

The salivary gland-specific apyrase of the mosquito *Aedes aegypti* is a member of the 5'-nucleotidase family

(insect vector/bloodfeeding/platelet antiaggregation/ATP diphosphohydrolase)

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ABSTRACT The saliva of hematophagous insects contains a variety of pharmacologically active substances that counteract the normal hemostatic response to injury in vertebrate hosts. The yellow-fever mosquito, *Aedes aegypti*, secretes an apyrase that inhibits ADP-dependent platelet aggregation. Apyrase was purified as an active enzyme from adult female salivary glands and subjected to tryptic digestion, and the resulting peptides were sequenced. The amino acid sequences obtained match the conceptual translation product of a cDNA clone isolated from an adult female salivary gland library. Sequence comparisons indicate similarities with a ubiquitous family of 5'-nucleotidases. The mosquito protein differs from other members of the family by lacking a carboxyl-terminal hydrophobic domain. The apparent conversion of a gene encoding an enzyme involved in a common metabolic event at the cellular level to a gene involved in the antihemostatic response of mosquitoes illustrates one way this particular insect has adapted to the challenges of bloodfeeding.

The name apyrase (ATP diphosphohydrolase, EC 3.6.1.5) was first used by Meyerhof (1) to describe a yeast enzyme that hydrolyzed the pyrophosphate bonds of nucleoside di- and triphosphates. Apyrase activity was found also in vertebrate tissues, but the existence of apyrase was challenged when Kalckar (2) showed that the activity in muscle resulted from the concerted action of an ATPase and adenylate kinase. However, true apyrase activity was purified to apparent homogeneity from plants, especially potato tubers and pea cotyledons (3), and more recently, apyrase has been recognized in a variety of vertebrate tissues (4–6) and in the salivary secretions of many hematophagous insects (7, 8). The functional significance of most of these enzymes remains unknown, but insect salivary apyrases inhibit platelet aggregation by destroying adenosine di- and triphosphates released from injured cells or by activated platelets.

The secreted salivary apyrase of the mosquito *Aedes aegypti* shares a number of similarities with vertebrate pancreatic and endothelial apyrases, including molecular weight, pH sensitivity, divalent cation dependence, and immunological cross-reactivity (9). However, the gene family to which this enzyme belongs was previously unknown. In this report, the salivary apyrase of *A. aegypti* is characterized as a member of the 5'-nucleotidase gene family.[¶]

MATERIALS AND METHODS

Mosquitoes. Adult mosquitoes (*A. aegypti*, Rockefeller strain) were reared in a 27°C controlled environment room at 80% relative humidity with a 16 hr light/8 hr dark cycle. All

animals used in these experiments were 2- to 7-day-old adult females. Mosquitoes were fed a 10% sucrose solution.

Purification, Activity, and Peptide Sequencing of Salivary Gland Apyrase. Salivary glands from adult female *A. aegypti* mosquitoes were dissected in phosphate-buffered saline (PBS), transferred to 10 mM Tris (pH 7.5), and stored frozen at –80°C. To purify active apyrase, 100 pairs of glands were homogenized with a Branson Sonifier (Model 450) at power setting 6 and 40% duty cycle to allow dissipation of heat. Sonicated glands were centrifuged at 14,000 rpm in a bench-top microcentrifuge, and the supernatant was transferred to a 30-kDa-cutoff Centricon filter (Amicon) and concentrated to 200 μ l. The retentate was chromatographed on a Cibacron Blue column (Alltech) with a 60-min gradient of 0.0–1.0 M NaCl in 20 mM Hepes (pH 6.8), run at 0.5 ml/min. Apyrase activity was monitored as the release of P_i from ATP and ADP (10, 11). Fractions with apyrase activity were concentrated and washed with 20 mM Hepes (pH 6.8) in a fresh Centricon filter, then chromatographed on a Macrosphere WCX 7- μ m weak cation-exchange column (Alltech) using the same mobile phase as before. To minimize nonspecific binding of enzyme to the tube walls, 0.5-ml fractions from the WCX column were collected into siliconized tubes with 50 μ l of bovine serum albumin (BSA) at 1 mg/ml, so that the final BSA concentration was 0.1 mg/ml. An aliquot of the initial homogenate and fractions collected from both columns were assayed for hydrolysis of ATP, ADP, and AMP.

