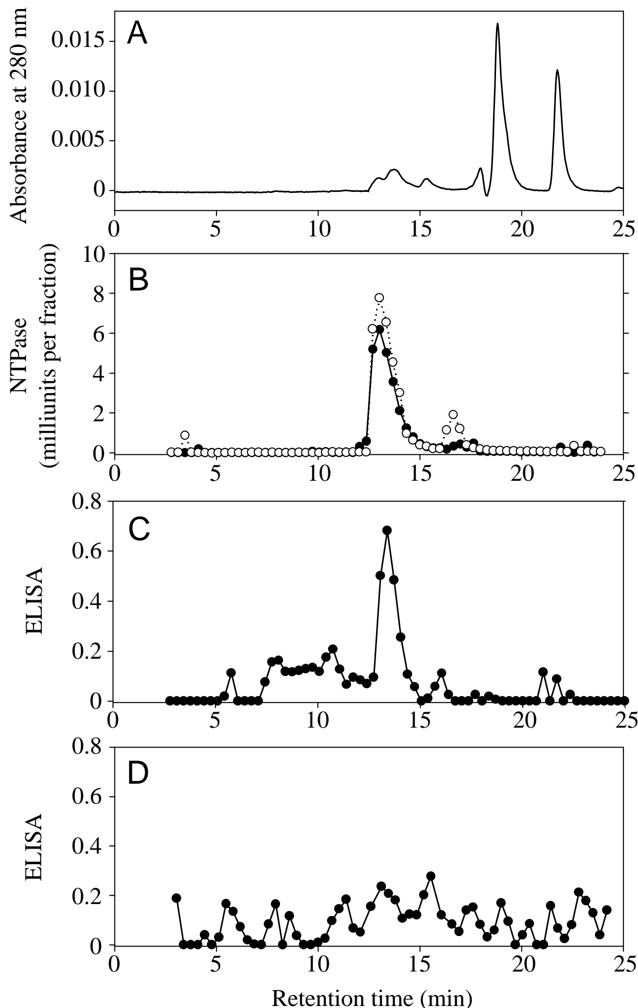

Fig. 3. Molecular sieving chromatography of 10 homogenized pairs of salivary glands from *Phlebotomus papatasi.* (A) Optical density at 280 nm. (B) ADPase activity (○) and ATPase activity (●) of the fractions. (C) ELISA results using rabbit anti-serum raised against the carboxy-terminal region of *Cimex lectularius* apyrase. (D) ELISA results using rabbit control serum. Activity in C and D is expressed as optical density units per milliliter.

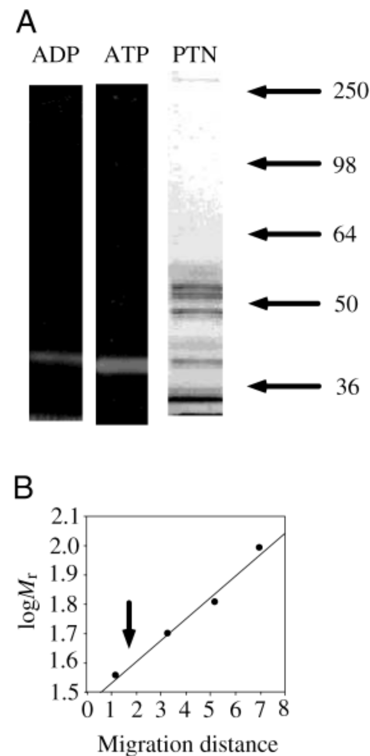

Fig. 4. SDS–PAGE gel electrophoresis of salivary homogenates of *Phlebotomus papatasi*. (A) ADP and ATP indicate lanes showing calcium phosphate precipitate (dark-field illumination) following incubation with either ADP or ATP, respectively. PTN indicates the lane stained with Coomassie Blue. Numbers and arrows indicate the positions of M_r markers (in kDa). (B) Graph showing the range of migration of apyrase activity as a function of the logM_r markers (30–98 kDa). The arrow marks the position of the apyrase band relative to molecular mass markers.

