Purification, Cloning, and Expression of an Apyrase from the Bed Bug Cimex lectularius

A NEW TYPE OF NUCLEOTIDE-BINDING ENZYME*

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An enzyme that hydrolyzes the phosphodiester bonds of nucleoside tri- and diphosphates, but not monophosphates, thus displaying apyrase (EC 3.6.1.5) activity, was purified from salivary glands of the bed bug, Cimex lectularius. The purified C. lectularius apyrase was an acidic protein with a pI of 5.1 and molecular mass of ~40 kDa that inhibited ADP-induced platelet aggregation and hydrolyzed platelet agonist ADP with specific activity of 379 units/mg protein. Amplification of C. lectularius cDNA corresponding to the N-terminal sequence of purified apyrase produced a probe that allowed identification of a 1.3 kilobase pair cDNA clone coding for a protein of 364 amino acid residues, the first 35 of which constituted the signal peptide. The processed form of the protein was predicted to have a molecular mass of 37.5 kDa and pI of 4.95. The identity of the product of the cDNA clone with native C. lectularius apyrase was proved by immunological testing and by expressing the gene in a heterologous host. Immune serum made against a synthetic peptide with sequence corresponding to the C-terminal region of the predicted cDNA clone recognized both C. lectularius apyrase fractions eluted from a molecular sieving high pressure liquid chromatography and the apyrase active band from chromatofocusing gels. Furthermore, transfected COS-7 cells secreted a Ca2+-dependent apyrase with a pI of 5.1 and immunoreactive material detected by the anti-apyrase serum. C. lectularius apyrase has no significant sequence similarity to any other known apyrases, but homologous sequences have been found in the genome of the nematode C. elegans and in mouse and human expressed sequence tags from fetal and tumor EST libraries.

Vertebrates protect themselves against excessive blood loss by activating blood clotting mechanisms that are induced by platelet aggregation at the site of injury. To feed effectively, blood-sucking arthropods have developed various mechanisms that counteract these host responses (1, 2). Since ADP released by injured cells and by aggregating platelets is one of the most important physiological mediators of platelet aggregation (3), saliva of most blood-feeding arthropods contains large amounts of apyrase (ATP:diphosphohydrolase, EC 3.6.1.5) that hydrolyzes ATP and ADP into AMP and P1 and thus inhibits platelet aggregation (4–6). After some initial controversy over whether apyrase activity resides in specialized enzymes or just represents a combination of enzymes (discussed in Refs. 1 and 7), apyrase enzymes from various plant and animal sources have been purified and characterized in sufficient detail (5, 8–18).

These studies revealed that, despite their common ability to hydrolyze ATP and ADP with approximately the same activity, apyrase preparations from different sources show wide variance in absolute activities, pH optima, and divalent cation requirements (4). Moreover, the first two apyrases to be sequenced, those from salivary glands of the mosquito Aedes aegypti (5) and from the parasitic protozoan Toxoplasma gondii (8) turned out to belong to completely different enzyme families. A. aegypti salivary apyrase is a member of the 5'-nucleotidase family of enzymes; unlike vertebrate 5'-nucleotidases, however, this apyrase is a soluble protein and does not hydrolyze the ester linkage of AMP (5). In contrast, T. gondii apyrase is almost identical to an NTPase (EC 3.6.1.15) from the same organism (8) and belongs to a large family of ecto-ATPases, also referred to as E-ATPases (7). In addition to recently characterized apyrases from potatoes (9), chickens (10, 11), rats (12, 13), and humans (14, 15), this family includes more specialized enzymes, such as chick gizzard ATPase (16) and yeast GDPase (19). Despite the lack of sequence similarity, apyrases from both families are active with either Mg2+ or Ca2+ and are commonly referred to as (Ca2+, Mg2+)-apyrases (20). Recently, we have found that the apyrase activity from the bed bug Cimex lectularius is strictly dependent upon Ca2+ ions and, unlike any previously described apyrase, cannot be activated by Mg2+, Mn2+, or Zn2+ (21).