Purified apyrase was incubated at 37°C in 1 ml of 1 mM ATP or AMP in pH 9.0 reaction buffer (10), and 100- μ l aliquots were removed at 0, 1, 2, 4, 8, 15, 30, 60, and 120 min. Controls without apyrase were treated in an identical manner. Aliquots were diluted 10-fold, and 20 μ l of each was analyzed for ATP, ADP, AMP, and adenosine by HPLC on a DEAE TSK column (Bio-Rad). The mobile phase was 0.125 M sodium phosphate (pH 3.5) for 15 min, followed by a 1-min gradient to 0.5 M sodium phosphate and a further 9 min at the higher salt concentration, run at 1 ml/min. Absorbance was monitored at 259 nm.

To purify sufficient active apyrase for protein sequencing, 1000 pairs of *A. aegypti* salivary glands were prepared by HPLC using the Cibacron Blue column followed by the WXC column as described above. In preparation for tryptic digests, salt was removed from the active apyrase by adjusting the pH to 2.0 with trifluoroacetic acid and chromatographing on a Hamilton PRP-[®] C₁₈ reverse-phase column (Alltech) developed with a 60-min gradient of 20–60% acetonitrile in 0.1% trifluoroacetic acid at 1 ml/min. Tryptic digestion of the purified apyrase and microsequencing of peptides were performed by Harvard Microchemistry (Harvard University) under the direction of W. Lane.

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[¶]The sequence reported in this paper has been deposited in the GenBank data base (accession no. L12389).

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Table 1. Purification of apyrase from 100 salivary gland pairs

Step	Apyrase activity		Protein, μ g	Yield, [†] %	Specific activity, units/mg	Fold purification [‡]
	Substrate	Units*				
Homogenate	ATP	35.8	361.2	100	99.1	
	ADP	35.8			99.1	
	AMP	0			0	
Cibacron	ATP	32.5	17.2	90.8	1886.6	19.0
	ADP	35.7		99.7	2075.6	20.9
	AMP	0			0	
WCX	ATP	9.4	5.3	26.3	1767.9	17.8
	ADP	6.0		16.8	1126.4	11.4
	AMP	0			0	

*Defined according to Ribeiro *et al.* (10), where 1 unit equals the release of 1 μ mol of orthophosphate per min, except that reactions were done at 37°C instead of 30°C.

[†]Calculated as the percent recovery of units.

[‡]Calculated by comparing fractionation steps with the homogenate.

Isolation and Characterization of the Apyrase cDNA. Differential screening of a cDNA library made from female salivary gland mRNA resulted in the recovery of clones with homology to genes expressed specifically in the adult female salivary glands (12). The cDNA insert of the clone λ SGG12 was isolated and ligated into plasmid vector pTZ18U (U.S. Biochemical) for sequencing by the dideoxy chain-termination method (13). An additional clone, λ SGG12-5, consisting of a cDNA made to the 5' end of the transcription product, was isolated by using a genomic fragment of DNA that overlapped both λ SGG12 and λ SGG12-5. λ SGG12-5 was cloned into pBluescript II SK(+/-) phagemid (Stratagene) for sequencing. Protein database searches were done with the National Center for Biotechnology BLAST Network services (14). Comparison and alignment of the apyrase with human and rat 5'-nucleotidases were performed with the multiple-sequence alignment programs (Genetics Computer Group, University of Wisconsin).

Preparation of Anti-Apyrase Antibodies and Immunoblot Analysis. The cDNA clone λ SGG12 was used to produce recombinant apyrase protein. The λ SGG12 open reading frame, excluding sequence 3' of the polyadenylation signal, was cloned between the *Xho* I and *Bam*HI sites of the bacterial expression plasmid pET His-Tag14b (Novagen) by means of appropriate linkers added to the λ SGG12 clone via PCR amplification. Bacterial protein was subjected to SDS/10% PAGE and the recombinant apyrase band was excised. The gel slice was processed (15) and used to generate antibodies in a rabbit. Serum was collected and the IgG fraction was isolated (16). For Western analysis of female salivary glands, purified apyrase, and bacterially expressed recombinant apyrase, proteins were separated by SDS/10% PAGE and transferred to nitrocellulose in modified Towbin buffer (17). Blocking was performed overnight with 1% polyvinylpyrrolidone in Tris-buffered saline containing 0.2% Tween 20. Western analysis and detection were adopted from the ECL Western blotting protocols provided by Amersham. The secondary antibody reagent, goat anti-rabbit IgG conjugated to horseradish peroxidase, was from Sigma. Both primary and secondary antibodies were used at a dilution of 1:10,000.

