Eur. J. Biochem. **270**, 4122–4132 (2003) © FEBS 2003 doi:10.1046/j.1432-1033.2003.03805.x

ATP N-glycosidase

A novel ATP-converting activity from a marine sponge *Axinella polypoides*

Tõnu Reintamm, Annika Lopp, Anne Kuusksalu, Tõnis Pehk and Merike Kelve

Laboratory of Molecular Genetics, National Institute of Chemical Physics and Biophysics, Tallinn, Estonia

A novel nucleosidase enzymatic activity was discovered in the marine sponge *Axinella polypoides*. This enzyme, designated as ATP N-glycosidase, converts adenosine-5′-triphosphate into adenine and ribose-5-triphosphate. The crude extract of *A. polypoides* was capable of hydrolysing 25 μmol ATP·min^{-1} per g wet weight of sponge. The catalytic activity of a sponge crude extract per mg total protein is comparable with specific activities of purified plant adenosine and bacterial AMP nucleosidases. The preferred substrate for the novel enzyme is ATP but any compound containing adenosine-5′-diphosphoryl fragment is also cleaved. The biochemical properties (K_m, K_{ip}, environmental requirements) of ATP N-glycosidase show similarities with previously described adenine-specific nucleosidases; however, the pattern of its biochemical characteristics does not match with that of any of those enzymes.

Keywords: adenosine nucleotide metabolism; ATP; *Axinella polypoides*; marine sponge; nucleosidase.

Most of the biological and chemical literature concerning marine sponges is primarily dedicated to the isolation and characterization of exotic secondary metabolites and studies of their biological activity (antibacterial, antifungal, anticancer, etc.) [1]. These works have been rooted and inspired by the discovery of unusual nucleosides in *Cryptotethya crypta*-arabinothymidine and -uridine [2] which have led to the development of pharmaceuticals with antiviral and anticancer action. We have shown the presence of 2′,5′-oligoadenylates (2-5A) in a marine sponge *Geodia cydonium* [3]. The synthesis of 2-5A from ATP in sponges proceeds independently from dsRNA [4], in contrast with higher animals (birds and mammals) [5]. There is an evolutionary gap in occurrence of this signal molecule between the sponges and birds, as no 2-5A synthetase genes have been found in completed insect, worm and fish genomes [6,7].

In the present study, a completely novel and unexpected ATP-utilizing activity in *Axinella polypoides* was found. The enzymatic activity, cleaving the most abundant high-energy nucleotide (ATP) into a free nucleobase without touching the energy–charge-carrying triphosphate moiety, seems to be in conflict with the current understanding of nucleotide utilization, salvage and catabolism in nature.

The capacity of the *A. polypoides* crude extract to utilize ATP in yet an undescribed direction is impressive. Its rate could be compared with the rate of ATP turnover in human muscle [8] and it masks any other ATP-utilizing activity potentially present in natural crude extracts. Such a fortunate circumstance enabled us to characterize the novel activity enzymatically without purification or enrichment of the crude extract. Substrate preferences and factors determining the reaction rate in the physiological concentration range were studied.

Whether the newly discovered enzyme, ATP N-glycosidase, participates in the purine nucleotide salvage pathway, regulation of cellular adenylate levels, signalling, or other mechanisms, remains to be established.

Materials and methods

Reagents and enzymes

Reagents and enzymes were purchased from commercial suppliers (Sigma, Fluka, Reanal, Fermentas, USB Corporation), except for those mentioned below. pppA2′p5′A was enzymatically synthesized by *Geodia cydonium* 2′,5′-oligoadenylate synthetase [4]. γ-P-(4-amino-*n*-butylamido)adenosine-5′-triphosphate (DAB-ATP) and (5′,5″)-diadenosine(α,ω)-oligophosphates (A5′p$_n$5′A, $n = 2$–5) were chemically synthesized according to the published

Correspondence to M. Kelve, Laboratory of Molecular Genetics, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, 12618 Tallinn, Estonia. Fax: +372 6398382. Tel.: +372 6398352, E-mail: merike@kbfi.ee

Abbreviations: DAB-ATP, γ-P-(4-amino-*n*-butylamido)adenosine-5′-triphosphate; cADPR, cyclic ADP-ribose; cADPRP, cyclic ADP-ribose 2′-phosphate; ADPR, β-P-(5-ribosyl) adenosine-5′-diphosphate (ADP-Ribose); ATPR, γ-P-(5-ribosyl) adenosine-5′-triphosphate; ATePR, δ-P-(5-ribosyl) adenosine-5′-tetraphosphate; APPR, ε-P-(5-ribosyl)adenosine-5′-pentaphosphate; FDPR, β-P-(ribosyl)-lactoflavin-5′-diphosphate; MTA, 5′-methylthio-5′-deoxyadenosine; SAH, S-adenosylhomocysteine; Ado, adenosine; 2–5 A, 5′-tri (di-, mono-)phosphorylated (2′,5′)oligoadenylates; (2′,5′)p$_3$A$_n$, 5′-triphospho(2′,5′)oligoadenylates; (2′,5′)A$_n$, (2′,5′)oligoadenylates; A5′p$_n$5′A, P^1,P ^{n}-bis(5′-adenosyl)oligophosphates; NDPR, β-P-(5-ribosyl)-1-β-D-ribofuranosylnicotinamide−5′-diphosphate.

Enzymes: snake venom phosphodiesterase (EC 3.1.15.1); alkaline phosphatase (EC 3.1.3.1); ribonuclease U2 (EC 3.1.27.4); purine nucleosidase (EC 3.2.2.1); 5′-methylthioadenosine/S-adenosylhomocysteine (MTA/SAH) nucleosidase (EC 3.2.2.9, EC 3.2.2.16); AMP nucleosidase (EC 3.2.2.4); adenosine nucleosidase (EC 3.2.2.9); ADP ribosyl cyclase (EC 3.2.2.5).

(Received 13 June 2003, revised 18 August 2003, accepted 26 August 2003)

methods [9,10]. Phosphodiesterase from the snake venom (*Vipera lebetina*) was a gift from J. Siigur (National Institute of Chemical Physics and Biophysics, Tallinn, Estonia).

Natural sponge material

The marine sponges *A. polypoides* (Porifera, Demospongiae, Ceractinomorpha, Halicondrida, Axinellidae) were collected near the Kalymnos Island (Greece). The material was kept in natural seawater during the transportation (< 24 h). Then it was frozen in liquid nitrogen and stored at −70 °C. All experiments, if not otherwise stated, were performed using this material.

The alternative sample of *A. polypoides* was generously provided by W.E.G. Müller (Johannes Gutenberg-Universität, Mainz, Germany) from his sponge collection (stored at −70 °C). The air-dried powder of *A. polypoides* was provided by W. Schatton (Klinipharm GmbH, Frankfurt, Germany).

Preparation of sponge extracts and their characterization

The sponge material, which had been mechanically powdered and thoroughly mixed at liquid nitrogen temperature, was used for the extraction of total RNA, the low molecular weight nucleotides and enzymes. The total RNA from a sample of *A. polypoides* was prepared and analysed by the Chomczynski method [11]. Low molecular mass nucleotides were extracted with 5% trichloroacetic acid (7 mL·g sponge^{-1}). The appropriately diluted trichloroacetic acid extract (5%) was analysed by HPLC and the ATP content was measured by the luciferase assay [12].

An extract with a maximal yield of ATP N-glycosidase activity and stable in storage was obtained using an extraction buffer, containing ≥100 mM KCl. All of the experiments described in the current work were performed using the single extract (hereafter referred to as 'crude extract'), which was prepared as follows. Two-hundred milligrams of the sponge powder (made from frozen sponge pieces from different body parts of several individuals collected from the same geographical location; each piece ≈ 0.5 g, total mass ≈ 5 g) was extracted with 0.1 M Mops pH 6.7, containing 0.1 M KCl (1200 μL) at room temperature for 30 min. The insoluble material was removed by centrifugation and 1100 μL of solution was collected. The protein content was estimated by the Bradford method [13]. The crude extract was kept unfrozen at 4 °C. The specific activity of the crude extract quantified by standard assay in parallel to each kinetic series yielded average deviation of 7.5%. No statistically significant decrease in the specific activity of this preparation was found throughout the biochemical characterization period (≈ 2 months).