Here we report purification, cloning, and heterologous expression of the salivary apyrase from C. lectularius. This enzyme is clearly not homologous to the salivary apyrase from A. aegypti (5). This means that, just as blood feeding has independently evolved in different groups of insects, salivary apyrase activity that usually accompanies blood sucking has been independently acquired by these two different insects. Moreover, we found no sequence similarity between C. lectularius apyrase and any previously characterized nucleotide-binding enzymes, which indicates that it belongs to a novel type of ATPases.

EXPERIMENTAL PROCEDURES

Insect Rearing—C. lectularius colonies were maintained at 27 °C and 65% humidity. Insects were fed every 10 days by exposing them to the shaved abdomen of an anesthetized rabbit. Salivary glands of insects of 8–10 days after feeding were dissected and stored in Hepes saline (10 mM Hepes at pH 7.0 in 150 mM NaCl) at −75 °C until needed. Before use, salivary glands were thawed and disrupted with a pestle, and the

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homogenate was cleared by centrifugation at 14,000 rpm for 5 min at 4 °C.

Apyrase Activity—Apyrase activity of the fractions from different stages of purification was assayed by measuring the release of P_i from ATP or ADP in 50 mM Tris-Cl buffer (pH 8.5), containing 0.1 mM NaCl, 5 mM MgCl_2, and 0.01% gelatin. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of orthophosphate/min at 37 °C.

ADP hydrolysis was also monitored by a platelet aggregation assay that was performed on a Thromtom microparticle reader (Molecular Devices, Menlo Park, CA) with a kinetic module (22) with further anti-platelet activity.

The procedure could be analyzed quickly and simultaneously for absorbance after a lag phase indicated aggregation of platelets. With 650 nm were taken at 60-s intervals. A rapid and sharp decrease in absorbance after a lag phase indicated aggregation of platelets. With 650 nm were taken at 60-s intervals. A rapid and sharp decrease in absorbance after a lag phase indicated aggregation of platelets.

A novel type of apyrase, defined as the amount of enzyme that released 1 μmol of orthophosphate/min at 37 °C.

The primers were then used as a template for the PCR reactions described below.

A synthetic peptide (KLVIEETKIDDHKYEGDVDF) based on the predicted C-terminal region of the apyrase clone was produced at the Laboratory of Molecular Structure, NIAID, National Institutes of Health (Twinbrook Facility, Rockville, MD). Five milligrams of the synthetic peptide were conjugated to 7 mg of keyhole limpet hemocyanin at the molar coupling ratio of peptide to carrier of 122 and used to immunize rabbits at Spring Valley Laboratories (Woodbine, MD). Preimmune sample was taken before the first injection, and immune serum samples were taken after two and three injections, respectively. Antibody specificity was verified by ELISA of crude salivary homogenate and prepurified apyrase with immune and preimmune sera.

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A novel type of apyrase was detected by submerging the gel into a solution of enzyme activity, which was complexed with calcium in the solution to form calcium phosphate precipitate in the surface of the gel.

ELISA of C. lectularius Apyrase—Salivary gland proteins eluted from the molecular sieving HPLC column were assayed with preimmune and immune serum (anti-apyrase) using conventional ELISA protocols. Dilutions of preimmune and immune serum were 1:500, and dilutions for the secondary antibody (anti-rabbit IgG peroxidase conjugate; Sigma) were 1:2000.