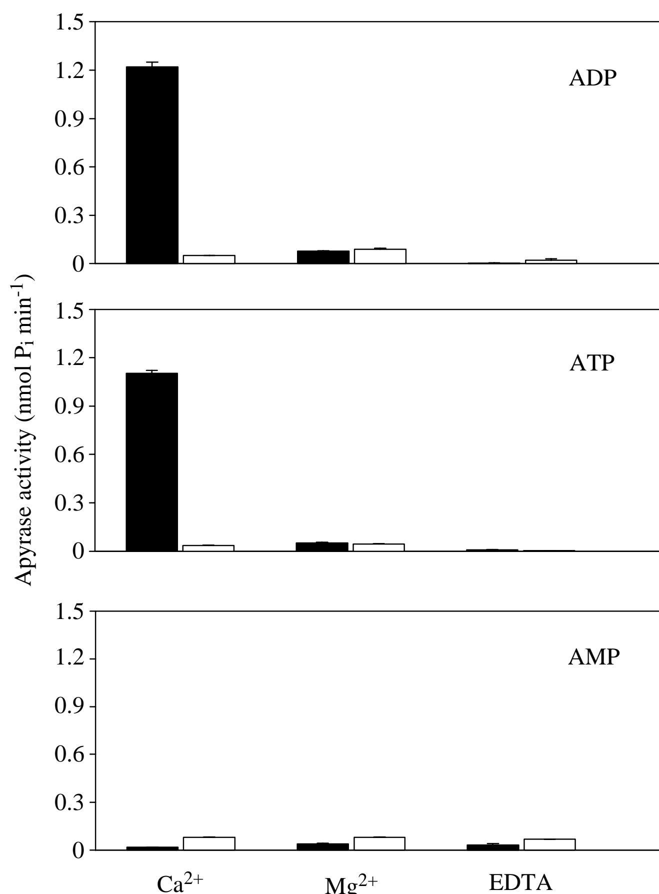

Fig. 5. Apyrase activity of cells transfected with *Phlebotomus papatasi* salivary apyrase (PpApy) cDNA. Supernatants of High Five cells transfected with PpApy cDNA (filled columns) or with control plasmid (open columns) were incubated with ATP, ADP or AMP in the presence of 5 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} $MgCl_2$ or 10 mmol l^{-1} EDTA. The columns indicate the mean + S.E.M. of triplicate measurements.

containing 20 pairs of homogenized salivary glands) to a PVDF membrane yielded the amino-terminal sequence underlined in Fig. 1, predicted by the cDNA sequence for *P. papatasi* (PpApy).

To test whether PpApy cDNA codes for a true apyrase enzyme, we transfected a pIB/V5-His expression plasmid carrying the PpApy cDNA (rPpApy). As a control, we used the PpApy cDNA in the reverse orientation of the pIB/V5-His expression vector (Ctl). Insect cells were transfected with these two constructs *in vitro*, and supernatants were tested for apyrase activity. While control cells did not secrete any detectable apyrase activity, the PpApy-transfected cells secreted Ca^{2+}-dependent ATPase and ADPase activities (Fig. 5). No AMPase activities were detected in any of the cell samples (Fig. 5). The ATPase and ADPase activities were inhibited in the presence of EDTA and were not activated by Mg^{2+}. In addition, the cells transfected with PpApy secreted an apyrase activity with a pI of 9.0, identical to the observed pI when assaying salivary homogenates of *P. papatasi* (Fig. 6).

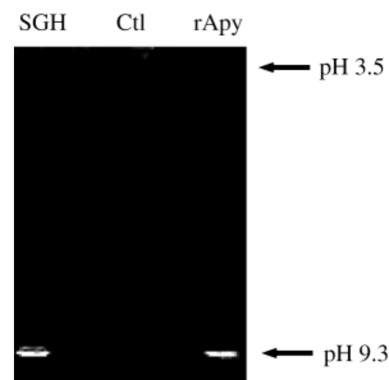

Fig. 6. Isoelectric focusing gel of recombinant *Phlebotomus papatasi* salivary apyrase (PpApy) and the native salivary apyrase from *Phlebotomus papatasi*. Supernatants of High Five cells transfected with PpApy cDNA (rApy) or with control plasmid (Ctl) and salivary gland homogenate of *P. papatasi* (SGH) were separated by isoelectric focusing gel electrophoresis with a pH gradient from 3 to 9. Apyrase activity was measured as described in the Materials and methods section.