RESULTS

Apyrase was purified in two separate experiments, one on a relatively small scale, to verify the purification protocol, and a larger effort to collect sufficient material for protein sequencing. To demonstrate the purification protocol, the enzyme was isolated from 100 pairs of salivary glands by using the Cibacron Blue affinity column and WCX column as described in *Materials and Methods*. Apyrase was separated from other salivary gland proteins in this two-step procedure. Ninety percent of

the initial apyrase activity was recovered after the Cibacron column purification, but this activity declined to 26% following the weak cation-exchange step.

Salivary gland extracts showed high levels of ATPase and ADPase activity, and these activities were coeluted in both the Cibacron Blue and the WCX HPLC step (Table 1). The purified enzyme rapidly hydrolyzed ATP, so that during the initial stages of the reaction both ADP and AMP were produced (Fig. 1). However, after 2 min, the rate of accumulation of ADP slowed, and between 4 and 8 min the amount of ADP decreased. Subsequent to this, AMP increased linearly. Adenosine was not found even after 120 min and no adenosine was produced when AMP was the only substrate available (D.E.C. and J.M.C.R., unpublished data).

In a separate experiment, apyrase was isolated from 1000 pairs of female glands. Homogenized glands were fractionated and proteins were separated as described above. However, in this experiment active fractions were desalted by reverse-phase HPLC in preparation for trypsin digestion. Only a single peak was observed after elution from the column (D.E.C. and J.M.C.R., unpublished data). The final yield of apyrase following reverse-phase HPLC was 0.024 μ g (0.363 pmol) per salivary gland pair.

Two peptides were recovered after tryptic digestion of 100 pmol of the purified apyrase and subjected to Edman sequencing. Both peptides were 24 aa long and their sequences were identical to portions of the conceptual translation product of the cDNA clone λ SGG12 (Fig. 2). The first peptide, LTLY-FDDTGEVQHWEGYPVFIDHK (yield, 42 pmol), matches a predicted tryptic fragment comprising aa 308–331, and the second peptide, QAEYYIVVPSYLADGKDGFSA MKR

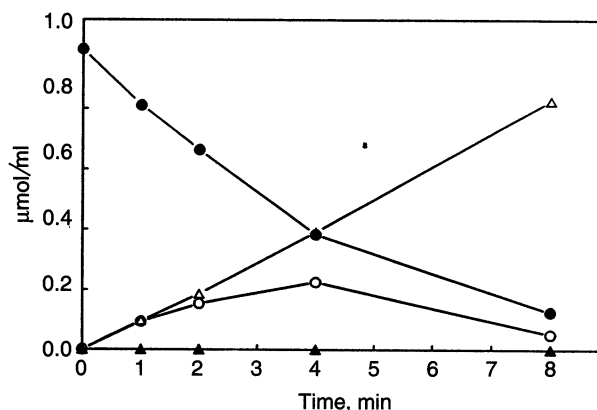


FIG. 1. Accumulation of AMP following hydrolysis of ATP (1 mM) by purified apyrase. Aliquots were analyzed at the indicated times for ATP (●), ADP (○), AMP (△), and adenosine (▲).

120
ATTTGTTAGTGAAGAAACATGGCTGGAAGACCGGTTCAGTGCAAGTATTTTTCTATACGTAGTGAGTGTGGCGGTGATTGCAAGGCCACGGATAATATGCCCCCTAATAAGGATGT
M A G R P G Y S A V I F L Y V V S V A V I A R A T D N M P P N K D V

240
ATCAAAGCTGTTTCCCTCACTTTGATTACATAAACGACTTGCATGCAAGGTCGAAGAGACCAATATGAAGTCCAACGCTTGTAACCAGAAGGATCAATGCATTGCCGAATCGCAAG
S K L F P L T L I H I N D L H A R F E E T N M K S N A C T Q K D Q C I A G I A R

