ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 11 (2001) 469–485

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

# Nucleotide ester-forming alcoholytic activities of nucleotide pyrophosphatases: implications for practical biotransformation, enzyme mechanisms and biological function

José Carlos Cameselle*, Antonio Agudo, José Canales, María Jesús Costas, Ascensión Fernández, Ana Flores, Miguel García-Díaz, Santiago González-Santiago, Juan López-Gómez, João Meireles Ribeiro, José María Vergeles

*Unidad de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Extremadura, Apartado 108, E-06080 Badajoz, Spain*

**Abstract**

Nucleotide pyrophosphatases (NPP) hydrolyze phosphoanhydride and phosphodiester derivatives of nucleoside 5′-monophosphates (NMP) yielding NMP as a product. In a water–alcohol mixture, the alcohol (R–OH) competes and substitutes for water as the splitting agent, so a mixture of NMP and NMP-O-alkyl ester (NMP-O-R) is formed. NPPs from snake venom, potato tuber and mammalian tissues have been studied in this regard. Snake and potato NPPs were considered as possible practical biocatalysts to synthesize NMP-O-Rs from various nucleotidic substrates and alcohols. Mammalian NPPs, mainly from human blood and rat liver, were studied considering the possibility that the alcoholytic reactions catalyzed by them could be biologically relevant. Valuable information on the active centers and catalytic mechanisms of NPPs was also obtained. 

*Keywords:* Alcohols; Nucleotide esters; Nucleotide pyrophosphatase; Phosphodiesterase; Enzyme mechanism

## 1. Introduction

Nucleotide pyrophosphatases (NPP; EC 3.6.1.9) are classified as broad-specificity enzymes that hydrolyze phosphoanhydride derivatives of 5′-nucleotides (NMP) yielding the corresponding NMP as a reaction product. Typical substrates are (di)nucleoside-oligophosphates, like $NAD^+$, $Ap_4A$ or ATP. The same enzymes hydrolyze also phosphodiester derivatives of 5′-nucleotides, an activity classified as phosphodiesterase I (PDE; EC 3.1.4.1) and usually assayed with 4-nitrophenyl-dTMP [1–4]. Typical reactions are:

$$Ap_4A + H_2O \rightarrow AMP + ATP \quad (1)$$

$$ATP + H_2O \rightarrow AMP + PP_i \quad (2)$$

$$\text{4-nitrophenyl-dTMP} + H_2O \rightarrow \text{dTMP} + \text{4-nitrophenol} \quad (3)$$

* Corresponding author. Tel.: +34-924-289470; fax: +34-924-289468.

*E-mail address:* camselle@unex.es (J.C. Cameselle).

1381-1177/01/$ - see front matter 
PII: S1381-1177(00)00013-8

Therefore, one can speak of NPP/PDE enzymes (below referred to just as NPP) catalyzing the general reaction

$$\text{NMP-O-X} + \text{H}_2\text{O} \rightarrow \text{NMP} + [\text{H}]\text{O–X} \quad (4)$$

NMP being any 5′-nucleotide and [H]O–X representing an (oligo)phosphate (either esterified or not) when NMP-O-X is a phosphoanhydride, or an alcohol when NMP-O-X is a phosphodiester. Some NPPs can also hydrolyze non NMP-containing substrates, like the artificial phosphodiester bis(4-nitrophenyl) phosphate or several phosphonate esters [5]. Anyhow, the biological leitmotiv of NPPs seems to be the ability to extract NMP from a large variety of NMP-containing substrates. In fact, at least animal NPPs catalyze their reaction through the formation of an NMP-enzyme covalent intermediate: first the OH group of a threonine side chain carries out a nucleophilic attack on the α-phosphorus atom of the substrate, thereafter NMP is released by hydrolysis [6–10].

Several years ago, while using snake venom NPP as an analytical tool, we found accidentally that it catalyzes also alcoholytic reactions [11]:

$$\text{NMP-O-X} + \text{HO–R} \rightarrow \text{NMP-O-R} + [\text{H}]\text{O—X} \quad (5)$$

In alcohol–water mixtures, both nucleophiles compete for the enzyme-bound NMP, and reactions (4) and (5) occur in parallel.

In recent years, we have been studying these and similar alcoholytic reactions catalyzed by other NPPs for several reasons: their potential use for practical synthesis of nucleotide derivatives (NMP-O-alkyl esters, NMP-O-R), the information that they give about enzyme mechanisms, and the possibility that they are related to the biological roles of NPPs. In this article, we summarize previous and new advances in this field.

## 2. Theory

With some NPPs, kinetic experiments were run at different alcohol concentrations to test competition between alcohol (A) and water (W) as acceptors of the enzyme-bound adenylate formed during the splitting of ATP. The equations used to account for such competition are shown below.

According to kinetic theory for competing substrates with hyperbolic kinetics, when an enzyme acts on a mixture of two alternative substrates (A and W, for instance), each will inhibit competitively the reaction on the other, with a $K_i$ value equal to its $K_m$. The rate equations are then (see e.g. Ref. [12], pp. 105–108):

$$v_A = \frac{V_{maxA}[A]}{K_{mA}\left(1 + \frac{[W]}{K_{mW}}\right) + [A]} \quad (6)$$

$$v_W = \frac{V_{maxW}[W]}{K_{mW}\left(1 + \frac{[A]}{K_{mA}}\right) + [W]} \quad (7)$$

In alcohol–water mixtures with NPPs, the ratio $R_{AW} = (v_A/v_W)/([A]/[W])$ is a useful measurement of the efficiency of the alcohol as adenylate acceptor relative to that of water, as it equals 1 for an alcohol with the same reactivity as water. In earlier work, a slightly different parameter, $E_A = (v_A/(v_A + v_W))/([A]/([A] + [W]))$, was used to quantitate the efficiency of the alcohols as water competitors [13]. One can reach the same conclusions by using either efficiency parameter. When Eqs. (6) and (7) apply, $R_{AW} = (k_{cat}/K_m)_A/(k_{cat}/K_m)_W$, the ratio of the specificity constants of both substrates. The same is true for $E_A$ but only when $v_A \ll v_W$ and $[A] \ll [W]$ (in fact under these conditions $E_A \approx R_{AW}$). Therefore, considering the competition between alcohol and water as substrates in NPP reactions according to Eqs. (6) and (7), $R_{AW}$ would be independent of the concentration of both competitors, and of any factor, such as enzyme (in)activation, affecting equally the alcoholytic and the hydrolytic reactions.

In some of the competition experiments run with snake NPP and in most of those with rat liver NPP, the experimental values of $R_{AW}$ decreased as alcohol concentration increased, indicating that the alcohol displayed a negative cooperativity-like behavior in contrast to water. To account for this, rate Eqs. (6) and (7) were modified, as in the Hill equation (see Ref. [12], p. 207), by introducing an empirical $h$

coefficient and substituting $K_{0.5A}^{h}$ and $[A]^{h}$ for $K_{mA}$ and [A], respectively. The resulting equations are:

$$v_A = \frac{V_{maxA}[A]^h}{K_{0.5A}^h\left(1+\frac{[W]}{K_{mW}}\right)+[A]^h} \quad (8)$$

$$v_W = \frac{V_{maxW}[W]}{K_{mW}\left(1+\frac{[A]^h}{K_{0.5A}^h}\right)[W]} \quad (9)$$

From here one can arrive at:

$$\log\frac{v_A/v_W}{[A]/[W]} = \log\frac{\left(k_{cat}/K_{0.5}^h\right)_A}{\left(k_{cat}/K_m\right)_W} + (h-1)\log[A] \quad (10)$$

Therefore, when Eqs. (8) and (9) apply, a plot of log $R_{AW}$ vs. log [A] is linear with a slope equal to $h-1$. When $h<1$, like in negative cooperativity, one can predict that $R_{AW}$ would decrease with the increase of alcohol concentration, as experimentally observed. However, interestingly, $R_{AW}$ is still independent of any factor that affects to the same extent both rates, $v_A$ and $v_W$. Observe that Eqs. (6) and (7) are particular cases ($h=1$) of Eqs. (8) and (9).

## 3. Experimental

### 3.1. Enzymes

Snake venom NPP was a commercial preparation from *Crotalus durissus* (Boehringer, currently Roche cat. No. 108260) as a suspension in 50% (by volume) glycerol. Before use, the enzyme was freed from glycerol by gel filtration chromatography in a Sephadex G-25 column [14]. Potato tuber NPP was obtained from potato tubers either as a crude ammonium sulfate fraction of tuber juice or as a highly purified preparation [15]. Bovine intestine NPP was from Sigma (cat. No. P6903) further purified by Sephadex G-25 gel filtration and chromatography on AMP-agarose [11]. Rat liver NPP was solubilized from a Triton X-100 extract of rat liver membranes by limited proteolysis with trypsin and it was partly purified as described [16]. Human serum NPP was partly purified from 15 ml of normal human serum by Sephacryl S-300 gel filtration (a column of 95 cm × 2.8 cm in 10 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$ was used; a single major peak of NPP was recovered with elution volume, $V_e \approx 300$ ml) followed by ion exchange chromatography on DEAE cellulose (a column of 17.5 cm × 1.8 cm in the same buffer, developed with a linear 0–300 mM gradient of NaCl; a single major peak of NPP was recovered around 100 mM NaCl). The initial rate of hydrolysis of 1 mM 4-nitrophenyl-dTMP in 25 mM sodium phosphate, pH 7.5, 2.3 mM $MgCl_2$, at 37°C, was used as the reporter activity. The active fractions of the second chromatography were pooled and concentrated by ultrafiltration (Amicon PM30 membrane) up to a final volume of 1 ml. The yield was low (about 10% of serum NPP activity) but the preparation was stable at 4°C for 2 months.