Western Blotting—Proteins separated by isoelectric focusing gel electrophoresis were transferred to a nitrocellulose membrane (Schleicher and

1 The abbreviations used are: HPLC, high pressure liquid chromatography; BSA, bovine serum albumin; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; ESI, expressed sequence tag.
& Schuell) by diffusion for 40 min at 37 °C, followed by blocking with 6% BSA in 20 mM Tris, pH 8.0, and 500 mM NaCl for 1 h. Primary antibody to C. lectularius salivary apyrase peptide was used at a dilution of 1:200 in 20 mM Tris, 500 mM NaCl and was incubated for 2 h at room temperature; preimmune serum was run as a control. After three washes with 20 mM Tris, pH 8.0, and 500 mM NaCl, the membrane was incubated with an anti-rabbit IgG peroxidase conjugate (1:10,000) for 1 h at room temperature. The membrane was then washed three times as described above. Bands were visualized using the 3,3’,5,5’-tetramethyl benzidine membrane peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Construction of the Expression Plasmid—The expression plasmid pCI-neo (Promega, Madison, WI) was used to express C. lectularius recombinant apyrase. Recombinant protein was made without the removal of the signal peptide to allow secretion of the produced protein into the medium.

RESULTS

Purification of C. lectularius Salivary Apyrase—The salivary apyrase activity of C. lectularius was previously found (21) to be exclusively dependent on Ca2+, to have an optimum pH of 8.5, and to hydrolyze ATP and ADP but not AMP. Additionally, the pl of the apyrase activity was determined to be 5.1. Based on this result, C. lectularius salivary homogenates were chromatofocused on a column eluting with a pH gradient from 6.3 to 4.0. A well isolated peak with apyrase activity eluted at pH 5.1, as expected (Fig. 1A). The active fractions hydrolyzed ATP ([Fig. 1B]) and ADP (Fig. 1C) but not AMP (Fig. 1D). The active fractions were further injected into a reverse-phase column, resulting in the separation of two UV-absorbing peaks (Fig. 2A). Aliquots from the reverse-phase fractions, dried in the presence of 10 μg BSA and resuspended in buffer, allowed apyrase activity to be detected in the small peak eluting at 45
An aliquot of the apyrase-containing peak was subjected to SDS-polyacrylamide gel electrophoresis and resulted in the presence of only one silver-stained band of approximately 40 kDa (not shown). The remaining material was enzymatically cleaved with trypsin, and three internal peptides with the following sequences were obtained: 1) TVEADDTETYFTAFDLEGK, 2) KFETQGANVII, and 3) ALSQEAYDSK. No N-terminal sequence was attempted during this purification step.

Later, we found that using strong cation exchange HPLC at the first step resulted in even more effective purification of C. lectularius salivary apyrase. After cation exchange chromatography, both apyrase and anti-platelet activities co-eluted (Fig. 3). Additionally, this fraction inhibited collagen-induced platelet aggregation when using a low dose (5 mg/ml) of collagen (not shown). The specific activity for ADP hydrolysis at this step was 379 units/mg protein (Fig. 3). The active apyrase fraction was submitted to reverse-phase chromatography. Aliquots of the fractions were dried in the presence of 10 mg of BSA and tested for apyrase activity. A well isolated peak with the same retention time as in the first purification strategy showed apyrase activity (data not shown). Edman degradation of this protein revealed the sequence YELGHASGETNANSKYPLTPVEENLKVFKIGVISDDKNASVKEKAGNYLVGSIGKBEWTKEGCACAATTGGATATTTATATGCTCCAACAGTAACATATGCT. Isolation of C. lectularius Apyrase cDNA Clone—The N-terminal amino acid sequence of the purified apyrase was used to design oligonucleotide primers for a PCR reaction using as a template cDNA of C. lectularius salivary glands. A single PCR product of 150 base pairs was obtained, and its sequence was found to correspond to the N-terminal region of the native C. lectularius apyrase. This PCR product was then labeled by digoxigenin and used to screen a C. lectularius salivary gland cDNA library. Two positive clones of approximately 1.3 kilobase pairs in length were obtained and sequenced. Their sequences turned out to be identical, and each contained a single open reading frame of 1092 base pairs.