HPLC analysis

All HPLC analyses were performed, using the C18 HPLC column (5 μm, 4.6 × 250 mm, Supelco, USA) and the Waters Model 600 chromatograph with a tunable wavelength detector (Model 486), controlled by the MILLENNIUM32 software (Waters, USA). Eluent A was 50 mM ammonium phosphate pH 7.0 and eluent B was 50% methanol in water. The flow rate was 1 mL·min^{-1} and the column temperature was 40 °C. The products were separated and analysed in a linear gradient of eluent B (1–60%, 30 min); the column was equilibriated with 1% eluent B before the next injection (10 min). Fast isocratic separations (8 or 20% of eluent B, 15 or 10 min) were used in the routine kinetic point analysis in appropriate cases. Retention times (min) of the adenosine nucleotide derivatives are listed in an ascending order: cADPRP (2.49), ADPRP (2.68), NDPR (2.89), unknown cADPR derivate (3.18), APPR (3.21), ATePR (3.35), ATP (3.60), ATPR (3.70), ADP (3.8), NADP + (3.84), 5′-AMP (4.00), cADPR (4.28), DAB-ATP (4.60), (2′,5′)p_3A_2 (4.6), ADPR (4.80), dATP (5.58), dADP (6.52), (2′,5′)p_3A_3 (6.60), A5′$p_5$5′A (6.70), 3′-AMP (7.8), A5′$p_4$5′A (8.0), dAMP (8.2), (2′,5′)p_3A_4 (8.95), A5′$p_3$5′A (9.1), Ade (9.24), NAD^+ (9.6), nicotinamide (10.4), (2′,5′)p_3A_5 (10.46), (2′,5′)p_3A_6 (11.25), (2′,5′)p_3A_7 (11.75), A5′$p_2$5′A (12.44), NADH (12.5), 2′-AMP (12.7), (2′,3′)cAMP (14.7), Ado (16.8), (3′,5′)cAMP (17.1), (2′,5′)A_5 (18.0), (2′,5′)A_4 (18.4), (2′,5′)A_2 (18.9), (2′,5′)A_3 (19.0), poly(A) (21.48), (3′,5′)A_3 (24.43), FDPR (26.3), FAD (27.9). The set of adenylate retention times has been derived from the chromatograms, which were internally or externally calibrated with ATP (3.6 ± 0.05) and Ado (16.8 ± 0.3).

Whenever possible, both the substrate and the product were quantified for the calculation of the reaction yield to exclude the partial loop filling method related error (≈ 10%). The HPLC raw data were recalculated according to different molar absorption coefficients of adenine and the substrates.

ATP N-glycosidase assay

Summing up the knowledge obtained during the work, a simple procedure was developed for the *A. polypoides* ATP N-glycosidase quantification.

Fifteen microlitres of 1 M KCl, 20 μL 5 mM ATP, pH 7.0 (25 °C), 10 μL 200 mM Mes, pH 5.3 (25 °C) and 50 μL deionized water were mixed and equilibriated at 37 °C. The reaction was started by adding 5 μL of the sponge extract, appropriately diluted with deionized water, to keep the half-decay of the substrate over 10 min. The reaction was monitored by HPLC with a 10-μL aliquot of the reaction mixture injected immediately at the time-point analysed.

A unit of ATP N-glycosidase activity is an amount of the enzyme which releases adenine at an initial rate of 1 μmol·min^{-1} under standard conditions (1 mM ATP, pH 5.0–5.5, 150–200 mM KCl, 37 °C). ATP decay by *A. polypoides* ATP N-glycosidase proceeds with pseudo-first order kinetics under the described assay conditions and the initial rates of the reaction were calculated from the progress curve of ATP decay, given that the concurrent reactions of ATP (and adenine) are slow. The accuracy of the assay was estimated by 10 parallel standard assays giving the initial rate with average deviation of 1.6%.

The ATP N-glycosidase activity in the *A. polypoides* crude extract could be observed under a variety of assay conditions. The reaction rate is dependent on pH and ionic strength (which could be adjusted equally with KCl or NaCl or $LiClO_4$). It should be noted that any additional component in the assay buffer capable of altering pH or ionic strength may therefore have an indirect influence on the reaction rate.

NMR measurements

NMR spectra were recorded with the Bruker spectrometer AMX500 at room temperature. The ^{1}H NMR signals are given, adjusted for the chemical shift of the residual water peak of 4.82 p.p.m. The ^{31}P signal chemical shifts were determined, using 85% H_3PO_4 as an external standard. ^{13}C chemical shifts are given relative to residual acetone (30.89 p.p.m. [14]), present in the sample NMR-B. Heteronuclear spectra were recorded with ^{1}H-saturation. The samples were prepared as follows. NMR-A: A 1-cm^2 piece of Hybond-N+ filter (Amersham) was soaked in 100 μL *A. polypoides* extract for 30 min at room temperature and washed several times with an excessive amount of deionized water. The filter was incubated with 1 mL 10 mM ATP pH 7.0 in 100 mM KCl at 37 °C until no more substrate could be detected by the HPLC-analysis. NMR-B: 1 mL 42 mM ATP pH 7.0 (25 °C) in 195 mM $LiClO_4$ was incubated with 50 μL *A. polypoides* crude extract at 37 °C for 29 h, monitoring the reaction by HPLC. After 29 h the HPLC analysis revealed the presence of 8% ATP, 8% ADP and 84% adenine in the reaction mixture. The phosphate-containing compounds were precipitated with acetone (20 vols). The precipitate was washed with acetone, dissolved in aqueous 0.5 M $LiClO_4$ and the precipitation procedure was repeated to remove any coprecipitated adenine. The precipitate was dissolved in 0.5 mL D_2O and the absence of adenine was confirmed by HPLC. The NMR-B sample contained acetone in trace amounts, serving as an excellent internal reference for ^{1}H and ^{13}C spectra (2.22 and 30.89 p.p.m., respectively [14]).

Results

Incubation of ATP with *A. polypoides* extract gives unexpected UV_{254} visible single product identified as adenine

When a panel of marine sponge extracts was assayed for their 2-5A synthetase activity [15], a different HPLC profile of products was obtained with the crude extract from *A. polypoides*. The substrate ATP was exhausted quickly, giving a single UV/visible product with a retention time of 9.24 min. No other peaks in addition to ATP and the unidentified product were detected in the HPLC profile with shorter incubation times where the reaction was incomplete. The HPLC retention time of the product did not match either that of ADP, AMP and adenosine or any of the 2-5A derivatives, or any other adenosine derivatives (see Materials and methods, HPLC analysis).

This peak was collected and its UV spectrum was found to be identical with that of the unmodified adenine chromophore (data not shown). This excluded the hypoxantine/inosine nucleosides/nucleotides as candidate products, which could be formed due to deaminase activity in the extract.

Because an apparent loss of the UV/visible material occurred during the reaction, an oligomeric product was suspected. The absence of terminal phosphoryl and adenosine-5′-phosphoryl groups, as well as a 3′,5′–internucleotidic linkage in the structure of unknown product, was shown by alkaline phosphatase, snake venom phosphodiesterase and ribonuclease U2 treatments, respectively [15]. The activity of the enzymes was qualitatively and quantitatively confirmed in parallel assays with their common substrates added.

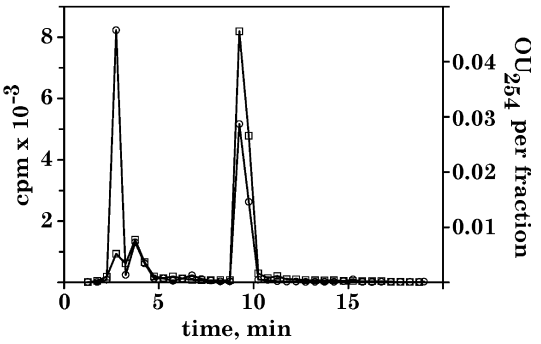

Fig. 1. HPLC analysis of products formed by *A. polypoides* extract from exogeneous ATP. A Hybond N+ filter, presoaked in *A. polypoides* extract, was incubated in a mixture containing 1 mM ATP (with [U-^{14}C]ATP as a tracer), 100 mM KCl, pH 7.0 at 37 °C. Ten microlitres of reaction mixture was subjected to HPLC fractionation. The radioactivity of the fractions (500 μL) was measured (○). The amount of the UV-absorbing material (OU_{254}) in the fractions (□) was determined by integration of the computer-stored UV_{254}-trace.

The initially most improbable candidate compound, adenine, was run in HPLC and found to have a retention time similar to that of the unidentified product from *A. polypoides*. An absolute match of adenine and the *A. polypoides* product was revealed by the peak shape analysis in the HPLC profile of a mixed probe.

Finally, ATP together with [U-^{14}C]ATP tracer were treated with the *A. polypoides* extract and the reaction mixture was analysed by HPLC (Fig. 1). UV_{254} trace showed two peaks: one at 3.6 min corresponding to residual ATP and another at 9.24 min corresponding to adenine. In addition to these two peaks, radioactivity was detected at 2.75 min. The ratio of radioactivity in peaks at 2.75 min and 9.5 min was 1.05, which approximately corresponds to the number of carbon atoms in ribose moiety and heterocycle. This experiment proved that ATP had been split into two molecules – adenine and a yet unidentified derivative of ribose.