The oligomeric dissociation of potato NPP tetramers to dimers was elicited by transient alkalinization according to Bartkiewicz et al. [17], either with crude or purified NPP. The enzyme was brought to near pH 10 by addition of 500 mM glycine/NaOH, pH 10, incubated at 0°C for 2 h, and neutralized to pH 7.8 by addition of 500 mM Tris/acetate, pH 6. Control samples received both buffers together at the end of the 2-h incubation at 0°C. Immediately after treatment alkalinized and mock-treated, 0.9-ml samples of NPP were chromatographed in a Sephacryl S-300 column equilibrated in 20 mM Tris-HCl, pH 7.8, 0.1 M KCl, 1 mM EDTA, 0.02% (by volume) Triton X-100. The chromatographies were developed with the same buffer at a flow rate of 4 ml/h. Tetramers and dimers of NPP were distinguished by the larger $V_e$ of the latter.

### 3.2. Assay of alcoholytic and hydrolytic activities of NPPs in alcohol–water mixtures

Except when otherwise indicated, alcoholytic activities were assayed by HPLC with 1–2 mM ATP (0.2 mM $Ap_2A$ in the case of potato NPP) as the substrate, under conditions of linearity with time and amount of enzyme. The incubations (performed at 37°C when not indicated otherwise) were stopped either by heating in a boiling bath for 2–6 min and freezing until HPLC analysis, or by direct injection

of a sample in the HPLC system. The substrate remaining after the reaction and the products specific for its alcoholysis and hydrolysis were quantitated by ultraviolet monitoring of HPLC chromatograms. The rates of alcoholysis ($v_A$) were determined always from the accumulation of the corresponding AMP-O-R. The rates of hydrolysis ($v_W$) of ATP were determined from the accumulation of AMP (plus adenosine and inosine, if necessary, in crude samples which were contaminated with phosphomonoesterases and adenosine deaminase). The rates of $Ap_2A$ hydrolysis were determined also from the amount of AMP formed (plus adenosine and inosine in crude preparations), but with the following corrections: the amount of AMP-O-R was substracted, as the alcoholysis of $Ap_2A$ gives rise to equimolar ester and AMP, and the result thus obtained was halved, as the hydrolysis of 1 mol of $Ap_2A$ yields 2 mol of AMP. With substrates other than ATP and $Ap_2A$, similar analysis and calculation procedures were implemented.

### 3.3. HPLC conditions

The analyses of NPP reactions in the presence of alcohols were carried out by reverse-phase HPLC in octadecylsilica (ODS) columns. The composition of the mobile phase in which the column was equilibrated (usually phosphate buffer, pH 7, with(out) tetrabutylammonium bromide (TBA) and/or methanol) and the type of elution performed (isocratic, gradient) depended on the compounds to be separated. Particular conditions for most combinations of nucleotidic and alcohol substrates can be found elsewhere [13–15]. For the analyses of samples containing AMP-O-seryl ester, a Hypersil ODS column (200 mm × 2.1 mm i.d.; Hewlett-Packard) with a precolumn guard (20 cm × 2.1 mm) of the same material was used. The column was equilibrated in 5 mM sodium phosphate, pH 7, and 20 mM TBA; the elution was accomplished at 0.5 ml/min with a 5-min isocratic wash followed by a 25-min linear gradient up to 300 mM phosphate in 20 mM TBA. Minor variations of elution conditions may be necessary depending on the age of the chromatographic column.

### 3.4. Identification of alcoholysis-specific products of NPP reactions

The nucleotidic products of alcoholysis catalyzed by snake NPP were identified as the corresponding NMP-O-Rs (see Fig. 1) by the following criteria: (i) HPLC retention times different to other known nucleoside(-phosphate) derivatives; (ii) alcohol-depen-

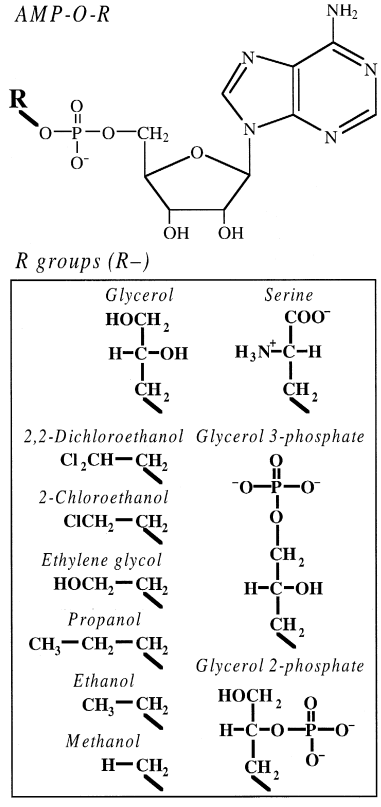


Fig. 1. Structures of the AMP-O-Rs formed by NPP-catalyzed alcoholytic reactions with primary alcohols. Two primary esters may be formed with glycerol, which would differ in the absolute configuration of glyceryl C-2, but they have not been detected independently. Therefore, what is described as AMP-O-glyceryl primary ester can be either one or a mixture of these two isomeric compounds. In addition to primary esters, the formation of secondary esters with the corresponding OH groups of glycerol and glycerol 3-phosphate has also been proved. Snake venom NPP catalyzes the formation of all of the AMP-O-Rs shown or mentioned. The same is true of rat liver NPP, except that chloroethanol and glycerol 3-phosphate have not been tested with this enzyme. In the case of potato tuber NPP, reactions only with methanol and ethanol have been investigated.

dent formation; (iii) resistance to alkaline phosphatase treatment (except for the esters formed by reaction with glycerophosphates as nucleophiles), in contrast to the standard nucleotides which yielded the corresponding nucleosides; (iv) susceptibility to extensive treatment with snake NPP in the absence of alcohol which converted them to the nucleotide (NMP-O-Rs, besides products, are (potential) substrates of NPPs, though not so efficiently split as ATP or $Ap_2A$ for instance); (v) ultraviolet spectrum typical of the corresponding nucleoside; (vi) $^{13}C$ and/or $^{1}H$ NMR spectra showing resonances attributable to the relevant alkyl group in addition to those expected for the nucleotidic moiety of the compound.

The same criteria were generally used to identify NMP-O-Rs formed by other NPPs, except that NMR spectra were not recorded. However, the NMP-O-Rs formed by the snake venom enzyme were used as HPLC standards in these cases.

## 4. Results and discussion

### *4.1. Snake venom NPP*

NMP-O-Rs (Fig. 1) are structural analogs of the natural ligands (ATP, ADP, AMP, adenosine, diadenosine polyphosphates) of purinergic receptors. So, NMP-O-Rs are potential (ant)agonists of purinoceptors or other receptors responding to nucleotides [18,19], and inhibitors of enzymes involved in the turnover of nucleotides [20,21]. Therefore, the synthesis of NMP-O-Rs by a commercially available biocatalyst, like snake venom NPP, is of interest.

#### *4.1.1. Alcoholytic reactions of nucleotidic substrates catalyzed by snake venom NPP: a versatile tool to produce NMP-O-Rs*

All the alcohols mentioned in Fig. 1 have been shown to participate in alcoholytic reactions of ATP catalyzed by snake NPP and yielding the corresponding AMP-O-R according to Eq. (5). This includes not only the primary OH groups [13], but also the secondary OH groups of glycerol and glycerol 3-phosphate [14]. The results of Fig. 2 show the formation of AMP-O-seryl ester from ATP and L-serine, a new addition to the list of reacting alcohols in NPP reactions.