Discussion

The salivary glands of hematophagous arthropods contain a very large diversity of compounds affecting vertebrate hemostasis, inflammation and immunity (Ribeiro, 1995). The compounds presumably help these animals to obtain a blood meal. It appears that, in their scramble for selecting these adaptive compounds, the arthropods visit their whole genome in search of molecules to enhance their feeding ability. For example, the anticlotting molecule of the mosquito *Aedes aegypti* is a 54 kDa inhibitor of coagulation factor Xa and a member of the serpin family of proteins (Stark and James, 1998), while *Anopheles albimanus* contain a 6.5 kDa anti-thrombin with no homology to other known proteins (Valenzuela et al., 1999). The vasodilatory molecules of these two mosquitoes are also unrelated (Champagne and Ribeiro, 1994; Ribeiro and Valenzuela, 1999). The blood-sucking bug *Rhodnius prolixus* has evolved several different salivary lipocalins that serve as anticlotting agents (Ribeiro et al., 1995), as carriers of nitric oxide (Champagne et al., 1995a), as histamine binders (Ribeiro and Walker, 1994) and as ADP binders (Francischetti et al., 2000), each affecting different aspects of host hemostasis or inflammation. The bed bug *Cimex lectularius* has evolved a completely different molecule to carry nitric oxide, a member of the inositol phosphate phosphatase protein family (Valenzuela and Ribeiro, 1998), not a lipocalin as in *Rhodnius prolixus*. Our earlier discovery of a novel Ca^{2+}-dependent salivary apyrase in *Cimex lectularius* (an enzyme destroying ADP, a mediator of platelet aggregation) (Valenzuela et al., 1998) could therefore reflect the discovery of a new family of apyrase enzymes or, alternatively, the finding of a unique enzyme that evolved from an unrelated protein.

In this paper, we present evidence that the salivary glands of the sand fly *P. papatasi* contain a cDNA encoding for the

abundant salivary apyrase present in this organ. This cDNA sequence belongs to the novel *Cimex* family of apyrases, which has no similarities to other known protein families. Because dipterans and hemipterans are at least 300 million years apart in evolutionary time (Labandeira and Sepkoski, 1993), the finding of these two very similar enzymes in these organisms (both confirmed by cDNA expression of the enzymatic activity) indicates that the *Cimex* family of apyrases is a *bona fide* family. Furthermore, the finding of sequences in *Homo sapiens,* the flies *Drosophila melanogaster* and *Lutzomyia longipalpis*, the nematode *Caenorhabditis elegans* and the protozoan *Cryptosporidium parvum* having high similarities to *Cimex lectularius* and *Phlebotomus papasi* apyrase indicates that this family of enzymes is widespread in animals.

The salivary apyrases of sand flies (Ribeiro et al., 1986; Ribeiro et al., 1989), as well as those of *Cimex lectularius* (Valenzuela et al., 1998), have a strict dependence on Ca^{2+}, while those of the CD39 family (Plesner, 1995) or the 5′-nucleotidase family (Zimmermann, 1992) work with either Ca^{2+} or Mg^{2+}. The search for novel members of the *Cimex* family of apyrases in vertebrates may thus exploit this characteristic, perhaps by screening tissue homogenates, or tissue homogenates submitted to chromatography, for their ability to hydrolyze ADP in the presence of Ca^{2+}, compared with the activity in the presence of Mg^{2+} and EGTA. Perhaps because *Cimex*-type apyrases are relatively small proteins (approximately 35 kDa), they tend to be relatively resistant to denaturation; the *Cimex lectularius* enzyme has been successfully reactivated after reverse-phase chromatography containing acetonitrile and trifluoroacetic acid (Valenzuela et al., 1998), while *Phlebotomus papasi* apyrase renatured well after SDS–PAGE electrophoresis (Fig. 5). This protein stability, together with their strict Ca^{2+}-dependence, could be exploited when assaying tissue homogenates for a *Cimex*-type apyrase activity.

In conclusion, it appears that apyrases, akin to several other enzymatic activities such as proteases and glycosidases (Davies and Henrissat, 1995; Rawlings and Barrett, 1993), are the product of convergent evolution and, as such, are coded for by several families of unrelated genes: the actin/heat shock 70/sugar kinase, the 5′-nucleotidase and the *Cimex* family of proteins. The *Cimex* family of apyrases is thus characterized by a strict Ca^{2+}-dependency and a unique sequence, and was probably acquired early in animal evolution because its sequence is found only in protozoans, worms, insects and humans, but not in bacteria and plants.

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