Adenine is not a result of a multistep conversion of ATP by phosphatases and N-glycosidases

The formation of adenine from ATP could be explained as a result of multiple known enzymatic activities, first of all by the combination of a relatively slowly acting phosphatase or ATPase and a relatively rapidly acting well-known AMP/adenosine nucleosidase. Thus, adenosine, AMP and ADP were incubated under the same conditions as ATP with the *A. polypoides* extract. Adenosine and AMP were not digested during the period, which was sufficient for ATP to be degraded almost completely; the release of adenine from ADP was significantly slower than that from ATP. This preliminary result completely excluded the possibility of the formation of adenine by the way of combined action of known enzymes. More detailed studies on these substrates will be described below.

The second product of ATP degradation in *A. polypoides* extract is ribose-5-triphosphate

The simplest reaction leading to the release of adenine from ATP is the hydrolysis of the N-glycosidic bond. If adenine results from hydrolysis of this bond the second reaction product has to be ribose-5-triphosphate. Here we show that the only way to interpret our results is to assign the NMR signals of the second reaction product to ribose-5-triphosphate.

Samples for the NMR analysis were prepared by treatment of a concentrated ATP solution (10–40 mM) with the crude *A. polypoides* extract either in solution (named NMR-B) or on a solid-phase support (Hybond-N+) (named NMR-A). The reaction rate for these reactions, performed on a preparative scale, decreased more rapidly than would be expected from the first-order-kinetics at lower substrate concentrations (0.1–5 mM). Only a small portion of adenine-releasing activity was adsorbed on the Hybond-N+ filter; therefore very long incubations (2 weeks for 10 mM ATP) were needed for the complete reaction. Still, the solid-phase approach was useful for NMR samples as the HPLC analysis revealed no concurrent dephosphorylation of the substrate in this sample. Presumably the ATP dephosphorylating enzymes had a lower adsorbing capacity to the Hybond-N+ than the ATP N-glycosidase, leading to occasional enrichment of the latter.

Ten signals were registered in the aliphatic region of NMR-A ^{13}C-spectrum (Fig. 2A). The comparison of their chemical shifts, ^{31}P–^{13}C coupling constants and anomer distribution (≈ two-thirds of β-anomer) with available data for the D-ribose-5-phosphate [16] revealed that they unambigously belonged to the 5-phosphorylated α- and β-D-ribofuranosides. The ^{1}H-NMR spectrum of NMR-A was almost unusable because of the large water signal and insufficient concentration. Still, the signals belonging to H-1 of ribose and aromatic protons of adenine could be detected, indicating a 1 : 1 ratio of adenine to D-ribose-5-triphosphates. The ^{31}P-NMR spectrum of NMR-A had three groups of multiplets assignable to α-, β- and γ-phosphates of the triphosphate monoester, while neither inorganic phosphate nor any other additional resolved signals were detected in NMR-A (Fig. 2B). However, the multiplet appeared to be more complex than expected from a single triphoshpate-containing compound.

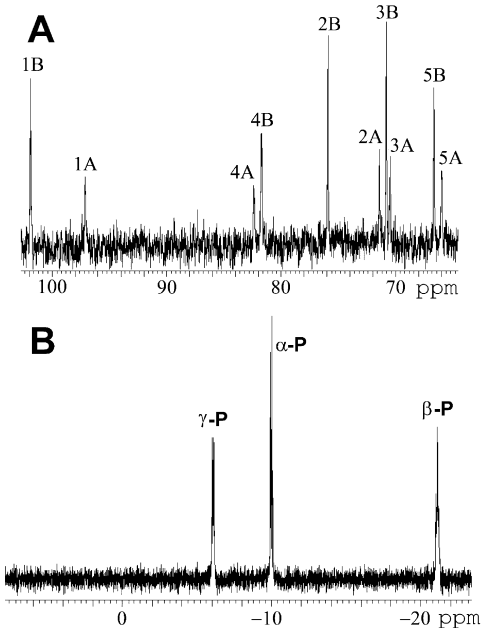

Fig. 2. NMR spectra of D-ribose-5-triphosphate. (A) ^{13}C-NMR spectrum of NMR-A. The assignment of signals in α- and β-anomers is shown. (B) ^{31}P-NMR spectrum of NMR-A.

The complete ^{13}C, ^{1}H and ^{31}P data for the D-ribose-5-triphosphate were obtained with the sample NMR-B (Table 1). The NMR-B sample contained a mixture of α- and β-D-ribofuranoside-5-triphosphates as the main product. The minor components (ATP, ADP and D-ribose-5-diphosphates, inorganic phosphate) were identified and quantified by one- and two-dimensional ^{31}P-NMR. It should be noted that no ^{13}C-NMR signal was resolved for the 5-diphosphorylated ribose. This indicates that the differences up to 1 p.p.m. (Table 1) between the reported ^{13}C-NMR data of the ribose-5-monophosphate and our data were probably caused by environmental differences in the spectra registration rather than by the influence of the number of phosphate groups. ^{1}H-NMR signals of α- and β-anomers of phosphorylated ribose were resolved by two-dimensional NMR. A small resolution between the ^{1}H signals of diphosphorylated and triphosphorylated compounds was evident, but these weak signals could not be assigned to particular positions in particular isomers because of the overall complexity of the spectrum.

It was possible to derive almost complete NMR data for ATP/ADP from the NMR-B spectra. The spectral characteristics of ATP and ADP obtained from NMR-B (Table 1) are included in Table 1 because they serve as fine-tuning internal standards for the ribose-5-triphosphate.

Thus, we can conclude that the second product formed by *A. polypoides* extract is the D-ribose-5-triphosphate (as a mixture of α- and β-anomers 1 : 2).

Preliminary kinetic studies of the hydrolysis of the N-glycosidic bond in ATP by the *A. polypoides* ATP N-glycosidase

Based on the results of product identification described above, the novel enzyme catalyses the reaction of hydrolysis of the N-glycosidic bond in ATP. This novel enzyme was named the ATP N-glycosidase.

The conversion of ATP catalysed by the ATP N-glycosidase present in the *A. polypoides* extract followed the exponential-like kinetics at the 1 mM substrate concentration (Fig. 3). Similar progress curves were registered within the whole range of substrate concentrations used for K_m determination (0.1–4 mM ATP). The K_m values ($K_m^{pH7} = 0.16$ mM and $K_m^{pH5} = 0.10$ mM) calculated from the initial rates were found to be smaller than the substrate concentration used (Fig. 4). The exponential form of progress curves at [S] > K_m could not be explained by enzyme degradation during the reaction, because no change in its activity was determined during the preincubation of the extract up to 4 h under assay conditions before the substrate was added (data not shown).

Table 1. ^{1}H, ^{13}C and ^{31}P-NMR data of the NMR-B sample. The differences in chemical shifts from those of the D-ribose-5-phosphate [16] are shown in brackets. The resolved and assigned signals are separated by slashes, signals unassigned to a particular molecule are separated by commas. NA, Not applicable; ND, not detected.

		β-D-ribose-5-triphosphate/ β-D-ribose-5-diphosphate		α-D-ribose-5-triphosphate/ α-D-ribose-5-diphosphate		ATP/ADP/P_i	
Nucleus		Chemical shift	Coupling constants	Chemical shift	Coupling constants	Chemical shift	Coupling constants
^{1}H	1H	5.23	$^{3}J_{HH} = 1.6$	5.40	$^{3}J_{HH} = 4.70$	6.13	$J_{HH} = 5.33$
	2H	4.04		4.17		4.78, 4.74	
	3H	4.37		4.26		4.58	
	4H	4.1		4.08		4.37	
	5H	(4.15,4.02)		(4.15,4.02)		4.21, 4.27	
^{13}C	1C	101.79 [−0.61]		97.07 [-0.43]		87.67, 87.34	
	2C	75.81 [−0.59]		71.35 [-0.55]		74.94, 74.86	
	3C	70.84 [−0.86]		70.48 [-0.82]		70.90, 70.60	
	4C	81.76 [−0.74]	$J_{CP} = 8.9$	82.40 [-1.20]	$J_{CP} = 8.3$	84.56, 84.38	$J_{CP} = 9.5$, 9.9
	5C	66.74 [0.14]	$J_{CP} = 6.2$	66.05 [0.25]	$J_{CP} = 5.3$	65.76/ND	$J_{CP} = 5.0$/ND
^{31}P	αP	−9.82/−8.92	$J_{PP} = 18.5/20.7$	−9.88/−9.03	$J_{PP} = 18.5/18.4$	−10.11/−9.23	$J_{PP} = 18.6/20.6$
	βP	−20.1/−5.73		−20.1/−5.81		−20.1/−5.78	
	γP	−5.52/NA	$J_{PP} = 18.6$	−5.55/NA	$J_{PP} = 18.5$	−5.46/NA	$J_{PP} = 18.5$
	p_i					1.86	

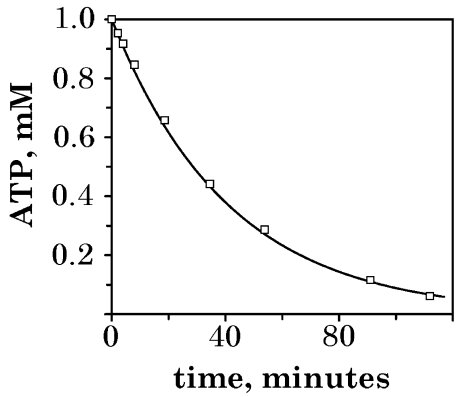

Fig. 3. Progress curves of ATP degradation by *A. polypoides* crude extract. ATP (1 mM), KCl (100 mM), pH 7.0, 37 °C, dilution of the crude extract 1 : 100. The almost perfectly fitted exponential line through the experimental points is shown.