Besides ATP, other nucleotidic substrates have been shown to participate at least in methanolytic reactions of snake NPP. This includes the nucleoside 5′-polyphosphates ADP, ATP, $p_4A$, GDP, GTP, $p_4G$, UDP, UTP, CDP, CTP and dTTP, the dinucleotide $Ap_2A$ and the phosphodiester 4-nitrophenyl-dTMP,

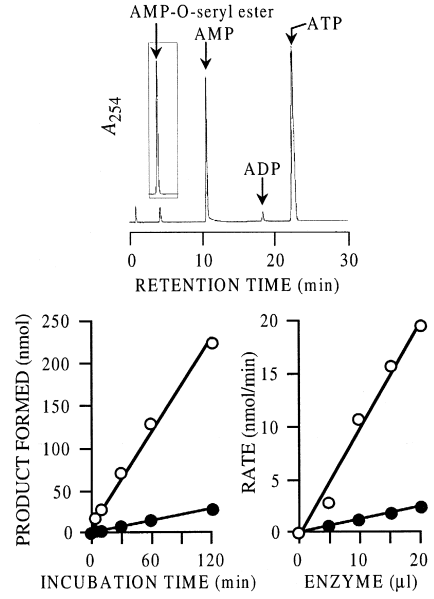


Fig. 2. Formation of AMP-O-seryl ester by NPPs. Upper panel: HPLC analysis (see Section 3.3) of a snake venom NPP reaction mixture (180 μl) incubated for 4 h, with 2 mM ATP, 0.67 M L-serine (Sigma, cat. No. S4500) and 4 μl of snake venom NPP in 5 mM sodium phosphate, pH 8. The enzyme stock solution contained 9 U/ml as standardized by its hydrolytic activity on 4-nitrophenyl-dTMP [14]. The insert shows the AMP-O-seryl ester peak with a ninefold expansion of the absorbance axis. This peak was not formed in the absence of serine, was resistant to alkaline phosphatase and was converted to AMP by further treatment with snake NPP in the absence of serine; after incubations in which alanine substituted for serine, no AMP-O-R formation was detected (results not shown). Lower panels: Dependency of ATP hydrolysis (○) and serinelysis (●) on the incubation length (with 2.5 μl of enzyme) and the amount of enzyme. The data were calculated as explained in Section 3.2 from HPLC chromatograms. Similar results were obtained with rat liver NPP (not shown).

which in all cases led to the formation of the corresponding NMP-O-methyl ester ([11] and results not shown).

*4.1.2. An ATP-regenerating system to increase the conversion of ATP to AMP-O-Rs*

So far, one of the limitations for the use of snake venom NPP for practical biotransformation is the low degree of conversion of nucleotidic substrate to NMP-O-R. This is so because the incubations are performed in monophasic mixtures of alcohol and water and there are constraints concerning the highest concentrations of alcohol that can be reached due to enzyme inactivation and/or low alcohol solubility. This is a problem with all the alcohols, but it is particularly strong with those showing low efficiencies as water competitors (propanol and ethanol), high denaturing potencies (chloroethanols) or low in water solubilities (glycerophosphates). In addition, the glycerophosphates display a negative cooperativity-like behavior (see Section 4.1.3) that poses further limits to conversion.

The degree of conversion could be increased—though at a cost, by the use of an ATP-regenerating system. With adenylate kinase and pyruvate kinase as auxiliary enzymes, and with a provision of phosphoenolpyruvate, the AMP formed by hydrolysis of ATP in snake NPP incubations was converted back to ATP (Fig. 3). The synthesis of AMP-O-glyceryl esters was studied under these conditions and compared to the same process in the absence of the regenerating system (Fig. 4). Without regenerating system, the rate of hydrolysis (i.e. AMP formation) was 2.6-fold higher than the rate of glycerolysis. In contrast, with the regenerating system, an 85% conversion of ATP to AMP-O-glyceryl esters was obtained after a 25-h incubation, with only a 1.4% buildup of AMP. The products of alcoholysis were separated from other components of the mixture by gel filtration chromatography on Sephadex G-25 in a system composed of three in-series columns: one of 74 cm × 1 cm, vertically layered, in which the sample was applied, and two of 110 cm × 1 cm each, horizontally layered. The chromatography was run with 500 mM NaCl. Although the primary and secondary AMP-O-glyceryl esters were not fully separated, two fraction pools were isolated: one containing only the primary ester(s) and the other containing both the primary and secondary ones (results not shown).

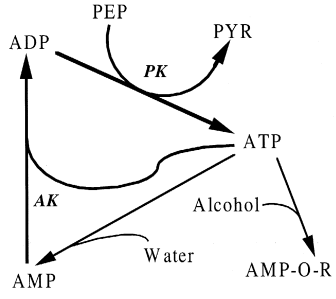


Fig. 3. Scheme of the ATP-regenerating system that increases the conversion of ATP to AMP-O-Rs. The thin arrows represent NPP-catalyzed reactions in an alcohol–water mixture; the formation of pyrophosphate as the second product of ATP alcoholysis and hydrolysis has been omitted. *AK*, adenylate kinase; PEP, phosphoenolpyruvate; *PK*, pyruvate kinase; PYR, pyruvate.

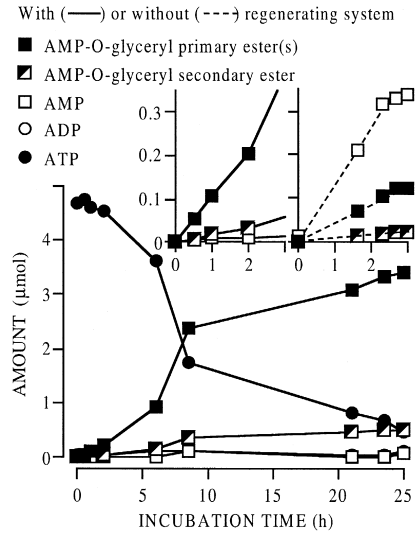


Fig. 4. Time course of the synthesis of AMP-O-glyceryl esters by snake venom NPP-catalyzed glycerolysis of ATP in the presence of the ATP-regenerating system. The reaction mixture (1 ml) was incubated at 37°C and initially it contained: 5 mM ATP, 4 M glycerol, 20 mM phosphoenolpyruvate, 5 mM $MgCl_2$, 20 U adenylate kinase (Boehringer cat. No. 127272), 4 U pyruvate kinase (Boehringer cat. No. 127418) and 13 μl of snake venom NPP (same stock solution as in Fig. 2) in 5 mM sodium phosphate buffer at pH 7.5. The addition of phosphoenolpyruvate was repeated after 6.5 and 11.5 h of incubation. Inserts: left panel, scale expansion of the major plot; right panel, reaction without regenerating system in a 0.7-ml mixture.

*4.1.3. Kinetic studies and the mechanism of snake venom NPP reactions*

This section refers to initial rate studies of alcoholysis and hydrolysis under different variable conditions (nucleotidic substrate, alcohol concentration, ionic additives, pH, temperature), to the effects of these variables on the relative proportions of alcoholysis and hydrolysis, and to the information that these experiments gave on the mechanism and active center of snake NPP.

*4.1.3.1. Studies with different nucleotidic substrates.* With all the nucleotidic substrates mentioned in Section 4.1.1 (which display different nitrogen bases, phosphate-chain lengths, and phosphoanhydride or phosphodiester linkage of the NMP moiety), the $R_{AW}$ ratios of methanol were very similar, around 3.5.

*4.1.3.2. Effects of changing alcohol concentration.* As stated in Section 2, the $R_{AW}$ ratio is a useful measurement of the efficiency with which an alcohol competes with water for the NPP-bound nucleotidylate, and a plot of log $R_{AW}$ vs. log [alcohol] should be linear (see Eq. (10)) with a slope related to the apparent cooperativity of the alcohol in the reaction. The plots of Fig. 5 illustrate the kind of experiment performed to test this point. With all the alcohols studied, the alcoholytic activity of snake NPP showed apparent saturation with a decrease of activity in some cases at the higher concentrations tested, whereas the hydrolytic activity was maximal in the absence of the alcohol and decreased as alcohol concentration was increased (except that low glycerol 2-phosphate concentrations activated the hydrolytic reaction). These results point to alcohol–water competition and enzyme (in)activation by some alcohols. Since $R_{AW}$ is independent of any factor that affects to the same extent alcoholysis and hydrolysis (Section 2), enzyme (in)activation does not preclude the application of Eq. (10). In fact all the log $R_{AW}$ vs. log [alcohol] plots for snake NPP were linear, with slope near zero (i.e. $h \approx 0.9$–$1.1$, indicative that alcohol and water competed with (near) hyperbolic kinetics; Table 1), with the following exceptions: (i) glycerol plots showed zero slope at concentrations lower than 2.5 M but inflected steeply downwards at higher concentrations; (ii) glycerol 2-phosphate plots were linear but with rather negative slopes, which indicated negative cooperativity-like behavior of the glycerophosphates.