360
AGTTTATCAAAAGATCAAGGATTTACTCAAAGAGTATGAGAGTAAAAATCCGATCTATCTCAATGCGGGTGATAACTTCCAGGGAACCTGTGGTACAATCTCTAAGGTGGAATGTGAC
V Y Q K I K D L L K E Y E S K N P I Y L N A G D N F Q G T L W Y N L L R W N V T

480
GGCGGATTTTATTA AAAAGCTTAAACCGCTGCCATGACTCTAGGAAACCACGAGTTTGATCATAACCGAAAGGATTGGCGCTTATCTGGCAGAACTGAATAAGAGGGAATCCAAC
A D F I K K L K P A A M T L G N H E F D H T P K G L A P Y L A E L N K E G I P T

600
CATTTGTCAAATCTAGTAATGAACAACGACCCGATTTGAAAAGTTCAAAAATTCAAAATCAATAAACTTACTGTTGGTAAGAGGAAGATAGGTATAATTGGTGCTGTATGATAA
I V A N L V M N N D P D L K S S K I P K S I K L T V G K R K I G I I G V L Y D K

720
AACGCATGAGATAGCCAAACGGGAAAGTTACTTTATCGAATGCCGTAGAAGCGGTGAGACGAGAGGCCGCTGCATTGAAGAAAGATAAAATCGATATTATTGTGGTCTTGCCCCTG
T H E I A Q T G K V T L S N A V E A V R R E A A A L K K D K I D I I V V L S H C

840
TAGCTACGAAGAGGACAAGAAAATTGCAGCTGAAGCCGGTGACGATATCGATGTAATTGTTGGCGCGCATTGCGATTCGTTCTTATATTACCAGGATTCCAAACAGCCGCATGATCAAA
S Y E E D K K I A A E A G D D I D V I V G A H S H S F L Y S P D S K Q P H D P K

960
GGCAAAAGTAGAAGGTCTTATCCAACGATTGTAGAAAGTAAAAACAAACGGAATTTCCAATCGTGAAGCAAAATCATTGCGTAAATATGTTGGTCGATTGACGCTTTACTTCGACGA
D K V E G P Y P T I V E S K N K R K I P I V Q A K S F G K Y V G R L T L Y F D D

1080
TACAGGTGAAGTGCAACACTGGGAGGGTTATCCTGTGTTTCGACCCACAAGGTTCAACAAGATCCACAAATTTTGAAGATCTGGTGCCTTGGCGCGAGAAGGTTGAAGCAATCGGATC
T G E V Q H W E G Y P V F I D H K V Q Q D P Q I L K D L V P W R E K V E A I G S

1200
AACCGTAGTTGGTGAGACTAAGATTGAATTGGATCGAGACTCCTGCCCGCATCAGGAATGTACATTGGGTGTTTTGTACGCTGACGGTTTTGCTGATCAATACACGAATGATACCTTCAG
T V V G E T K I E L D R D S C R D Q E C T L G V L Y A D G F A D Q Y T N D T F R

1320
ACCGTTTGAATCATTCAAGCAGCAATTTTCGAATCCGATCAAAGTTGGAAGATTACGAATGGTGACATTACGAGGCCGACCATTCGGTCCACAGCGGATCTGATTGATTGAA
P F A I I Q A G N F R N P I K V G K I T N G D I I E A A P F G S T A D L I R L K

1440
GGGTGCTGACATATGGGACGTGGCCGAGCATTATTCGCGCTGGACGATGAGGGTCGTACAAATGCTTGCAAGTATCGGGACTGAGGATTGTTATCGATATCAGTAAGCCGATTAGGAG
G A D I W D V A E H S F A L D D E G R T N C L Q V S G L R I V I D I S K P I R S

1560
TAGGGTAAGAAAATTGAAGTTATGGACTATACAAATCCCAAGTCTGATGAATTGAAACCATTTGGATAAACAAGCAGAGTACTACATAGTTGTCCCATCATATCTGGCCGATGGAAGAA
R V K K I E V M D Y T N P K S D E L K P L D K Q A E Y Y I V V P S Y L A D G K D

1680
TGGATTTTCTGCAATGAAAGGGCGCAGCGCAAGCGCAACTGGTCTTTGGATTCCGATGTTTTCAAAAATTATGTGGAAGAAATTAAGAAAGTAGATAACCTTAAGTTGGGTAGAGTAAT
G F S A M K R A T A R R T G P L D S D V F K N Y V E K I K K V D N L K L G R V I