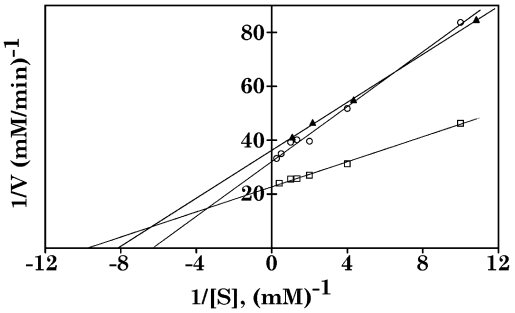

Fig. 4. Lineweaver–Burk plots of *A. polypoides* ATP N-glycosidase activity on ATP and ADP. The initial rates of each reaction containing *A. polypoides* crude extract in a dilution of 1 : 100 were found from the progress curves, assuming pseudo first-order kinetics. ATP was investigated at two pH values: at pH 7. ± 0.1 (100 mM KCl, 37 °C, $K_m = 0.158$ mM, $v_{max} = 0.031$ mM·min^{-1}, ○) and at pH 5.3 ± 0.1 (20 mM Mes, 170 mM KCl, 37 °C, $K_m = 0.102$ mM, $v_{max} = 0.044$ mM·min^{-1}, □). ADP was assayed at pH 5.1 ± 0.2 (20 mM Mes, 170 mM KCl, 37 °C, $K_m = 0.122$ mM, $v_{max} = 0.027$ mM·min^{-1}, ▲). pH for each reaction mixture at the assay temperature was determined.

Competitive inhibition by a product with $K_{ip} \approx K_m$ [17] predicts pseudo-first order kinetics at substrate concentrations above K_m. The inhibition of the ATP N-glycosidase by adenine was examined. K_{ip} for adenine, estimated from the decrease of the initial reaction rate by addition of adenine to 1 mM ATP at pH = 7.0, appeared to be close to the K_m value (Fig. 5). The progress curves obtained in the assays for K_m determination (Fig. 4, pH 7) and for adenine inhibitory effect (Fig. 5) were analysed together, using the procedure described in [17]. Similar values of K_m (0.15 mM) and K_{ip} (0.15 mM) were obtained for the ATP N-glycosidase.

At very high substrate concentrations (> 10 mM ATP) the kinetic model $K_m \approx K_{ip}$ was incomplete to simulate the progress curves, as the reaction rate decreased even faster than predicted by this model. Thus the kinetics of ATP glycohydrolysis by the *A. polypoides* enzyme is actually more complex than described by the relatively simple $K_m^{ATP} \approx K_{ip}^{Ade}$ scheme.

The reaction rate was cross-dependent on ionic strength and pH. The optimal pH was about 5 and the optimal salt concentration was 100–250 mM (Fig. 6). Alteration of the environmental condition did not lead to a drastic change of the K_m^{ATP} and K_{ip}^{Ade} ratio, as far as it could be judged by progress curve shapes. The enzyme activity was not substantially altered by the presence of 10 mM EDTA, 140 mM mercaptoethanol or the inorganic phosphate.

The enzyme appeared to be relatively stable. The temperature dependence of the reaction (Fig. 7) showed that the denaturation of the enzyme started above 60 °C. The reaction catalysed by the ATP N-glycosidase was described by a single activation energy (ΔH_a) of 11.6 kcal·mol^{-1} in the temperature range 10–60 °C. Heating of the extract for 10 min at 92 °C resulted in

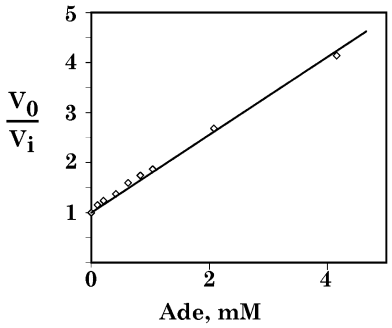

Fig. 5. Inhibition of *A. polypoides* ATP N-glycosidase by adenine. The reaction mixtures contained 1 mM ATP pH 7.0, 100 mM KCl, *A. polypoides* crude extract (dilution 1 : 100) and various concentrations of adenine. Initial rates were calculated from progress curves, assuming pseudo first-order kinetics. The K_{ip}^{Ade} calculated from the equation $K_{ip}^{Ade} = K_m^{ATP}/\text{slope} \times (1/(K_m^{ATP} + [S]))$ ($K_m^{ATP} = 0.158$ mM) is equal to 0.176 mM.

a complete irreversible loss of activity. The complete and unrecoverable loss of ATP N-glycosidase activity was also observed when the sponge was treated with trichloroacetic acid.

ATP N-glycosidase from *A. polypoides* is capable of releasing adenine from a wide range of substrates containing an adenosine-5′-diphosphoryl fragment

When any of the nucleotide triphosphates GTP, ITP, CTP, UTP, dGTP, dCTP or dTTP was incubated together with the *A. polypoides* extract instead of the substrate ATP, no heterocycle release was observed (detection limit ≈ 0.1%) during 8–10-fold half-hydrolysis periods of ATP. Longer incubations could not be used due to a dephosphorylating activity present in the extract.

Various natural adenine ribosides were assayed as substrates for the ATP N-glycosidase (Table 2). The assays were performed under conditions optimized for ATP and adenine release was monitored and quantified by HPLC. In several cases where the substrate contained two chromophores (A5′p_n5′A, FAD, NAD^+), UV_{254}-visible intermediates or products complementary to adenine were detected. The retention times for those compounds (see above) are consistent with a proposed structure.

Pseudo-first-order progress curves similar to ATP were characteristic of a few substrates (Table 2). These substrates should have their K_m in the same range as ATP to satisfy the condition $K_m \approx K_{ip}^{Ade}$ and form a group of good substrates for the ATP N-glycosidase. This group includes ATP, A5′p_n5′A ($n = 3$–5) and ADP.

A special K_m study was performed for ADP as a substrate. The K_m of ADP (0.12 mM) was found to be close to the K_m of ATP (0.10 mM), and correspondingly to K_{ip}^{Ade} (Fig. 4).

The progress curves of the other substrates exhibit a $K_m > K_{ip}^{Ade}$ character. The β-P-5′-ribosides of ADP (ADPR, NAD^+, NADH and FAD) were hydrolysed between three and six times slower than ATP. Under conditions where the reaction rate of ATP was maximal, adenine release was observed from AMP at the rate of

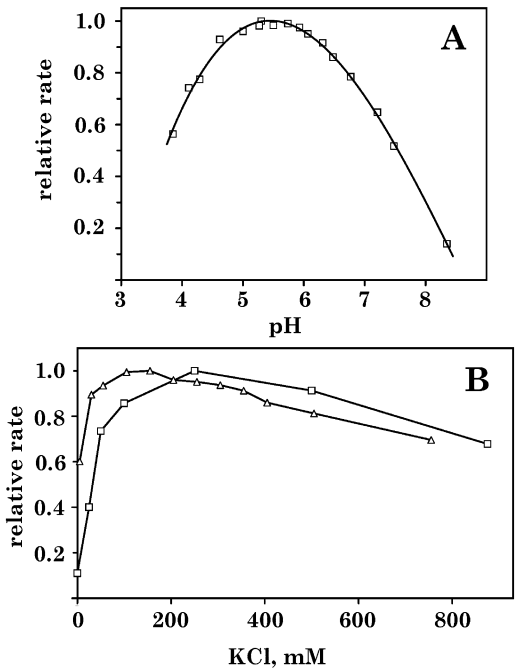

Fig. 6. Influence of pH and ionic strength on N-glycohydrolysis rate of ATP. (A) pH-dependence. The reactions were performed at 37 °C with 1 mM ATP containing 250 mM KCl and 20 mM buffer (acetate, Mes, Mops or bicarbonate) and *A. polypoides* crude extract (1 : 100). The actual pH of each final mixture at 37 °C was determined and used as an abscissa value. The reaction rates were calculated from progress curves, assuming a pseudo first-order kinetics, and normalized to the highest registered value (pH 5.3, $v = 0.0384$ mM·min^{-1}). The progress curves were exponential in the whole pH range analysed, independently from the buffer. The curve drawn through the experimental points is arbitrary. (B) Ionic strength dependence. The assay mixture contained 1 mM ATP and *A. polypoides* crude extract (1 : 100). The concentration of KCl was varied in the pH 7.0 (□) and pH 5.2 (△) series. The pH of each reaction mixture was measured at the assay temperature (37 °C). Variations in the pH within the series were found to be negligible. The initial rates calculated from the progress curves were normalized to the highest rate observed within the series (pH 7.0 series: 250 mM KCl $v = 0.0267$ mM·min^{-1}; pH 5.2 series: 155 mM KCl $v = 0.0394$ mM·min^{-1}).