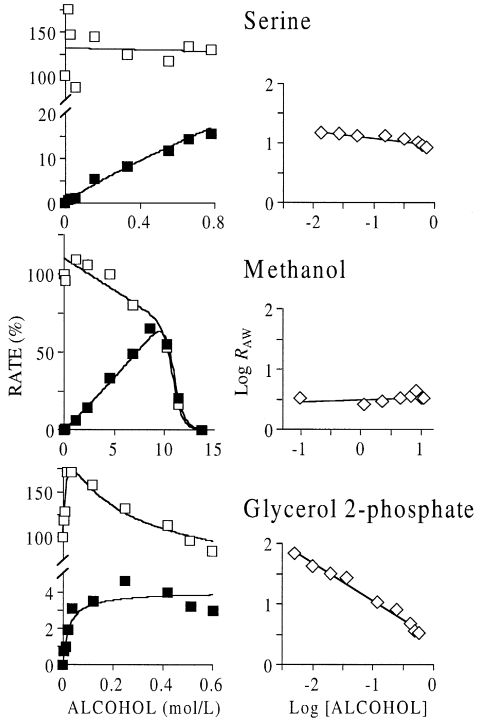


Fig. 5. Competition between alcohols and water in NPP reactions. Examples are shown corresponding to reaction mixtures with snake venom NPP, ATP as nucleotidic substrate and varying concentrations of the indicated alcohol. Left panels: The rates of alcoholysis (■) and hydrolysis (□) were measured by HPLC and expressed as percent of the hydrolytic rate observed in the absence of alcohol. Assays in the presence of serine were performed as in Fig. 2. The rate data of the methanol and glycerol 2-phosphate plots were taken from Refs. [13,14]. Experimental data were fitted directly to Eqs. (8) and (9) (serine) or to similar equations in which the terms $V_{max}$ are multiplied by a factor representing the nondenatured enzyme fraction at each alcohol concentration (methanol) or by a factor that accounts for the enzyme activation by the alcohol (glycerol 2-phosphate) (see Ref. [43] for details). Right panels: $R_{AW}$ ratios, calculated as described in Section 2, were fitted to Eq. (10). From these plots, values of the Hill-type coefficients $h$ ($h$ = slope + 1) were obtained for each alcohol (Table 1).

The $R_{AW}$ ratios of the alcohols that showed little or no concentration dependency ($h \approx 0.9$–$1.1$; Table 1) varied from 0.3 (propanol) to 15 (serine), whereas

Table 1
Apparent degree of cooperativity in the kinetics of alcohols as water competitors in NPP reactions

| Alcohol | Snake venom NPP | | Rat liver NPP | |
|---|---|---|---|---|
| | $h$ | Alcohol concentration range (mol/l) | $h$ | Alcohol concentration range (mol/l) |
| Methanol | 1.0[a] | 0.1–11.5 | 0.7[b] | 0.3–10 |
| Ethanol | 1.0[a] | 0.1–6.1 | 0.6[b] | 0.3–10 |
| Propanol | 0.9[a] | 0.1–2.5 | 0.8[b] | 0.2–2.0 |
| Ethylene glycol | 0.9[a] | 0.1–12.5 | 0.8[b] | 0.3–10 |
| 2-Chloroethanol | 1.0[a] | 0.02–2.0 | n.d. | – |
| 2,2-Dichloroethanol | 0.9[a] | 0.05–1.0 | 0.7[b] | 0.15–1.0 |
| Glycerol[c] | 0.9[a] | 0.05–2.5 | 1.0[b] | 0.005–0.67 |
| Glycerol 2-phosphate | 0.4[d] | 0.005–0.60 | 0.3[b] | 0.005–0.67 |
| Glycerol 3-phosphate[c] | 0.5[d] | 0.025–0.78 | n.d. | – |
| Serine | 0.9 | 0.014–0.78 | 1.0 | 0.005–0.67 |

The values of the empirical coefficient $h$ (Section 2, Eqs. (8) and (9)) were determined experimentally as in Fig. 5, for the indicated alcohol concentration ranges.
n.d., not determined.
[a] Calculated from earlier published rate data [13].
[b] From Ref. [43].
[c] Data only for the reaction with the primary OH groups.
[d] Calculated from earlier published rate data [14].

the $R_{AW}$ ratio of glycerophosphates ($h \ll 1$) was as high as 70 at the lowest concentrations tested.

*4.1.3.3. Effects of ionic additives*. The $R_{AW}$ ratios of methanol, glycerol, glycerol 2-phosphate and serine, each at a 110 mM concentration, were measured in the presence of two sodium salts (Table 2). The results indicated that 275 mM sodium phosphate decreased more than 50% the $R_{AW}$ of glycerol 2-phosphate but not of the other alcohols. Even stronger was the effect of sodium chloride on glycerol 2-phosphate $R_{AW}$, but this salt produced also smaller effects on the $R_{AW}$ values of methanol and serine.

Table 2
Effects of ionic additives on the $R_{AW}$ ratios of snake venom NPP with different alcohols and ATP as nucleotidic substrate

| Alcohol | Addition | | |
|---|---|---|---|
| | None | 275 mM $P_i$ | 750 mM NaCl |
| Methanol | $3.2 \pm 0.4$ | $3.2 \pm 0.6$ | $2.5 \pm 0.5^{**}$ |
| Glycerol[a] | $5.0 \pm 0.8$ | $6.0 \pm 1.5$ | $5.1 \pm 0.9$ |
| Glycerol 2-phosphate[a] | $10.9 \pm 1.3$ | $4.9 \pm 1.9^{***}$ | $3.3 \pm 1.5^{***}$ |
| Serine | $12.5 \pm 1.4$ | $12.6 \pm 4.0$ | $9.4 \pm 2.8^{*}$ |

$R_{AW}$ values (Section 2) are given as mean ± S.D. ($n = 10$) as determined at 110 mM alcohol with the indicated addition.
Statistical significance of differences versus no-addition controls: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, according to a Mann–Whitney test performed with InStat (GraphPad Software).
[a] Re-elaborated from published data [14].

*4.1.3.4. Effects of changing pH or temperature*. The $R_{AW}$ ratio of methanol was measured with $Ap_2A$ as the nucleotidic substrate in the ranges of pH 6–11 and temperature 0–37°C. The results indicate that $R_{AW}$ is independent of these parameters [15]. In addition, with ATP and 4-nitrophenyl-dTMP the $R_{AW}$ ratios for methanol assayed at 37°C and 0°C were also similar. The behavior of snake NPP in this concern is compared later with potato NPP (Section 4.2.2).

*4.1.3.5. Inferences about the mechanism and the active site of snake venom NPP*. All the experiments carried out to study the methanolysis of different nucleotidic substrates and the alcoholysis of ATP with different alcohols agree with the splitting of the NMP-enzyme intermediate being the limiting step of the catalytic route of snake NPP alcoholysis. For the alcohols with electrically neutral R groups and water, when log $R_{AW}$ (or log $E_A$; see Section 2) is plotted against $pK_a$, an interesting biphasic and direct corre-

lationship can be observed between the efficiency of the alcohol and the acidity of the primary OH group. Since this is opposed to what one would expect from OH nucleophilicities, it points to a rate-determining, general-base catalysis step in snake NPP reactions, which facilitates the attack of the nucleophile on the enzyme-bound nucleotidylate [13].

Interestingly, water fits very well among alcohols in the efficiency vs. acidity correlationship. Therefore, there is no reason to think that snake NPP distinguishes between water and alcohols with a neutral R group through binding to the active site. Things are different when glycerophosphates, particularly glycerol 2-phosphate, are considered: (i) the efficiencies ($R_{AW}$ ratios) of these phosphorylated alcohols can be very high at low concentrations, actually much higher than those of glycerol, whereas the $pK_a$ of the primary OH groups of glycerophosphates should be higher, if something, than that of glycerol [14]; (ii) glycerophosphates showed an apparent negative cooperativity behavior; (iii) the efficiency of glycerol 2-phosphate is diminished by phosphate. All these aspects point in the same direction: that glycerol 2-phosphate (and possibly the 3-phosphate too) is recognized by snake NPP through an electrostatic interaction between the phosphoryl group and a subsite of the active center.

### 4.2. Potato tuber NPP

As a practical biocatalyst, snake venom NPP is relatively expensive and it is obtained from a toxic source. Bovine intestine NPP is a commercially available alternative known to have methanolytic activity [11], but it is still more expensive than the snake NPP if one takes into account the current prices of both enzymes (sold on a per-unit basis relative to the activity on bis(4-nitrophenyl) phosphate [22]) and their ratios of activities on ATP/bis(4-nitrophenyl) phosphate (much lower for the bovine [23] than for the venom NPP [2,24]). Therefore, finding alternative sources of NPPs with alcoholytic capabilities could be commercially important.

Potato tuber is a readily available, nontoxic source material which decays very slowly, is simple to store and preserve, and contains an active NPP [25], although with a substrate specificity partly different to typical NPP/PDE enzymes (Eq. (4)). Potato NPP hydrolyzes dinucleoside diphosphates, like $NAD^+$ and $Ap_2A$, and the phosphodiester 4-nitrophenyl-dTMP, yielding 5′-nucleotides as products, but it does not hydrolyze 3′-5′ phosphodiesters like ApA or dTpdT ([25] and results not shown), which are hydrolyzed by NPPs of animal origin [23,24,26]. On the other hand, it hydrolyzes nucleoside triphosphates in a dual way, splitting either the α–β phosphoanhydride linkage, like the regular NPP/PDE enzymes, or the β–γ one [25], such that with ATP as a substrate a mixture of AMP and ADP are formed as products. The β–γ splitting has not been observed with snake NPP, but it has been seen with some mammalian NPPs that are autophosphorylated with ATP in a reaction possibly involved in the control of the regular α–β splitting activity [8–10].