Analysis of C. lectularius Apyrase cDNA Sequence—We sequenced 1354 base pairs of the isolated apyrase clone, which contained an open reading frame coding for 364 amino acids (Fig. 4). The sequences of three internal tryptic peptides of C. lectularius apyrase were all represented in the deduced protein, and its sequence from Tyr-36 to Thr-85 coincided with the N-terminal sequence of purified C. lectularius apyrase obtained by Edman degradation (Fig. 4, underlined). The first 35 amino acids of the predicted protein were thus considered to form the signal peptide and the remaining 329 amino acids to constitute the mature protein. Indeed, sequence analysis of the unprocessed protein using the SignalP program (24) identified the N-terminal part of this open reading frame as a potential signal peptide and predicted the cleavage site between the residues 35 and 36. No other cell targeting signals have been detected in this sequence by the PSORT program (25), indicating that the product of this gene was secreted outside the cells. The analysis of the predicted processed protein indicated a molecular mass of 37,558 Da and isoelectric point of 4.95 with a minus 10.64 charge at pH 7.0. Search of the nonredundant protein data base at the National Center for Biotechnology Information (Bethesda, MD) using the gapped BLASTP program (26) did not find any statistically significant similarity between the predicted sequence of the C. lectularius salivary apyrase and sequences of any characterized enzymes, including other apyrases (5, 14–16).
other arpyrases, such as CD39 (12, 15), potato apyrase (9), or A. aegypti salivary apyrase (5) using the MACAW (27), BLAST 2.0 (26), and Lalign (28) programs also did not reveal any significant similarity or conserved sequence motifs.

Anti-apyrase Antibody Recognized C. lectularius Salivary Apyrase—Because the sequence of the C. lectularius apyrase was not similar to other arpyrases, we made antibodies against the predicted sequence of the putative carboxyl-terminal region (KVLIEETKIDDHKYEGVDFV) of the C. lectularius apyrase to investigate whether antigenic activity would co-elute with apyrase activity in different protein separation protocols. Accordingly, when salivary gland homogenate of C. lectularius was subjected to molecular sieving HPLC column, a chromatographic procedure different from those used above to purify the protein, the apyrase activity co-eluted with immunoreactive activity (Fig. 5). Western blot analysis also confirmed recognition by the anti-serum of antigen co-localizing with apyrase activity in isoelectric focusing gels (Fig. 6). An antiserum against the C-terminal region predicted by CL-Apy cDNA thus recognized an antigen co-eluting with apyrase activity by two independent protein separation methods.

Expression of Cimex Apyrase cDNA in COS-7 Cells—Evidence presented so far strongly indicates that the 1.3-kilobase pair cDNA described in this work represents a salivary cDNA expressing a protein with apyrase activity. However, it could be derived from a co-purified molecule deprived of apyrase activity. In order to test whether the obtained CL-Apy cDNA sequence would translate into a protein with apyrase activity, we expressed it in COS-7 cells. While control COS-7 cells supernatants displayed (Ca\(^{2+}\), Mg\(^{2+}\))-dependent nucleotidase activity (Fig. 7, white bars), CL-Apy cDNA-transfected cells additionally produced an apyrase activity that was exclusively Ca\(^{2+}\)-dependent (Fig. 7, dark bars) and with the same pI as the native enzyme (Fig. 8). Moreover, Western blots of transfected COS-7 cell supernatants yielded antigenic material recognized by the anti-apyrase antiserum generated against the C-terminal region of the putative apyrase gene product (Fig. 9). The immunoreactive band resulting from the recombinant protein had an apparent molecular weight slightly larger than that observed with the native antigen (Fig. 9) and could be the result of protein modification (e.g. glycosylation, see below) by the host cells. We conclude that the CL-Apy cDNA depicted in Fig. 4 codes for a protein displaying apyrase activity.