> 1/8 of ATP (Table 2). A faint, but still reliably detectable adenine release from adenosine was also observed (> 300 times slower than in the case of ATP).

No release of adenine was observed from (2′,5′)p_3A_2, poly(A), adenosine-rich oligodeoxyribonucleotides, cAMP or 2′(3′)-AMP.

Possible involvement of ATP N-glycosidase in the NAD^+/cADPR signalling pathway

The results on cleavability of the two substances included in Table 2 should be presented in a greater detail.

The adenine release studies from NAD^+ and $NADP^+$ were interfered by a huge ADP ribosyl cyclase activity in *A. polypoides* [18]. The cADPR formation rate calculated from the earliest time-point of the NAD^+ reaction (Fig. 8A) was 182 μmol·min^{-1}·mg^{-1}. The cyclization reaction did not exhaust the NAD^+ ($NADP^+$) completely

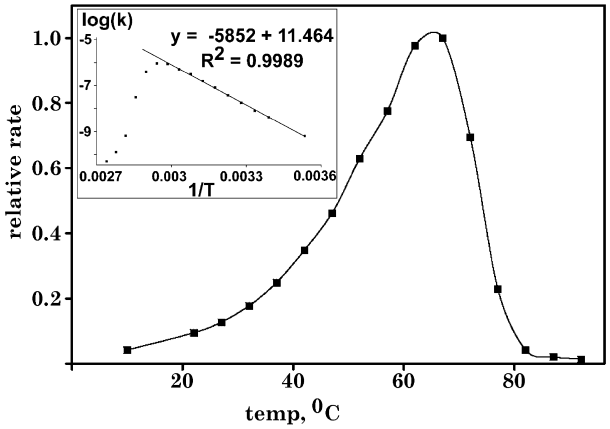

Fig. 7. Temperature dependency of ATP N-glycohydrolysis by *A. polypoides* crude extract. ATP (1 mM; 20 mM Mes pH 5.3, 250 mM KCl) was incubated with the crude extract (dilution 1 : 100) at different temperatures for 10 min. The initial rates, assuming pseudo first-order kinetics, were calculated. The initial rates in the main graph were normalized to the highest observed rate within the series (67 °C, $v = 0.145$ mM·min^{-1}). The temperature points from 10 to 62 °C were used for the slope calculation on the Arrhenius plot.

Table 2. The initial rates of adenine release from different substrates by *A. polypoides* extract. The assays were performed in optimal conditions for ATP (pH 5.3, I = 0.15–0.25 mM, $[S_0] \approx$ 1–2 mM, 37 °C) with a 100-fold diluted crude extract (3 μg total protein·mL^{-1}).

Substrate	Adenine release (μmol·min^{-1}·mg protein^{-1})
ATP[a]	12.5
ADP[a]	9.2 (6.5[b])
AMP	1.43 (< 0.1[b])
Ado	0.038
DAB-ATP[a]	(12.5[b])
dATP	9.98 (1.5[b])
A5′p$_5$5′A[a]	5.20[c]
A5′p$_4$5′A[a]	3.73[c]
A5′p$_3$5′A[a]	5.04[c]
A5′p$_2$5′A	2.34[c]
FAD	3.25
ADPR	4.17
NADH	3.63
NAD+	1.99
NADP+	0.3
2′(3′)-AMP	0
(3′,5′)cAMP	0
poly(A)	0
(2′,5′)p$_3$A$_2$	0

[a] Progress curves of these substrates follow a pseudo first order kinetics within the accuracy of the experiments. [b] Estimated from the mixed substrate assay with ATP. [c] These substrates were assayed at the concentration ≈ 0.16–0.22 mM A5′p$_n$5′A (0.32–0.44 mM of adenine base), close to the K_m of ATP. For comparison with other substrates the values should be multiplied by ≈ 2.

under conditions used (high substrate concentration, pH 5.2), since an equilibrium was established between the cyclization reaction and its backward reaction (Fig. 8A). It

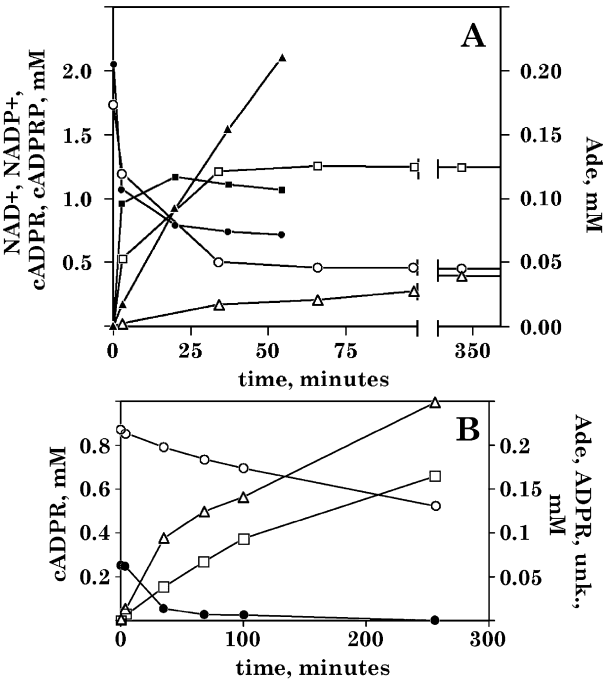

Fig. 8. Progress curves of NAD+, NADP+ and cADPR, incubated with *A. polypoides* crude extract. The substrates were incubated with the *A. polypoides* crude extract (1 : 100) (20 mM Mes pH 5.3, 170 mM KCl, 37 °C), 10 μL aliquots of the reaction mixture were analysed by HPLC. (A) Comparison of the progress curves of the NAD^+ and $NADP^+$ reaction mixtures. The compounds observed in the NAD^+ reaction are shown with filled symbols and those in the $NADP^+$ reaction with open symbols. Circles, NAD^+/$NADP^+$; squares, cADPR/cADPRP; triangles, adenine. Note the different scale used for adenine. (B) Progress curves of cADPR reaction. ○, cADPR; ●, ADPR, △, Ade; □, an unidentified compound. Extinction coefficient ε = 15 400 of the unidentified compound (retention time 3.18 min) was assumed in its quantification.

was uncertain how much adenine was formed directly from NAD^+ and how much could originate from cADPR. The latter could be considered as an alternative source of adenine. Direct release of adenine from cADPR is impossible (two N-glycosidic bonds to cleave), but ADPR, a product of the ADP ribosyl cyclase hydrolytic activity [18,19], has been shown to be a substrate of the ATP N-glycosidase (Table 2).

The formation of adenine from cADPR was studied (Fig. 8B). The overall rate of cADPR consumption (0.4 μmol·min^{-1}·mg^{-1}) showed that cADPR was a minor source of adenine in the NAD+ reaction. The formation of adenine from cADPR should be under the kinetic control of cADPR N1-glycosidic bond cleavage since the N-glycosidic bond hydrolysis of ADPR is a much faster reaction (Table 2). This is also evident from Fig. 8B, since the degradation of the contaminant ADPR (≈ 7%), present in the commercial preparation of cADPR, was more effective than that of the parent compound.

Still, cADPR was consumed in a parallel process resulting in an unknown compound (Fig. 8B). A lower extinction ratio of 260/290 nm of this unknown 'cADPR derivative' than even that of cADPR [20] indicates that the

N1-glycosidic bond in this compound is probably preserved. The exact nature of this novel cADPR metabolite remains to be determined. Defining this compound as a 'cADPR derivative' was useful for the identification of the NDPR, an ATP N-glycosidase hydrolysis product of NAD^+.

The initial rate of adenine release from NAD^+ (occurring relatively slowly compared to the NAD^+ cyclization) is about a magnitude higher than that from $NADP^+$ (Fig. 8A, Table 2). The fact that release of adenine from $NADP^+$ stopped before reaching K_{ip}^{Ade} [the kinetic points at 255 (not shown in Fig. 8A) and 343 min were almost identical] in contrast to any other substrate analysed, questioned the direct action of the ATP N-glycosidase on $NADP^+$. Thus, the cleavability of $NADP^+$ by the ATP N-glycosidase (Table 2) is very probably overestimated.