We have investigated the alcoholytic capabilities of potato tuber NPP, aiming at first to obtain an easy to prepare, cheap catalyst for these reactions (Section 4.2.1). Given the peculiarities displayed by the alcoholytic activity, we studied also the alcoholytic behavior of purified potato NPP (Section 4.2.2), aiming to foresee a model of its catalytic action (Section 4.2.3).

#### *4.2.1. Semipreparative synthesis of AMP-O-methyl ester catalyzed by a simple to obtain, ammonium sulfate fraction of NPP-rich potato juice*

A crude ammonium sulfate fraction (35–50% saturation) of potato juice catalyzes the methanolysis and ethanolysis of $Ap_2A$ (but not of the much cheaper ATP, which in the presence of methanol yields only AMP and ADP as detectable nucleotidic products). In addition, to ensure that the alcoholytic activity on $Ap_2A$ represents a significant fraction of the hydrolytic activity, the incubation has to be performed at relatively high pH and low temperature ([15], and see below). Due to these peculiarities, crude potato NPP may be only of moderate interest as a practical biocatalyst. However, as it is quite active and simple to obtain, a semipreparative demonstration experiment was carried out. In a 7-ml reaction mixture, 1 mM $Ap_2A$ was incubated at pH 9 and 0°C with 1.75 ml of crude NPP in 5 M methanol. After 97 h, 95% of $Ap_2A$ was converted

to 0.17 mM AMP-O-methyl ester, 0.76 mM AMP, 1.3 mM adenosine and 0.06 mM inosine. The temperature was raised to 37°C and the incubation continued for a further 8 h to allow $Ap_2A$ exhaustion and AMP dephosphorylation (catalyzed by a phosphomonoesterase or nucleotidase activity also present in the crude fraction of potato juice) without affecting the yield of AMP-O-methyl ester. This product, after ion-exchange chromatography, was found essentially free of $Ap_2A$, AMP, adenosine and inosine [15].

One of the limitations for scaling-up the above procedure is the high cost of $Ap_2A$. In this regard, recently we have tested cheaper substrates and have found that NADH or FAD (but not $NAD^+$ or ADP) are efficiently methanolyzed by purified potato tuber NPP yielding AMP-O-methyl ester (we cannot yet discard that NMNH-O-methyl ester or FMN-O-methyl ester are also formed to some extent).

*4.2.2. Factors that affect the alcoholytic activity of purified potato tuber NPP*

For these experiments, potato tuber NPP was purified to a very high degree following a published procedure [25] along which the hydrolytic activities on 4-nitrophenyl dTMP and $Ap_2A$, and the methanolytic activity on $Ap_2A$ were shown to copurify [15].

*4.2.2.1. Effects of pH and temperature*. The $R_{AW}$ ratio of purified potato NPP, in the presence of 5 M methanol, increases with pH and with lowering temperature, as the methanolytic and the hydrolytic activities of the enzyme display different pH–activity profiles in the range of pH 6–10 (assayed at 0°C) and different temperature–activity profiles in the range 0°C–37°C (assayed at pH 9) [15]. The different responses to pH can be taken to mean that the dissociation of a group with a basic $pK_a$ is a requisite for the catalysis of methanolysis but not for hydrolysis. The different responses to temperature indicate that potato NPP-catalyzed methanolysis and hydrolysis course with different activation energies. The effects of pH and temperature are in agreement with the occurrence of two different catalytic pathways (see the legend to Fig. 7). This contrasts with snake venom and rat liver NPPs: with these enzymes, methanolysis and hydrolysis responded in parallel both to changes of pH and to changes of temperature (Sections 4.1.3 and 4.3.2).

*4.2.2.2. Studies with different nucleotidic substrates*. The methanolytic activity of purified potato NPP differed from its hydrolytic activity also in their profile of nucleotidic substrate specificity. Based on experiments carried out at pH 9.4 and 0°C, i.e. the optimal conditions for the methanolytic activity on $Ap_2A$, one can say that the following substrates were methanolyzed to yield AMP-O-methyl ester with the efficiencies ($R_{AW}$ ratios) mentioned in parenthesis: $Ap_2A$ (5), NADH (3.5 or higher), FAD (5), ADP-ribose (1.5) and ATP (1). Concerning the latter substrate, two aspects must be remarked: one is that ATP methanolysis was not observed with crude NPP (Section 4.2.1), another is that the $R_{AW}$ ratio given above was obtained when only the accumulation of AMP (not ADP, which is also formed in large amounts) was computed to calculate the hydrolytic activity. Actually, when AMP + ADP were computed together as hydrolytic products the $R_{AW}$ ratio is only 0.1. Finally, $NAD^+$, ADP and 4-nitrophenyl-dTMP were hydrolyzed by purified potato NPP but very little, if something, methanolyzed to AMP-O-methyl or dTMP-O-methyl esters. This is at least partly in contrast to snake venom and rat liver NPPs, which methanolyzed 4-nitrophenyl-dTMP to dTMP-O-methyl ester with similar efficiency as other substrates, e.g. ATP.

*4.2.2.3. Effect of oligomeric dissociation*. Bartkiewicz et al. [17] showed that potato tuber NPP is a tetramer of 75-kDa subunits that can dissociate into catalytically active dimers with relative substrate preferences different to the tetramer. Therefore, we studied the influence that oligomeric dissociation could have on the alcoholytic behavior of the enzyme. This experiment was carried out both with crude (Section 4.2.1) and purified NPP. Dissociation of native tetramers to active dimers was carried out by transient alkalinization (Section 3.1). Treated and mock-treated samples were then submitted to gel filtration chromatography, where NPP tetramers and dimers could be (partly) separated and their alcoholytic and hydrolytic activities assayed (Fig. 6). To judge from the chromatographic profiles of the hydrolytic activities on 4-nitrophenyl-dTMP and $Ap_2A$, tetramer dissoci-

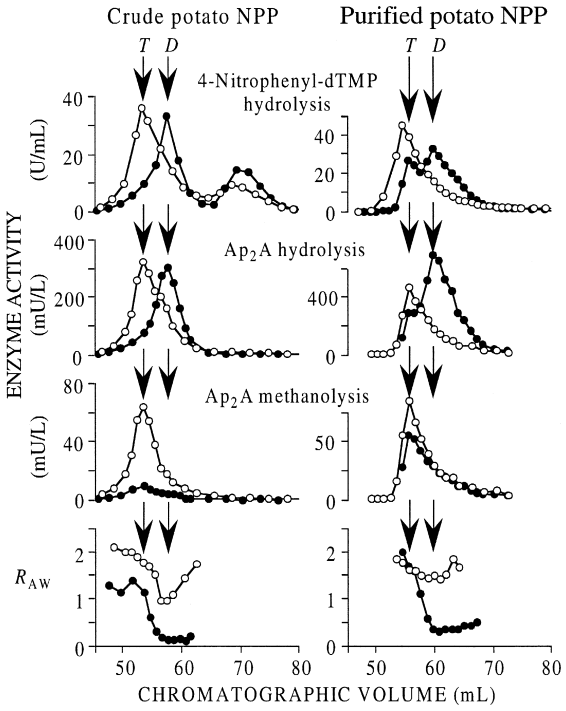


Fig. 6. Methanolytic and hydrolytic activities of potato tuber NPP tetramers and dimers: gel filtration chromatography of crude and purified NPP samples treated by transient alkalinization to elicit the dissociation of native NPP tetramers to dimers. Treated samples (●) and mock-treated controls (○) were chromatographed in Sephacryl S-300 columns of 94 cm×1 cm (crude NPP) or 101 cm×1 cm (purified NPP) (see also Section 3.1). The activities measured in the chromatographic fractions were: hydrolysis of 4-nitrophenyl-dTMP at pH 6 and 37°C, hydrolysis and methanolysis of $Ap_2A$ at pH 7.8 and 0°C in 5 M methanol (assays as in Ref. [15]). The $R_{AW}$ ratio is defined in Section 2 and was calculated from the rates of $Ap_2A$ methanolysis and hydrolysis in 5 M methanol. The arrows mark the $V_e$ corresponding to NPP tetramers (T) and dimers (D). They corresponded to about 400 and 200 kDa, respectively, as determined with protein markers (not shown) and in good agreement with published data for the two oligomeric forms of potato NPP [17].

ation to dimers was successful both with crude and purified NPP, although it was more complete in the first case. Concerning the methanolytic activity on $Ap_2A$, the results indicated that the oligomeric dissociation did not alter the relative shape of its elution profile, although the amount of methanolytic activity was diminished (more markedly in the crude than in the purified NPP sample). In agreement with this view, the $R_{AW}$ ratios calculated for methanol throughout the chromatographic fractions with significant $Ap_2A$ methanolytic and hydrolytic activities, led to the following picture (Fig. 6): (i) the mock-treated, crude NPP sample showed a relative minimum near the $V_e$ corresponding to the dimer, indicating that this crude sample contained some active dimer; (ii) the treated, crude NPP sample showed instead a sharp decrease of $R_{AW}$ immediately after the $V_e$ corresponding to the tetramer, reaching near-null $R_{AW}$ values in the fractions where the dimer appeared; (iii) the mock-treated, purified NPP sample showed a practically constant $R_{AW}$ value, indicating the practical absence of NPP dimers in the purified enzyme, (iv) the treated, purified NPP sample showed a sharp decrease of $R_{AW}$ from the fraction corresponding to maximum tetramer content to that with maximum dimer content. All these results indicated that, whereas NPP tetramers and dimers were similarly capable upon catalyzing hydrolytic reactions, only the tetramer was active catalyzing the methanolysis of $Ap_2A$.