Sequence Analysis of Cimex Apyrase—As noted above, sequence similarity searches of the National Center for Biotechnology Information protein data base did not show any statistically significant matches between C. lectularius salivary apyrase and any previously characterized enzymes. In fact, the only protein in the data base that showed any significant (p < 0.1) similarity to C. lectularius apyrase (Fig. 10) was an unknown protein from the nematode worm Caenorhabditis elegans (National Center for Biotechnology Information accession no. 868189), discovered in the course of the C. elegans sequencing project at Washington University (St. Louis, MO). Analysis of the GenBank™ expressed sequence tag (EST) data base, however, showed that proteins homologous to C. lectularius apyrase are also encoded in mouse and human genomes (Fig. 10) and are actually expressed in these organisms. Most of the human and mouse EST, however, corresponded only to the C-terminal part of the C. lectularius apyrase, which could be due to mRNA degradation or incomplete reverse transcription in the process of making these ESTs.

The alignment of the predicted protein sequences (Fig. 10) shows several highly conserved regions that could be related to the catalytic activity of C. lectularius apyrase. In particular, conserved Aasp residues in the conserved motif DDBTG, located in a short loop between two strands, could play a role in binding Ca\(^{2+}\) ions, which are required for C. lectularius apyrase activity (Fig. 6). Remarkably, none of the conserved regions highlighted in Fig. 10 corresponds to any previously

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2 T. Tatusov and T. L. Madden, unpublished; available at the National Center for Biotechnology Information World Wide Web site at http://www.ncbi.nlm.nih.gov/BLAST.
described sequence motif. Conversely, scanning the *C. lectularius* apyrase sequence against the PROSITE and Pfam data bases (29, 30) identified potential glycosylation, phosphorylation, and *N*-myristoylation sites but did not reveal any previously described nucleotide-binding sequence motifs. A direct comparison of the *C. lectularius* apyrase with several recently described generalized ATP-binding motifs, such as ATP-grasp (31) or P-ATPase (32), also failed to identify its nucleotide-binding motif. These observations indicate that *C. lectularius* apyrase and its homologs constitute a novel family of enzymes that contains new type of nucleotide-binding site.

**DISCUSSION**

We have purified, cloned, and expressed a soluble apyrase from the salivary glands of the bed bug, *C. lectularius*. Since the sequence of this enzyme is substantially different from that of any other reported apyrase, special efforts were undertaken to verify that the sequenced cDNA clone (Fig. 4) actually codes for *C. lectularius* apyrase.

The sequence obtained for *C. lectularius* apyrase was based on two independent purification protocols (chromatofocusing and reverse-phase HPLC or strong cation exchange and reverse-phase HPLC) that yielded different segments of the primary protein structure, all contained within the translated cDNA sequence. An antiserum against the putative carboxyl-terminal peptide, obtained from the information contained in the cDNA clone, recognized the apyrase-containing fractions in two different immunoassays (ELISA of samples from molecular sieving fractionation and the Western blot of salivary homogenate separated by isoelectric focusing gel electrophoresis). The final confirmation of the validity of the cDNA as coding for an apyrase was indicated by the expression and secretion by transfected COS-7 cells of a Ca\(^{2+}\)-dependent apyrase with molecular weight, pI, and immunoreactivity similar to the native *C. lectularius* protein.

Although somewhat unexpected, the identification of a novel type of apyrase in *C. lectularius* is hardly surprising. The existence of enzymes that have arisen independently to have a common activity has been repeatedly observed before in different enzyme groups, *e.g.* proteases (33) and glycosidases (34) (see Refs. 35 and 36 for discussion). A comprehensive analysis of such analogous, as opposed to homologous, proteins shows that they are found in at least 5% of all of the enzyme classification nodes for which sequences are currently available (36). In many cases, such analogous enzymes seem to evolve by recruitment of enzymes acting on different but related substrates, *i.e.* by minor structural change of a protein that leads to a novel substrate specificity or even a new class of reactions.
(36, 37).