A. polypoides contains unusually strong ADP ribosyl cyclase activity. Our data indicate that the cADPR signalling pathway in *A. polypoides* could be modulated by the ATP N-glycosidase as both downstream (ADPR) and upstream (NAD^+) compounds of cADPR are its substrates.

Biochemical characterization of *A. polypoides*

The extraction of enzymes from *A. polypoides* yielded a crude extract of 0.3 mg protein·mL^{-1} (2 mg protein per 1 g frozen animal). This crude extract contained 12.5 μmol·min^{-1}·ml^{-1} (25 μmol·min^{-1}·g wet $weight^{-1}$) of ATP N-glycosidase activity and 250 μmol·min^{-1}·ml^{-1} (500 μmol·min^{-1}·g wet $weight^{-1}$) ADP ribosyl cyclase activity, measured under the conditions of the ATP N-glycosidase assay (the 500-fold dilution of the crude extract was necessary for the adequate estimation of the initial reaction rate). The nucleotide-5′-triphosphate dephosphorylating activity of the crude extract was estimated to be ≈ 0.2 μmol·min^{-1}·mg^{-1} (dTTP, dGTP), adenosine was formed from 2′(3′)-AMP at 0.02 μmol·min^{-1}·mg^{-1}. No adenosine nucleotide/nucleoside/nucleobase deaminase activities were observed in any assay performed.

The extract prepared from an alternative sample of frozen *A. polypoides* showed a similar level of ATP N-glycosidase activity per g of animal wet weight, dominating similarly over alternative routes of ATP utilization. The ATP N-glycosidase activity yield from the air-dried *A. polypoides* sample (in spite of its lower water content) was lower by more than a magnitude (per g sample) as compared to frozen samples.

Most, if not all sponges harbour microorganisms, such as bacteria and fungi, within their tissues. In contrast to *G. cydonium*, *A. polypoides* contained only few bacteria (Fig. 9, lanes 5 and 4, respectively). This result speaks in favour of the animal origin of the ATP N-glycosidase.

The ATP content of *A. polypoides*, estimated by the sensitive luciferase assay, was 1.5 nmol·g^{-1} frozen animal. It was not possible to detect any adenine, ATP, ADP or AMP in the trichloroacetic acid extract by the HPLC method used, as interfering peaks of unknown nature with close retention times were present.

Discussion

Here we report that the marine sponge *A. polypoides* contains an enzymatic activity which hydrolyses the N-glycosidic bond of ATP, leaving the energy-rich triphosphate moiety intact. Special care was taken to prove that the ribose–triphosphate moiety of ATP was not altered during the reaction and was left as ribose-5-triphosphate.

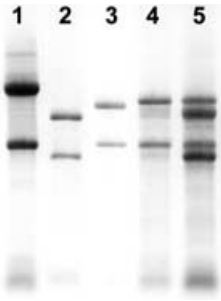

Fig. 9. Ribosomal RNA of *A. polypoides*. The samples were analyzed in a 1.2% agarose-formaldehyde gel and stained with ethidium bromide. Markers for eukaryotic and prokaryotic rRNA are shown in lanes 1 (*Homo sapiens*), 2 (*Escherichia coli*) and 3 (*Saccaromyces cerevisiae*). In comparison with the marine sponge *G. cydonium* (lane 5), *A. polypoides* (lane 4) contains only few bacteria.

On the basis of our experiments we assumed similar reactions with other adenylates as substrates. The formation of UV-absorbing products, complementary to adenine and with expected chromatographic properties, were registered when substrates A5′p_n5′A, NAD^+ and FAD were assayed. Thus the unique enzyme from *A. polypoides* may be applied to preparative synthesis of otherwise hardly obtainable compounds, containing the D-ribose-5-oligophosphoryl group.

The data in Table 2 prove that ATP is a preferred substrate for the novel enzyme. No other natural substrate was degraded to adenine more efficiently than ATP at the millimolar concentration. ADP and dATP, both of which exhibited rates of the adenine release similar to ATP in separate reactions, were clearly discriminated when assayed in mixtures with ATP. ADP and dATP were degraded with about 2- and 8.5-fold lower rates than ATP, respectively. AMP, which released adenine at an initial rate of 1/8 of that of ATP in an individual assay, remained nearly unchanged within the time required for the complete degradation of ATP in a mixed assay.

We also performed preliminary kinetic studies of the ATP N-glycosidase. The kinetic scheme considering product inhibition ($K_m^{ATP} \approx (K_m^{ADP}) \approx K_{ip}^{Ade} \approx 0.1$–$0.2$ (mM) described adequately the shapes of progress curves of individual reactions of ATP and ADP in a millimolar range. We propose that the inhibition by adenine is the main factor that determines progress curve shapes in a millimolar concentration range for more stable substrates than ATP (ADP) analogues.

Our experiments with canonical nucleotides showed that the enzyme completely ignored pyrimidine derivatives and also purine derivatives having 6-oxy substituents. ITP, which differs from ATP only in a substituent in position 6 of the purine heterocycle, was neither a substrate nor an inhibitor of the ATP N-glycosidase (as revealed in a mixed assay with ATP). Thus, nonadenosine nucleotides were discriminated by the ATP N-glycosidase at the binding level. Taking into account that $K_m^{ATP} \approx K_{ip}^{Ade}$, a conclusive role of 6-aminopurine in substrate binding to the enzyme could be proposed. However, the role of the other parts of the ATP molecule is

also important. K_m^{AMP} and K_m^{Ado} ($\approx$ 1.5 and $\approx$ 4.5 mM, respectively) estimated from the progress curve shapes were a magnitude higher than those of ATP and ADP. A relatively small contribution of the γ-phosphate to the binding affinity was deduced from the observation that the $K_m \approx K_{ip}^{Ade}$ condition was satisfied only for ATP analogues, having a substituent at the γ-phosphate (DAB-ATP, A5′p$_3$5′A, A5′p$_4$5′A and A5′p$_5$5′A), or lacking the γ-phosphate (ADP). K_m of substituted at the β-phosphate analogues of ADP (A5′p$_2$5′A, ADPR, NADH, FAD, NAD^+), estimated from the progress curve shapes, were three- to fivefold higher than K_{ip}^{Ade}. Substitution of the 2′-OH group of ATP with 2′-H had a similar impact on K_m (dATP $K_m \approx 0.45$ mM), while modifications of the 2′- or 3′- group of ribose by a phosphate group led to a significant decrease in cleavability of the substrate by the ATP N-glycosidase.

(2′,5′)p$_3$A$_2$ was completely resistant to the ATP N-glycosidase. The 5′-terminal adenylate in (2′,5′)p$_3$A$_2$ is resistant because of the 2′-substituent. Unacceptance of a bulky substituent at the phosphate OH-group in 5′-AMP is evident from the stability of the 2′-terminal adenylate in (2′,5′)p$_3$A$_2$. This explains the stability of adenylates in RNA towards the ATP N-glycosidase, which was confirmed using polyadenylic acid as a substrate.

Discontinuity of the binding affinity in N6-aminopurine derivative series ATP < ADP $\ll$ AMP < Ado $\gg$ Ade indicates that the binding modes of substrates and inhibitors may be different. The equivalency of substrate and inhibitor binding was questioned in a recent study on the v_{max}-mutant of the purine nucleosidase from *Trypanosoma vivax*, complexed with its native substrate inosine [21]. In the enzyme–substrate complex inosine was present in *anti* conformation in contrast with the inhibitor 3-deazaadenosine *syn* conformation [22], while the relative orientation of the ribose to the enzyme was preserved, i.e. the orientation of the heterocycle in the active site of the enzyme was changed by $\approx$ 180°.

The ATP N-glycosidase-catalysed degradation of ATP was indifferent to the addition of Mg^{2+} or a chelator of a divalent metal. This was assayed in an EDTA concentration (10 mM) sufficient to keep the substrate free from any divalent metal which could originate from the crude extract.

Ca^{2+}-containing nucleosidases use the metal ion to coordinate both the 2′- and 3′-OH groups of a substrate [21–23]. However, the attempts to demonstrate the requirement in a metal ion, using divalent metal chelator inhibitory assays, have partially or completely failed because of the too high affinity of the metal ion to the enzyme [24,25]. The almost absolute stability of natural 2′-deoxynucleosides due to their mode of ribose binding, against the action of nucleosidases, having a nucleoside hydrolase fold [22], is therefore a good preliminary characteristic in distinguishing nucleoside hydrolases from nucleosidases, having a nucleoside phosphorylase/hydrolase fold [26,27]. The latter do not require any divalent metal for ribose binding, thus more easily accepting the absence of the 2′-OH group as well as other variations in the ribose structure. The acceptance of dATP as a substrate (Table 2) is in favour of the point of view that the ATP N-glycosidase is not a member of the nucleoside hydrolase family.