### *4.2.3. A model for the oligomeric transitions and catalytic action of potato tuber NPP*

The results described in Section 4.2.2 can be summarized by saying that clear-cut differences exist between the methanolytic and the hydrolytic activities of potato tuber NPP in their responses to assay pH or temperature, in their relative magnitudes with different substrates, and in their relative magnitudes as catalyzed by tetrameric or dimeric enzyme. At first sight, one is tempted to think that this complex behavior may be the result of a two-enzyme mixture: either one enzyme specifically catalyzing methanolysis with a basic pH optimum, and another specifically catalyzing hydrolysis of $Ap_2A$ with a broader pH optimum (e.g. pH 6–9), or one enzyme responsible for hydrolysis with an acidic or near neutral pH optimum, and another catalyzing methanolysis and hydrolysis with a basic pH optimum. However, there is much evidence, discussed elsewhere [15], against this and for $Ap_2A$ methanolysis and hydrolysis being activities of a single enzyme, perhaps with two interconvertible forms.

In fact, before investigating the two oligomeric forms of potato NPP, we hypothesized that perhaps they would explain the responses of the methanolytic

and hydrolytic activities on $Ap_2A$ to pH and temperature. As high pH favors the dissociation of tetramers to dimers [17], if the dimer were the methanolytically active form of potato NPP, it would account for the increase of methanol efficiency as pH augmented. The decrease of efficiency with increasing temperature at pH 9 could then be explained by the known instability of the dimer particularly at high pH [17]. However, contrary to our expectations, the results indicated that the tetramer was methanolytically active and the dimer was not (Section 4.2.2, Fig. 6). Therefore, the tetramer-to-dimer transition alone cannot explain the pH and temperature effects, and it constitutes a novel factor that affects the methanolytic behavior of potato NPP. Trying to summarize all the enzymatic features considered by us, we have arrived at the model depicted and explained in Fig. 7 and legend.

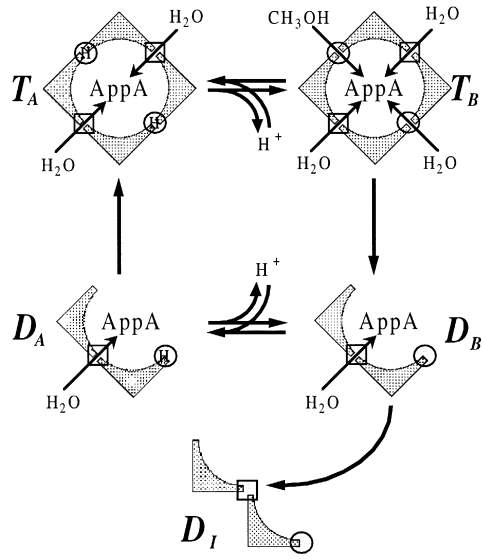


Fig. 7. Model of potato tuber NPP oligomeric transitions and catalytic actions on $Ap_2A$. The scheme encompasses the following elements and phenomena. (i) The tetrameric (T) and dimeric (D) forms of the enzyme first described by Bartkiewicz et al. [17] and studied in this work (Fig. 6). (ii) The occurrence of two different catalytic pathways for $Ap_2A$ splitting, to explain the differences between the methanolytic and hydrolytic activities on $Ap_2A$ in their responses to assay pH or temperature: a hydrolytic-only pathway (squares) in which methanol cannot substitute for water, and a methanolytic and hydrolytic pathway (circles) in which water and methanol can possibly compete. The inactivity or activity of these two pathways in each enzyme form is indicated, respectively, by filled symbols or by empty ones crossed by arrows. (iii) The participation of an acid–base dissociable group which must be dissociated for the methanolytic and hydrolytic catalytic pathway to be active (empty circles); (de)protonation of this group gives rise to two acid–base pairs ($T_A$ or $D_A$, conjugate acid; $T_B$ or $D_B$, conjugate base). (iv) The transitions $T_B \rightarrow D_B$ and $D_A \rightarrow T_A$ that represent, respectively, the dissociation of tetramers to dimers at alkaline pH and the reassociation of dimers at acidic pH, both described by Bartkiewicz et al. [17]; $T_B \rightarrow D_B$ is the transition that in this work is shown to cause the loss of the methanolytic activity of NPP (Fig. 6). (v) The inactivation process $D_B \rightarrow D_I$ that stands for the high reported instability of NPP dimers, particularly as pH and temperature are increased [17]. Finally, notice that the only enzyme form methanolytically active is $T_B$.

### *4.3. Mammalian (including human) NPPs*

The interest of studying the alcoholytic reactions of mammalian NPPs, more than in their practical application, relies in their possible functionality in these organisms. In fact, the biological role(s) of mammalian NPPs are largely unknown. On the one hand, being located in the outer surface of many cell types and also as circulating enzyme in blood serum, NPPs are involved predominantly in the turnover of extracellular dinucleoside polyphosphates which are secreted from various sources and show an array of regulatory activities [3,27,28]. On the other hand, several proteins displaying the typical hydrolase activities of NPPs have been implicated in the pathogenesis of insulin resistance [29], tumor motility [30] and bone and cartilage mineralization [31]. Interestingly, the latter aspect is linked to the generation of pyrophosphate from ATP, but the two others may not be related to the catabolism of nucleotidic substrate pools. The so-called PC-1 NPP inhibits insulin receptor phosphorylation even after a point mutation that renders it devoid of hydrolytic activity [32], which seems to speak for the (in)direct effect of a protein-to-protein contact. Also, the capability of mammalian NPPs to act as phosphotransferases (protein kinases) has been indicated [33,34] though it is under debate [4,8,9].

The alcoholytic reactions of mammalian NPPs could be biologically important at least in two ways: as a source of nucleotide esters following alcohol ingestion and/or as a reflection of the general ability of NPPs to act as nucleotidyl transferases.

*4.3.1. NPP-related alcoholytic activities producing AMP-O-Rs in mammalian tissues and potential relevance of the products*

Crude membrane preparations of mammalian origin were described 20 years ago to catalyze the formation of AMP-O-Rs from ATP and methanol, ethanol, propanol, ethylene glycol or glycerol [35–38]. After the finding that these reactions are catalyzed by snake venom NPP, we investigated their catalysis by mammalian NPPs and found that bovine intestine [11], rat liver and human serum NPP (see below) have associated alcoholytic activities. Also, we have assayed and detected alcoholytic activities in crude blood fractions, although ATP could not be used as a substrate due to the heavy interference by phosphomonoesterase activity. Instead, $Ap_2A$, a phosphomoesterase-resistant dinucleotide, was successfully used to detect the presence of alcoholytic activities in serum, lymphocytes, polymorphonuclear leukocytes and blood platelets, with methanol in all cases, with ethanol only in serum and lymphocytes (Table 3 and Fig. 8).

To test whether the alcoholytic activities of human serum could be actually due to a typical NPP/PDE, the major serum activity responsible for 4-nitrophenyl-dTMP hydrolysis was partly purified as described in Section 3.1 and, afterwards, chromatographed in a Sephacryl S-300 column (86 cm × 1 cm) equilibrated in 10 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$. NPP activities were assayed both with 4-nitrophenyl-dTMP and with ATP, in the latter case in the presence of either 3 M methanol or 1 M ethanol. In all cases, the activities detected (4-

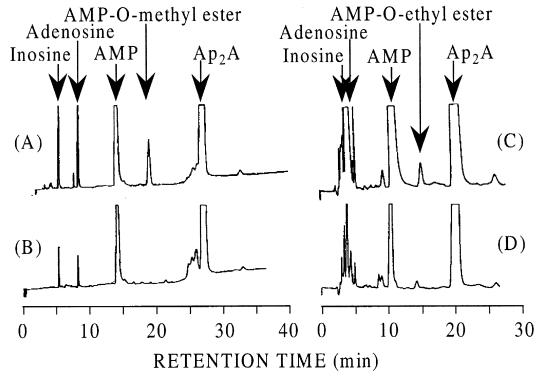


Fig. 8. HPLC detection of AMP-O-methyl and AMP-O-ethyl ester formation catalyzed by a lysate of human lymphocytes. The chromatograms correspond to the experiment described in Table 3 and to reaction mixtures with (A) 3 M methanol, (C) 3 M ethanol, or (B, D) controls incubated without alcohol. A column of Hypersyl ODS of 150 mm × 3.9 mm was used. For A and B, the column was equilibrated in 5 mM sodium phoshate, pH 7, 28 mM tetrabutylammonium bromide (TBA), and the elution was accomplished with a 5-min isocratic wash in this buffer followed by a linear 5–300 mM phosphate gradient of 40 min at the same pH and TBA concentration. For C and D, the column was equilibrated in 5 mM sodium phoshate, pH 7, 23 mM TBA, 15% (by volume) methanol, and the elution was isocratic with this buffer.