1741
AGTTTGAAGGGTTGCAATGTACTTAGTGACCCAAATAAAGACGTTTCAGCCTGTAAAAAAAAAAAAAAAAAAAA
V C K G S K C T

FIG. 2. Nucleotide sequence and conceptual translation product of cDNA clones λ SGG12 and λ SGG12-5, homologous to the *A. aegypti* apyrase gene. The numbering refers to the nucleotide sequence. λ SGG12-5 corresponds to nt 1–476, and λ SGG12 corresponds to nt 471–1741, with the overlap occurring at the shared *EcoRI* site at nt 471–476. The partial amino acid sequences resulting from Edman degradation analysis of the purified apyrase are underlined. In addition, the stop codon and the polyadenylation signal are underlined.

(yield, 30 pmol), is identical to a predicted fragment including aa 498–521. These results indicate that the cDNA clone λ SFG12 contains sequences identical to the apyrase gene. The corresponding gene has been designated *Apv*.

Antibodies raised to recombinant apyrase detected a single protein band at ≈ 68 kDa in female salivary gland extracts and

in the purified apyrase preparation (Fig. 3). These data indicate further that the cDNA corresponds to the *Apy* gene. In addition, the gene corresponding to this cDNA clone was shown by hybridization *in situ* to be expressed specifically in the distal-lateral lobes of the salivary glands of adult female mosquitoes (C.T.S. and A.A.J., unpublished data), a pattern

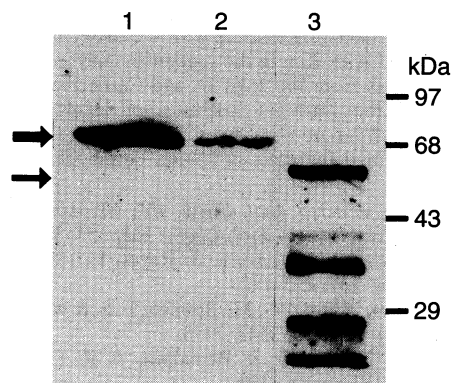


FIG. 3. Antibody detection of apyrase in salivary gland homogenates and chromatographically purified enzyme. Antibodies to recombinant apyrase were used to detect apyrase (large arrow) in one pair of female salivary glands (lane 1), purified apyrase from ~50 pairs of glands (lane 2), and the bacterially expressed material (lane 3) (small arrow). Smaller crossreacting materials in lane 3 are breakdown products of the high molecular mass form.

that overlaps the localization of the apyrase enzyme activity (18).

The cDNA clone λ SGG12 was sequenced and shown to be 1270 nt long with an open reading frame of 1227 nt. Further analysis of λ SGG12 indicated that it was not a full-length cDNA, most likely due to an *EcoRI* site left unprotected by methylation during construction of the library. λ SGG12 was used to identify and isolate a clone from an *A. aegypti* genomic library. A fragment from this genomic clone overlapping λ SGG12 and sequence 5' of the cDNA was used to rescreen the cDNA library. Clone λ SGG12-5, 477 nt long, was recovered. Sequence comparison of λ SGG12, λ SGG12-5, and the

genomic clone verified the identity of the fragments (C.T.S. and A.A.J., unpublished data) and demonstrated that we could recreate a transcription product of 1741 nt (Fig. 2). Although it is likely that a small portion of 5' untranslated region has not been recovered, this product is within the size range, 1.6–1.8 kb, of an RNA transcript detected in Northern analyses (C.T.S. and A.A.J., unpublished data).

Conceptual translation of the cDNA revealed a single, large open reading frame initiating with a methionine codon and encoding a protein of 63 kDa. This is smaller than the size predicted by the biochemical analysis of apyrase, 66 kDa (9), and that observed in our Western analysis, 68 kDa (Fig. 3), and most likely indicates that the final protein product is modified after translation. The putative protein has two sites, aa 112–114 and 390–392 (NVT and NDT, respectively), that could function as signals for asparagine-linked glycosylation (19).