The comparison of enzymatic properties of the ATP N-glycosidase from *A. polypoides* with a selected set of N6-aminopurine riboside nucleosidases, which are independent from divalent metals, is given in Table 3.

The bacterial 5′-methylthioadenosine/S-adenosylhomocysteine (MTA/SAH) nucleosidase (EC 3.2.2.9, EC 3.2.2.16) is the only nucleosidase independent from a divalent metal, having a known three-dimensional structure, which is similar to nucleoside phosphorylases [26,28]. Similar to the ATP N-glycosidase, the MTA/SAH nucleosidase: (a) accepts a range of substrates differing in the size of their 5′-substituents [29] and (b) cleaves the 2′-deoxy derivative of its preferred substrate [30]. The affinity of the MTA/SAH

Table 3. Comparison of enzymatic properties of ATP N-glycosidase from *A. polypoides* with other adenine-releasing nucleosidases. ND, Not determined.

	Enzyme				
	ATP N-glycosidase *Axinella polypoides* (animal[a])	Adenosine nucleosidase *Hordeum vulgare* (plant[a])	AMP nucleosidase *Azotobacter vinelandii* (bacterium[a])	MTA/SAH nucleosidase *Escherichia coli* (bacterium[a])	NAD^+ nucleosidase *Aspergillus niger* (fungus[a])
K_m (mM)	0.1	0.002	0.1	MTA 0.00043 SAH 0.0043	3
K_{ip}^{Ade}(mM)	0.1	0.004	0.53	0.3	ND
pH optimum	5.2	4.7–5.4	7.8	< 4.5; 6.0	4.0–4.5
Me^{2+}	Independent	Independent	MgATP Activator	Independent	Independent
$v_{max} \gg 12.5$ ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	30.5	34	373	55	
Thermostability	t_{opt} = 60–70 °C	Half-denaturation 10 min at 45 °C; (60 °C with adenine)	> 2 h at 60 °C	t_{opt} = 37–42 °C; unstable at 55 °C	> 2 h at 37 °C
ΔH_a ($kcal \cdot mol^{-1}$)	11.6	6.5[b]	10.6, 18.5	3.5[b]	ND
References	This work	[36]	[33,34]	[29,30]	[38]

[a] Source organism. [b] Calculated from figures given in the articles cited.

nucleosidase to its products ($K_{ip}^{Ade} = 0.3$ mM $\ll K_{ip}^{MTR}$ [29]) is comparable with that of the ATP N-glycosidase. However, the product inhibition ($K_{ip}^{Ade} \gg K_m^{MTA} = 0.43$ μM) is obviously not characteristic of this enzyme [29].

The AMP nucleosidase (EC 3.2.2.4) has a nucleoside phosphorylase/hydrolase fold predicted by the sequence homology [31]. This enzyme is inefficient in releasing adenine from dAMP ($v_{max}^{AMP}/v_{max}^{dAMP} = 77$), but strongly binds dAMP ($K_m^{dAMP} < K_m^{AMP}$) [32]. Though the AMP nucleosidase binds ATP, the complex of ATP with Mg, MgATP, acts as an allosteric activator and not as a substrate. AMP nucleosidase is not able to hydrolyse either IMP or Ado, having $K_i^{IMP}/K_m^{AMP} = 4.75$ and $K_i^{Ado}/K_m^{AMP} = 175$, respectively [32,33]. The inhibition of the AMP nucleosidase by adenine is a complex process with the most pronounced competitive component [34]. K_{ip}^{Ade} is fivefold higher than K_m^{AMP} for this enzyme.

No information about the primary structure is available for the adenosine nucleosidase (EC 3.2.2.9) [35]. The enzyme purified from barley leaves [36] is active on dAdo, but not on Ino and it is inhibited by the adenine ($K_{ip}^{Ade}/K_m^{Ado} = 2$) similarly to the ATP N-glycosidase. However, the nucleosidase from *Lupin luteus* has a relative activity of 100 : 27 : 7 on the substrates Ado/Guo/Ino [37].

The NAD^+ adenosine nucleosidase from *Aspergillus niger* [38] has the most pronounced overlap in the substrate range with the ATP N-glycosidase. This enzyme has been classified as EC 3.2.2.1 due to its substrate preferences (Ino > IMP > AMP > Ado∼α-NAD^+ > NAD^+ > GMP > Guo). No primary structure information is available for this enzyme but the reported resistance to EDTA and the acceptance of 2′- or 3′-phosphorylated substrates [38] make its assignment to the nucleoside hydrolase type of proteins rather problematic. Unfortunately the substrates of our interest (ATP, ADP, dATP, etc.) have not been studied for this enzyme.

The present classification of nucleosidases (EC 3.2.2.-) is misleading and should be revised. This will be possible when the information about the structure of plant nucleosidases, fungal nucleosidases and the sponge ATP N-glycosidase becomes available.

The most amazing aspects of the usage of ATP by the *A. polypoides* extract are not only the presence of a novel enzymatic activity, but also the unprecedented high potency of ATP utilization. The rate of ATP consumption by the extract of *A. polypoides* (12.5 μmol·min^{-1}·mg^{-1}) was more than a magnitude higher than that of the extract of *G. cydonium* (0.39 μmol·min^{-1}·mg^{-1} at 37 °C, 2′,5′-oligoadenylates as the main products formed [15]). Among the adenine-specific nucleosidases the activity of *A. polypoides* crude extract is of the same order as the specific activities of the purified barley adenosine nucleosidase, the AMP nucleosidase or the NAD^+ adenosine nucleosidase (Table 3). The potency of the ATP N-glycosidase for ATP degradation, according to appropriate recalculations for conditions simulating natural ones (pH, temperature) per g animal wet weight (4.375 μmol·min^{-1}), still significantly exceeds the ATP formation rate in a sponge (estimated from oxygen utilization of 0.146–0.56 μmol·min^{-1}·g wet weight^{-1} [39]). Moreover, the ATP N-glycosidase acts on the precursor of the ATP formation, ADP, as well. Thus, the access of the enzyme to its substrate should be locally restricted or its action should be transient.

Another unusually potent activity, converting a high-energy nucleotide – the ADP ribosyl cyclase – was characterized in parallel in the crude extract of *A. polypoides*. The ADP ribosyl cyclase activity in *A. polypoides* has been described previously [18]. The authors referred to it as a huge activity but it was still over two magnitudes lower than that found in the present work (Fig. 8). Even considering the different temperatures of the assays (the difference in the cADPR forming rate at 14 °C was found to be 6.5 times slower than that at 37 °C; data not shown), and possible variations arising from other assay conditions (pH), it is clear that Zocchi *et al.* [18] had revealed only a part of the huge ADP ribosyl cyclase activity present in the whole animal body. Two different carriers of the ADP ribosyl cyclase activity in *A. polypoides*, a cell-associated and a secreted form, were reported in a later publication by Zocchi *et al.* [40]. We suppose that the ADP ribosyl cyclase activity quantified in the current study was mainly presented by the secreted form of the enzyme.

The extracellular location of the ATP N-glycosidase provides a possible explanation for its paradoxical substrate specificity, combined with its high enzymatic capacity. NAD^+, but not ATP, was detected in the seawater surrounding *A. polypoides* [40]. The absence of ATP and products of its usual degradation (ADP, AMP, Ado) has been taken as a proof for a directional efflux of NAD^+ from the organism [40]. However, the absence of ATP in this experiment may be explained by the ATP N-glycosidase activity outside the cell. On the other hand, if the preferred *in vitro* substrates are absent, the ATP N-glycosidase may be functional on its alternative substrates (e.g. NAD^+ and ADPR). It should be mentioned that the *Aspergillus niger* NAD^+ adenosine nucleosidase was discovered as the enzyme producing nicotinamide ribose diphosphate ribose (NDPR), found in media surrounding mould [37]. Secretion of NDPR was proposed, since the cADPR synthesis in the outer membrane of the cell (topological paradox [41]) was unknown at that time and the NAD^+ was thought to be solely a cellular ingredient.

We have no data on the localization of the ATP N-glycosidase yet. Therefore the given hypothesis about the extracellular function of the ATP N-glycosidase is only one of the numerous alternative guesses, which could be proposed on the basis of the known importance of ATP in cells.

Acknowledgements

We wish to thank W. Schatton and W.E.G. Müller for supplying us with the sponge material. The study was supported by the European Commission (Project Sponge) and the Estonian Science Foundation (grant no. 4221).

References

1. Guyot, M. (2000) Intricate aspects of sponge chemistry. *Zoosystema* **22**, 419–431.
2. Bergmann, W. & Feeney, R.J. (1950) The isolation of a new thymine pentoside from sponges. *J. Am. Chem. Soc.* **72**, 2809–2810.