The selective pressure to counteract host hemostasis mechanisms, coupled with independent adaptation to hematology in different groups of blood-sucking arthropods makes their salivary glands a place where the recruitment scenario could be particularly advantageous. Indeed, while apyrases from plants, vertebrates, and the protozoan parasite T. gondii are closely related to ATPases, the enzyme from the mosquito A. aegypti appears to have evolved from 5'-nucleotidases (5). Structurally, shifting from AMP to ADP, the substrate specificity of the enzyme that still catalyzes the same reaction (cleaving of the terminal phosphate) should not take too many amino acid residues (A, I, L, V, M, F, Y, or W) with a propensity to fold β-strand. Secondary structure elements predicted by the PHD program (41) are indicated as H (α-helices), E (β-strands), and L (loop regions); positions of conserved Pro and small residues (G, A, or S) are indicated by asterisks.

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REFERENCES

1. Ribeiro, J. M. C. (1995) Infect. Agents Dis. 4, 143–152
2. Law, J. H., Ribeiro, J. M. C., and Wells, M. A. (1992) Annu. Rev. Biochem. 61, 87–111
3. Marcus, A. J., and Safier, L. B. (1993) FASEB J. 7, 516–522
4. Ribeiro, J. M. C. (1997) Annu. Rev. Entomol. 32, 463–478
5. Champagne, D. E., Smartt, C. T., Ribeiro, J. M. C., and James, A. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 694–698
6. Champagne, D. E., and Valenzuela, J. G. (1996) in The Immunology of Host- Ectoparasitic Arthropod Relationships (Wikel, S. K., ed), pp. 85–106, CAB International, Walingford, Oxon, UK
7. Plesner, L. (1995) Int. Rev. Cytol. 158, 141–214
8. Asai, T., Miura, S., Sibata, S. L., Okabayashi, H., and Takeuchi, T. (1995) J. Biol. Chem. 270, 11391–11397
9. Handa, M., and Guidotti, G. (1996) Biochem. Biophys. Res. Commun. 218, 916–923
10. Strobel, R. S., Nagy, A. K., Knowles, A. F., Buegel, J., and Rosenberg, M. D. (1996) J. Biol. Chem. 271, 16323–16331
11. Nagy, A. K., Knowles, A. F., and Nagami, G. T. (1996) J. Biol. Chem. 271, 1076–1081
12. Wang, T. F., Rosenberg, P. A., and Guidotti, G. (1996) Mol. Biol. Cell. 7, 1078–1092
13. Kegel, B., Braun, N., Heine, P., Malszewski, C. R., and Zimmermann, H. (1997) Neuropeptides 31, 1189–1200
14. Christoforidis, S., Papamarkaki, T., Galaris, D., Kellner, R., and Tsolas, O. (1997) J. Biol. Chem. 272, 11391–11397
15. Kettlun, A. M., Mancilla, M., Valenzuela, M. A., and Verjovski-Almeida, S. (1997) Biochem. J. 321, 691–698
16. Kaczmarek, E., Kozlowski, E., Sevigny, J., Siegel, J. B., Anrather, J., Beaudoin, A. R., Bach, F. H., and Robson, S. C. (1996) J. Biol. Chem. 271, 1076–1081
17. Vasconcelos, E. G., Ferreira, S. T., de Carvalho, T. M. U., de Souza, W., Kettlun, A. M., Mancilla, M., Valenzuela, M. A., and Verjovski-Almeida, S. (1996) J. Biol. Chem. 271, 22139–22145
18. Sevigny, J., Levesque, F. P., Gendrin, G., and Beaudoin, A. R. (1997) Biochem. Biophys. Acta 1334, 73–88
19. Abejón, J., Yanagisawa, K., Mandon, E. C., Hausler, A., Moremen, K., Hirschberg, C. B., and Robins, P. W. (1993) J. Cell Biol. 122, 307–323
20. Wang, T. F., and Guidotti, G. (1996) J. Biol. Chem. 271, 9989–9991
21. Valenzuela, J. G., Chuff, M. O., and Ribeiro, J. M. C. (1996) Insect Biochem. Mol. Biol. 26, 557–562
22. Bednar, B., Condra, C., Gould, R. J., and Connolly, T. M. (1995) Thromb. Res. 77, 453–463

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3. Champagne and J. M. C. Ribeiro, unpublished data (GenBank accession number U700377).