Table 3
Detection of alcoholytic activities in human blood fractions

| Blood fraction | Methanolysis | | Ethanolysis | |
|---|---|---|---|---|
| | Rate (mU/g protein) | $R_{AW}$ | Rate (mU/g protein) | $R_{AW}$ |
| Serum | 3.0 ± 0.1 | 5.0 ± 0.7 | 0.25 ± 0.04 | 0.6 ± 0.1 |
| Lymphocyte lysate | 69 ± 11 | 3.9 ± 0.3 | 6.1 ± 0.6 | 0.4 ± 0.1 |
| Polymorphonuclear leukocyte lysate | 9.4 ± 1.5 | 1.6 ± 0.2 | n.d. | n.d. |
| Platelet lysate | 6.2 ± 1.0 | 1.8 ± 0.1 | n.d. | n.d. |

The activities were assayed with 0.5 mM $Ap_2A$ as the nucleotidic substrate in 50 mM Tris–HCl, pH 7.5, 3 M methanol or ethanol, 5 mM $MgCl_2$, and 10 mg/ml Triton X-100. Four incubations were run in each case with two different volumes of blood fraction and for two different time lengths. Long incubations at 37°C, up to 48–72 h, were needed in some cases and strict linearity could not be observed. However, the products of $Ap_2A$ hydrolysis (AMP and its degradation products adenosine and inosine) and alcoholysis (AMP-O-methyl or –ethyl ester) accumulated in a time- and fraction-volume-dependent fashion. Only the rates of alcoholysis are given. The $R_{AW}$ ratios (see Section 2) were calculated as described in Section 3.2 from the rates of alcoholysis and hydrolysis. Means ± S.D. are given for the four incubation mixtures. Controls carried out without alcohol showed no accumulation of AMP-O-R. One unit (U) is 1 μmol of substrate split per min.

n.d., not determined.

Table 4
Comparison of AMP, AMP-O-methyl ester and AMP-O-ethyl ester as rat liver NPP inhibitors and controls to check for possible AMP-O-R conversion to AMP by the same enzyme

| Test | AMP | AMP-O-methyl ester | AMP-O-ethyl ester |
|---|---|---|---|
| (A) $K_i$ value (competitive inhibition) | 3 μM | > 300 μM | 30 μM |
| (B) Rate of conversion to AMP by rat liver NPP (relative to ATP) | – | 13% | 7% |
| (C) Percent inhibition of the hydrolysis of 0.12 mM 4-nitrophenyl-dTMP at 25 μM inhibitor: | | | |
| without alkaline phosphatase | 60% | n.d. | 25% |
| with alkaline phosphatase | 0% | n.d. | 20% |

(A) The activity of rat liver NPP was assayed in Tris–HCl, pH 7.4, with 0.006–1.1 mM 4-nitrophenyl-dTMP as the substrate, by continuously recording the increment of $A_{405}$ concomitant to the liberation of nitrophenol ($\varepsilon = 11{,}100\ M^{-1}\ cm^{-1}$ at pH 7.4). To discard that NPP inhibition by AMP-O-methyl ester could be due to conversion to AMP, control tests were carried out (B) measuring the rates of conversion of ATP, AMP-O-methyl and AMP-O-ethyl ester to AMP (assayed by HPLC) and (C) comparing the effects of alkaline phosphatase (5 U/ml; Boehringer, cat. No. 108146) on the inhibition by AMP and AMP-O-methyl ester.
n.d., not determined.

nitrophenyl-dTMP or ATP hydrolysis, and ATP methanolysis or ethanolysis) coeluted in a single, broad and assymmetric peak ($V_e \approx 39$–$60$ ml, with the maximum at $V_e \approx 44$ ml; results not shown). The calculated $R_{AW}$ ratios for methanol and ethanol throughout the peak profile were 3–4.5 and 0.5–1.1, respectively.

We did not try to correlate the alcoholytic activities found in cell lysates with NPPs, but in our view it is very likely that they correspond to these enzymes which are known to be present in white cells [4,39–41]. Actually, the $R_{AW}$ ratios given in Table 3 for methanol and ethanol are within the value ranges seen with human serum, rat liver and snake venom NPPs.

Although $Ap_2A$ does not seem to be a physiological substrate, $Ap_nA$ ($n = 3$–$6$), are present in secretion granules [28,42].[1] Assuming substrates in micromolar amounts, it seems a reasonable hypothesis that in the presence of, for instance, near 0.1 M ethanol (which is typical of acute alcoholism) AMP-O-ethyl ester can be formed in nanomolar amounts. An important aspect of this hypothesis is that AMP-O-ethyl ester can exhibit a long half-life, as it is resistant to phosphomonoesterases and it is not a good NPP substrate ([13] and see also Table 4).

As stated, AMP-O-Rs are potential (ant)agonists of purinergic receptors or effectors of enzymes involved in the turnover of the natural agonists. However, to our knowledge, these possible effects have not yet been studied. To evaluate what difference a simple alkyl group could make on the recognition of a nucleotide by a protein site, and given the known inhibition of NPPs by AMP, we carried out estimations of the inhibitory potency of AMP-O-methyl and AMP-O-ethyl ester on rat liver NPP (Table 4, test A). AMP and AMP-O-ethyl ester behaved as inhibitors, whereas the effect of AMP-O-methyl ester was very weak if something. As a necessary control, since AMP-O-ethyl ester can be converted to AMP by rat liver NPP itself (though not faster than the much less inhibitory AMP-O-methyl ester; Table 4, test B), it was confirmed that the inhibition by AMP was abolished in the presence of alkaline phosphatase while the inhibition by AMP-O-ethyl ester was not (Table 4, test C). The different inhibitory potencies of the three adenosine derivatives make clear that a single methylene group can make a significant difference in binding and biological effect. Therefore, the possible effects of AMP-O-Rs on specific protein sites recognizing nucleotides remain an interesting field to be explored.

[1] Note added in proof: recently $Ap_2A$ was found in human myocardium granules by Luo et al., FASEB J. 13 (1999) 695-705.

*4.3.2. Rat liver NPP as an efficient adenylyl transferase recognizing alcohols through multiple active-site interactions*

The first evidence that NPPs could act as transferases recognizing non-water acceptors as substrates through specific binding interactions was the behavior of glycerophosphates in snake venom NPP reactions (see Section 4.1.3). This made it interesting to explore the behavior of mammalian NPPs in this concern, since as explained above, the biological role of these enzymes is still unclear but current knowledge offer very interesting perspectives (see the beginning of Section 4.3). For this study we chose to investigate rat liver NPP and carried out kinetic experiments like those earlier performed with the snake enzyme (see Section 4.1.3, Fig. 5, Tables 1 and 2), including the same set of alcohols except 2-chloroethanol and glycerol 3-phosphate. The detailed results of this research have been published elsewhere [43] and only a brief general summary is presented below.

All of the alcohols investigated as water competitors acted as adenylate acceptors in the concentration ranges indicated in Table 1. In contrast to snake venom NPP, the efficiencies ($R_{AW}$ ratios) decreased as alcohol concentration increased, except for glycerol and serine, which showed near constant $R_{AW}$ values. These results suggested a negative cooperativity-like behavior of most of the alcohols, quantitated by values of $h \approx 3$–$0.8$ (Table 1). The effects of ionic additives were tested like with snake venom enzyme (Table 2) only with glycerol 2-phosphate, glycerol and serine with similar results. The $R_{AW}$ ratio for reactions with methanol was determined at different pH values and at different temperatures, and it was found independent on these factors, like snake NPP but contrary to the potato enzyme. A very interesting difference between the snake and rat NPPs concerned the magnitude of their respective $R_{AW}$ values for the alcohols with uncharged R group: at a determined alcohol concentration, for instance 0.3 M, all of them were larger for the rat than for the snake NPP by a factor of about 2. Henceforth, the correlation between alcohol efficiency and acidity ($\log R_{AW}$ vs. $pK_a$ plot, see Section 4.1.3) showed the same characteristics with both enzymes, but the plots were parallel rather than coincident. Interestingly, whereas water fits very well among alcohols in the snake NPP plot, it does not in the rat one. This indicates that rat liver NPP distinguishes between water and neutral alcohols by an additional favorable interaction with alcohols which must involve the methylene group present in all of them.