3. Kuusksalu, A., Pihlak, A., Müller, W.E.G. & Kelve, M. (1995) Identification of the reaction products of (2′-5′) oligoadenylate synthetase in the marine sponge. *Eur. J. Biochem.* **232**, 351–357.
4. Lopp, A., Kuusksalu, A., Reintamm, T., Müller, W.E.G. & Kelve, M. (2002) 2′,5′-Oligoadenylate synthetase from a lower invertebrate, the marine sponge *Geodia cydonium*, does not need dsRNA for its enzymatic activity. *Biochim. Biophys. Acta* **1590**, 140–149.
5. Justesen, J., Hartmann, R. & Kjeldgaard, N.O. (2000) Gene structure and function of the 2′-5′-oligoadenylate synthetase family. *Cell. Mol. Life Sci.* **57**, 1593–1612.
6. Kumar, S., Mitnik, C., Valente, G. & Floyd-Smith, G. (2000) Expansion and molecular evolution of the interferon-induced 2′-5′ oligoadenylate synthetase gene family. *Mol. Biol. Evol.* **17**, 738–750.
7. Gough, J., Karplus, K., Hughey, R. & Chothia, C. (2001) Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure. *J. Mol. Biol.* **313**, 903–919.
8. Rolfe, D.F.S. & Brown, G.C. (1997) Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol. Rev.* **77**, 732–758.
9. Ng, K.E. & Orgel, L.E. (1987) The action of a water-soluble carbodiimide on adenosine-5′-polyphosphates. *Nucleic Acids Res.* **15**, 3573–3580.
10. Purmal′, A.A., Drutsa, V.L. & Shabarova, Z.A. (1988) Introduction of tri- and tetraphosphate internucleotide bonds into oligodeoxyribonucleotides. *Bioorg Khim.* **14**, 1183–1187.
11. Chomczynski, P. & Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.
12. Lundin, A. (2000) Use of firefly luciferase in ATP-related assays of biomass, enzymes, and metabolites. *Methods Enzymol.* **305**, 346–370.
13. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
14. Gottlieb, H.E., Kotlyar, V. & Nudelman, A. (1997) NMR chemical shifts of common laboratory solvents as trace impurities. *J. Org. Chem.* **62**, 7512–7515.
15. Reintamm, T., Lopp, A., Kuusksalu, A., Subbi, J. & Kelve, M. (2003) Qualitative and quantitative aspects of 2-5A synthesizing capacity of different marine sponges. *Biomol. Eng.* **20**, 389–399.
16. Serianni, A.S., Pierce, J. & Barker, R. (1979) Carbon-13-enriched carbohydrates: Preparation of triose, tetrose and pentose phosphates. *Biochemistry* **18**, 1192–1199.
17. Duggleby, R.G. (2001) Quantitative analysis of the time courses of enzyme-catalyzed reactions. *Methods* **24**, 168–174.
18. Zocchi, E., Carpaneto, A., Cerrano, C., Bavestrello, G., Giovine, M., Bruzzone, S., Guida, L., Franco, L. & Usai, C. (2001) The temperature-signaling cascade in sponges involves a heat-gated cation channel, abscisic acid, and cyclic ADP-ribose. *Proc. Natl Acad. Sci. USA* **98**, 14859–14864.
19. Cakir-Kiefer, C., Muller-Steffner, H. & Schuber, F. (2000) Unifying mechanism for *Aplysia* ADP-ribosyl cyclase and CD38/NAD(+) glycohydrolases. *Biochem. J.* **349**, 203–210.
20. Gu, Q.-M. & Sih, C.J. (1994) Cyclic ADP-ribose: Synthesis and structural assignment. *J. Am. Chem. Soc.* **116**, 7481–7486.
21. Versées, W., Decanniere, K., Van Holsbeke, E., Devroede, N. & Steyaert, J. (2002) Enzyme–substrate interactions in the purine-specific nucleoside hydrolase from *Trypanosoma vivax*. *J. Biol. Chem.* **277**, 15938–15946.
22. Versées, W., Decanniere, K., Pellé, R., Depoorter, J., Brosens, E., Parkin, D.W. & Steyaert, J. (2001) Structure and function of a novel purine specific hydrolase from *Trypanosoma vivax*. *J. Mol. Biol.* **307**, 1363–1379.
23. Shi, W., Schramm, V.L. & Almo, S.C. (1999) Nucleoside hydrolase from *Leishmania major*. Cloning, expression, catalytic properties, transition state inhibitors, and the 2.5-Å crystal structure. *J. Biol. Chem.* **274**, 21114–21120.
24. Ribeiro, J.M.C. & Valenzuela, J.G. (2003) The salivary purine nucleosidase of the mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol.* **33**, 13–22.
25. Ogawa, J., Takeda, S., Xie, S.-X., Hatanaka, H., Ashikari, T., Amachi, T. & Shimizu, S. (2001) Purification, characterization, and gene cloning of purine nucleosidase from *Ochrobactrum anthropi*. *Appl. Env. Microbiol.* **67**, 1783–1787.
26. Lee, J.E., Cornell, K.A., Riscoe, M.K. & Howell, P.L. (2001) Structure of *E. coli* 5′-methylthioadenosine/S-adenosylhomocysteine nucleosidase reveals similarity to the purine nucleoside phosphorylases. *Structure* **9**, 941–953.
27. Pugmire, M.J. & Ealick, S.E. (2002) Structural analyses reveal two distinct families of nucleoside phosphorylases. *Biochem. J.* **361**, 1–25.
28. Lee, J.E., Cornell, K.A., Riscoe, M.K. & Howell, P.L. (2003) Structure of E. coli MTA/AdoHcy nucleosidase inhibitor complexes provide insight into the conformational changes required for substrate binding and catalysis. *J. Biol. Chem.* **278**, 8761–8770.
29. Cornell, K.A., Swarts, W.E., Barry, R.D. & Riscoe, M.K. (1996) Characterization of recombinant *Eschericha coli* 5′-methylthioadenosine/S-adenosylhomocysteine nucleosidase: analysis of enzymatic activity and substrate specificity. *Biochem. Biophys. Res. Comm.* **228**, 724–732.
30. Allart, B., Gatel, M., Guillerm, D. & Guillerm, G. (1998) The catalytic mechanism of adenosylhomocysteine/methylthioadenosine nucleosidase from *Escherichia coli* – chemical evidence for a transition state with a substantial oxocarbenium character. *Eur. J. Biochem.* **256**, 155–162.
31. Leung, H.B. & Schramm, V.L. (1984) The structural gene for AMP nucleosidase. Mapping, cloning, and overproduction of the enzyme. *J. Biol. Chem.* **259**, 6972–6978.
32. DeWolf, W.E., Fullin, F.A. & Schramm, V.L. (1979) The catalytic site of AMP nucleosidase. Substrate specificity and pH effects with AMP and formycin 5′-PO_4. *J. Biol. Chem.* **254**, 10868–10875.
33. DeWolf, W.E., Emig, F.A. & Schramm, V.L. (1986) AMP nucleosidase: kinetic mechanism and thermodynamics. *Biochemistry* **25**, 4132–4140.
34. Heppel, L.A. (1963) Adenylic acid ribosidase. *Methods Enzymol.* **6**, 117–124.
35. Auer, C.A. (2002) Discoveries and dilemmas concerning cytokinin metabolism. *J. Plant Growth Regul.* **21**, 24–31.
36. Guranowski, A. & Schneider, Z. (1977) Purification and characterization of adenosine nucleosidase from barley leaves. *Biochim. Biophys. Acta* **482**, 145–158.
37. Abusamhadneh, E., McDonald, N.E. & Kline, P.C. (2000) Isolation and characterization of adenosine nucleosidase from yellow lupin (*Lupinus luteus*). *Plant Sci.* **153**, 25–32.
38. Kuwahara, M. & Fuji, T. (1978) Purification and some properties of NAD-degrading purine nucleosidase from *Aspergillus niger*. *Can. J. Biochem.* **56**, 345–348.
39. Reiswig, H.M. (1974) Water transport, respiration and energetics of three tropical marine sponges. *J. Exp. Mar. Biol. Ecol.* **14**, 231–249.
40. Zocchi, E., Basile, G., Cerrano, C., Bavestrello, G., Giovine, M., Bruzzone, S., Guida, L., Carpaneto, A., Magrassi, R. & Usai, C. (2003) ABA- and cADPR-mediated effects on respiration and filtration downstream of the temperature-signaling cascade in sponges. *J. Cell Sci.* **116**, 629–636.
41. Zocchi, E., Franco, L., Guida, L., Benatti, U., Bargellesi, A., Malavasi, F., Lee, H.C. & De Flora, A. (1993) A single protein immunologically identified as CD38 displays NAD+ glycohydrolase, ADP-ribosyl cyclase and cyclic ADP-ribose hydrolase activities at the outer surface of human erythrocytes. *Biochem. Biophys. Res. Commun.* **196**, 1459–1465.