Comparison of the conceptual translation product with protein databanks revealed that it had sequence similarities to a family of 5'-nucleotidases including those from rat and human (Fig. 4). The putative protein displayed conservation in location and spacing of all seven domains known to be present in enzymes exhibiting 5'-nucleotidase activity (20). Furthermore, amino acid differences in the domains result principally from conservative changes at the nucleotide and amino acid level (for example, multiple substitutions of isoleucine for leucine and valine). However, domains 2 and 4 in the mosquito protein are more charged than their mammalian counterparts. Most striking is the absence of a hydrophobic carboxyl-terminal domain in the mosquito protein. This region is present in the 5'-nucleotidase family and is the one that is substituted for a glycosylphosphatidylinositol (GPI) membrane-anchoring moiety during posttranslational modification (20–22). The mosquito apyrase does have the conserved serine residue (aa 559) to which the GPI anchor is attached covalently

	SIGNAL PEPTIDES		1	2	
Apy	<u>MAGRPGYSAVIFLYVSVAVIARA</u>	TDNMPPNKDVSKLFLPTLIHINDLHARFEETNMKSNACTQKQDCIAGIARVYQKIKDLLKEYESKNPIYLNAGDNF	100		
Human	<u>MCPRAARAPATLLALLGAVLWPAAGA</u>	-----WE IL T V S L Q SED SK VNASR MG V LFT VQQIRR--AEP VLL D QY	87		
Rat	<u>MRPAAATAPKWLALLSALLPLWPTAKS</u>	-----WE IM T VHS L Q SDD TK LNASL VG V LFT VQQIR --EP VLL D QY	89		
	3	4			
Apy	QGTLYNLLRWNVTDADFIKKLPAA <u>MTLGNHEFDHT</u> PKGLAPYLAELNKEGIPTIVANLVNNDPDLKSSKIPKSIK-LTVGKRKIGIIGVLYDKTHEIA	199			
Human	I FTVYKGAEV H MNA RYD A NGVE IEP --KEAKF ILS IKAKGPLASQI GLYLPY V P DEVV V YTSKE PFLS	185			
Rat	I FTVYKGLEV H MNL GYD A NGVE IDP --RNVKF ILS IKARGPLAPQI GLYLPY V S GEVV V YTSKE PFLS	187			
	5				
Apy	QTG-KVTLNNAVEAVRREAAALKDKDIDIVVLSHCSYEEDKKIAEAGDDIDVIGAHSHSFLYSPDSKQPHDKPKDVEGPYPTIVESKNKRKIPIVQA	298			
Human	NP TNLVFEDEIT LQP VDK TNLVVK IA G SGF M L - QKVRGV V G NT TGNP----PS EVPA K F T DDG V V	280			
Rat	NP TNLVFEDE T LQP VDK TNLVVK IA G SGF M L - QKVRGV V G TNT TGNP----PS EVPA K F T DDG V V	282			
	6				
Apy	<u>KSFGKYVGR</u> LTLYFDDTGEVQHWEQPVFIDHKVQDDPQILKDLVPHREKVEATGSTVVGETKIELD--RDSCRDQECTLGVLVADGFAD---QYTNDTF	393			
Human	YA L Y KIE ER N ISSH N ILLNSSIPE S KA INK I LDNYSTQEL K IVY GSSQ FR NM N IC AMINNNLRH DEM	380			
Rat	YA L Y KVE K N VTSY N ILLNSTIRE AA KA INQ I LDNYSTQEL R IVY NGSAGE FR NM N IC AMINNNLRHPDEM	382			
	7				
Apy	R---PFAIIQAGNFRNPIKV---GKITNGDIIIEAAPFGSTADLIRLKAGDIWDVAEHSFALDDEGRNTCLQVSGLRIVIDISKPIRSRVKKIEVMDYNTN	487			
Human	WNHVSVC LNG GI S DERNN T WENLA AVL G F VQ STLKAF VHYRGQSTGEF G IHV Y L RKP GD V LD L-C KC	479			
Rat	WNHVSVC VNG GI S DERNN T WENLA AVL G F VQ STLKAF VHYRGQSTGEF G IHV Y RKPWD VQLK L-C KC	481			
	7				
Apy	KSDELKPLDQAEYYIVVPSYLADGKDGFSAMKRATARR-TGPLDSDFVKNYVEIKKVDNLKGRVIVCKGSKCT*	562			
Human	RVPSYD KMDEV KVIL NF N G QMI DELL HDS DQ IN VST IS M VIYPAVE IKFST H HGSFSLIFLSLWAVIFVLYQ*	574			
Rat	RVPIYE EMDKV KV L VN G QMI DELLKHDS DQ IS VSE IS M VIYPAVE IKFSAA HYQGSFPLIILSFWAVILVLYQ*	576			

FIG. 4. The mosquito apyrase (Apy) shows similarity to the 5'-nucleotidase family of proteins. Comparisons with the human placental and rat liver amino acid sequences are shown here. Amino acids in the vertebrate sequences that are identical to the mosquito apyrase are represented by blanks. The hyphens represent gaps introduced to maximize sequence alignment. The seven conserved regions of enzymes possessing 5'-nucleotidase activity are numbered and overlined. Known and predicted hydrophobic amino-terminal signal sequences are underlined. Stars indicate the carboxyl termini of the proteins.

in the 5'-nucleotidases. The lack of the hydrophobic domain in the mosquito apyrase correlates with the fact that it is a secreted salivary protein (23).

The mosquito apyrase and mammalian 5'-nucleotidases have secretory signal regions at their amino-terminal ends. The mammalian signal peptides were identified by sequencing the amino-terminal regions of the mature proteins (21, 22). The mosquito signal sequence is predicted from the hydrophobicity of the amino-terminal end. The amino terminus of the mosquito protein is blocked and the exact cleavage site remains to be determined.

The mosquito apyrase shows a region of 6 aa, GKYYVGR, identical to a sequence in two UDP-sugar hydrolases of bacterial origin, enzymes that possess 5'-nucleotidase activity (20, 24–26). Furthermore, this region overlaps the conserved domain 6 in 5'-nucleotidases (Fig. 4). A number of nucleotide-binding domains have a sequence where glycine moieties flank from three to five other amino acids (27, 28), and it is possible that this region is the expected nucleotide-binding domain of the apyrase.

DISCUSSION

We have shown that the cDNAs λ SGG12 and λ SGG12-5 represent *Apy*, the gene encoding the salivary gland apyrase in *A. aegypti*. This apyrase shares sequence similarity with a family of 5'-nucleotidases. All seven conserved regions observed in 5'-nucleotidase are found in the mosquito apyrase. However, apyrase lacks significant AMPase activity as indicated by the absence of adenosine as a product of AMP hydrolysis. This corroborates previous reports of the substrate affinities of *Aedes* apyrase (10). The higher levels of apyrase activity reported here can be ascribed to a higher assay temperature (37°C vs. 30°C) than that used previously (10). The specific activity of the purified apyrase is about twice that of the apyrase from *Rhodnius prolixus* (29) and is >10-fold greater than the apyrase from vertebrate pancreas (30) or endothelium (5).

The observation that apyrase is similar to 5'-nucleotidase extends earlier suggestions that the evolutionary history of the nucleotidase family has been characterized by increasing substrate specialization (20). The ancestral state is apparently represented by a bacterial enzyme that combines 5'-nucleotidase, UDP-sugar hydrolase, and apyrase activities (31). A similar enzyme in the yeast *Saccharomyces oviformis* hydrolyzes ribo- and deoxyribonucleoside 5'-phosphates, NAD, NADH₂, FAD, and ATP (32). An enzyme from the tick *Boophilus microplus* has sequence similarity to bacterial 5'-nucleotidase and hydrolyzes nucleoside 5'-mono-, di-, and triphosphates and UDP-glucose (33). We propose that duplications of the broad-substrate-spectrum ancestral gene were followed by divergent selection to produce apyrases (specialized to hydrolyze the pyrophosphate bond of nucleoside pyrophosphates), 5'-nucleotidases (specialized for the ester bond), and UDP-sugar hydrolases. It is possible that a gene for a single enzyme such as the 5'-nucleotidase is the common progenitor for salivary apyrases in the various arthropod taxa. Alternatively, genes encoding enzymes with different cellular functions may have been selected to fill the new roles necessitated by the evolution to hematophagy. Given the importance of bloodfeeding insects in contributing to the burden of disease on humanity, and the critical role of apyrases in facilitating bloodfeeding, further analysis of the mosquito apyrase is likely to provide insights of broad interest.

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