## 5. Conclusions

Snake venom NPP is a versatile tool for the synthesis of NMP-O-Rs: a large variety of esters with different nucleoside and alkyl moieties can be easily synthesized in small amounts using different combinations of e.g., a nucleoside 5′-triphosphate and alcohol. ATP conversion to AMP-O-R can be increased with an ATP-regenerating system.

In the active center of snake NPP, primary alcohols with neutral R groups compete freely with water for an enzyme-bound nucleotidylate. The result of this competition is determined mainly by the rate of a proton transfer from the OH nucleophile to a basic catalytic group. However, the phosphate groups of glycerophosphates interact favorably with a (cationic) subsite which, in the competition with water, confers to these alcohols an advantage independent on the intrinsic acidity of the OH nucleophile.

A simple to obtain preparation of potato tuber NPP can be also used for the synthesis of some AMP-O-Rs, but with more stringent reaction requirements than snake NPP. Potato tuber NPP behaves as if it had two different catalytic pathways for the splitting of nucleotidic substrates with different relative preferences for water and methanol, and with different responses to pH, temperature and oligomeric dissociation.

The presence of alcoholytic activities yielding AMP-O-Rs has been demonstrated in mammalian tissues easily exposed to ingested alcohols: intestine, blood and liver. Hence, the potential exists in mammals to form AMP-O-Rs as a consequence of chronic or acute alcohol intoxication (e.g., with methanol, ethanol or ethylene glycol). These AMP-O-Rs could have specific effects on nucleotide-recognizing protein sites.

The characteristics of the active center of snake NPP are shared by rat liver NPP with the additional feature of another subsite interacting favorably with

the methylene group common to all primary alcohols. This ability of rat NPP to discriminate any alcohol from water indicates that it is well fit to act as transferase. We put forward the hypothesis that it can be an adenylylating agent in the membrane where it is naturally located, perhaps with other proteins as acceptors. In this concern, particularly interesting is the new observation that the lateral chain of the free amino acid serine is an acceptor of adenylate transfer in snake venom and rat liver NPP reactions with very high efficiencies: $R_{AW} = 16$ and 27, respectively, at 10 mM serine.

We envisage the following future lines of action: (i) search for new NPPs with alcoholytic activities which can help to overcome the shortcomings of the snake venom and the potato tuber ones as practical biocatalysts, (ii) search for strategies to minimize the hydrolytic activity of these enzymes in the presence of alcohols, (iii) search for effects of NMP-O-Rs on receptors and enzymes, (iv) test rat liver and other (mammalian) NPPs as adenylate transferases to peptide models and to proteins.

## Acknowledgements

The research reported here has been supported by grants from C.I.C.Y.T. (SAF94-0194; SAF97-0209), D.G.E.S. (PM97-0022), Consejería de Educación y Juventud, Junta de Extremadura y Fondo Social Europeo (EIA94-09; IPR98C009) and Consejería de Bienestar Social (Proyectos de Investigación Sociosanitaria), Junta de Extremadura, Spain.

## References

[1] H.G. Khorana, in: P.D. Boyer, H. Lardy, K. Myrbäck (Eds.), 2nd edn., The Enzymes vol. 5, Academic Press, New York, 1961, pp. 79–94, Chap. 6.
[2] W.E. Razzell, Methods Enzymol. 6 (1963) 236–258.
[3] A. Guranowski, A. Sillero, in: A.G. McLennan (Ed.), $Ap_4A$ and Other Dinucleoside Polyphosphates, CRC Press, Boca Raton, 1992, pp. 81–133, Chap. 5.
[4] J.W. Goding, R. Terkeltaub, M. Maurice, P. Deterre, A. Sali, S.I. Belli, Immunol. Rev. 161 (1998) 11–26.
[5] M. Landt, R.A. Everard, L.G. Butler, Biochemistry 19 (1980) 138–143.
[6] J.S. Culp, H.J. Blytt, M. Hermodson, L.G. Butler, J. Biol. Chem. 260 (1985) 8320–8324.
[7] J.S. Culp, L.G. Butler, Arch. Biochem. Biophys. 246 (1986) 245–249.
[8] S.I. Belli, F.A. Mercuri, A. Sali, J.W. Goding, Eur. J. Biochem. 228 (1995) 669–676.
[9] C. Stefan, W. Stalmans, M. Bollen, Eur. J. Biochem. 241 (1996) 338–342.
[10] T. Clair, H.Y. Lee, L.A. Liotta, M.L. Stracke, J. Biol. Chem. 272 (1997) 996–1001.
[11] M. García-Díaz, M. Avalos, J.C. Cameselle, Eur. J. Biochem. 196 (1991) 451–457.
[12] A. Cornish-Bowden, Fundamentals of Enzyme Kinetics, Portland Press, London, 1995.
[13] M. García-Díaz, M. Avalos, J.C. Cameselle, Eur. J. Biochem. 213 (1993) 1139–1148.
[14] J.M. Vergeles, M. García-Díaz, J.C. Cameselle, Eur. J. Biochem. 233 (1995) 442–447.
[15] A. Agudo, J.M. Ribeiro, J. Canales, J.C. Cameselle, Biotechnol. Bioeng. 59 (1998) 62–67.
[16] J. López-Gómez, M.J. Costas, J.M. Ribeiro, A. Fernández, A. Romero, M. Ávalos, J.C. Cameselle, FEBS Lett. 421 (1998) 77–79.
[17] M. Bartkiewicz, E. Grzybowska, H. Sierakowska, D. Shugar, Biochim. Biophys. Acta 830 (1985) 313–319.
[18] M.P. Abbrachio, G. Burnstock, Pharmacol. Ther. 64 (1994) 445–475.
[19] B.B. Fredholm, M.P. Abbracchio, G. Burnstock, J.W. Daly, T.K. Harden, K.A. Jacobson, P. Leff, M. Williams, Pharmacol. Rev. 46 (1994) 143–156.
[20] H. Zimmermann, Trends Pharmacol. Sci. 20 (1999) 231–236.
[21] A.U. Ziganshin, C.H.V. Hoyle, G. Burnstock, Drug Dev. Res. 32 (1994) 134–146.
[22] Sigma Catalog, 1999, p. 836.
[23] S.J. Kelly, D.E. Dardinger, L.G. Butler, Biochemistry 14 (1975) 4983–4988.
[24] W.E. Razzell, H.G. Khorana, J. Biol. Chem. 234 (1959) 2105–2113.
[25] M. Bartkiewicz, H. Sierakowska, D. Shugar, Eur. J. Biochem. 143 (1984) 419–426.
[26] J.C. Cameselle, M.J. Costas, M.A. Günther-Sillero, A. Sillero, J. Biol. Chem. 259 (1984) 2879–2885.
[27] L.L. Kisselev, J. Justesen, A.D. Wolfson, L.Yu. Frolova, FEBS Lett. 427 (1998) 157–163.
[28] M.T. Miras-Portugal, J. Gualix, J. Pintor, FEBS Lett. 430 (1998) 78–82.
[29] B.A. Maddux, P. Sbraccia, S. Kumakura, S. Sasson, J. Youngren, A. Fisher, S. Spencer, A. Grupe, W. Henzel, T.A. Stewart, G.M. Reaven, I.D. Goldfine, Nature 373 (1995) 448–451.
[30] H.Y. Lee, T. Clair, P.T. Mulvaney, E.C. Woodhouse, S. Aznavoorian, L.A. Liotta, M.L. Stracke, J. Biol. Chem. 271 (1996) 24408–24412.
[31] M. Lotz, F. Rosen, G. McCabe, J. Quach, F. Blanco, F. Dudler, J. Solan, J. Goding, J.E. Seegmiller, R. Terkeltaub, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 10364–10368.
[32] A. Grupe, J. Alleman, I.D. Goldfine, M. Sadick, T.A. Stewart, J. Biol. Chem. 270 (1995) 22085–22088.
[33] Y. Oda, M.-D. Kuo, S.S. Huang, J.S. Huang, J. Biol. Chem. 266 (1991) 16791–16795.

[34] M. Uriarte, W. Stalmans, S. Hickman, M. Bollen, Biochem. J. 306 (1995) 271–277.
[35] B. Petrack, D. Ma, F. Sheppy, J. Biol. Chem. 249 (1974) 3661–3663.
[36] B.I. Gold, L. Volicer, Biochem. Pharmacol. 25 (1976) 1825–1830.
[37] J. Ryan, G.N. Rogers, D.G. Toscano, D.R. Storm, J. Biol. Chem. 252 (1977) 1719–1722.
[38] Z. Kiss, J. Hanoune, Biochim. Biophys. Acta 538 (1978) 458–472.
[39] E.R. Abney, W.H. Evans, R.M.E. Parkhouse, Biochem. J. 159 (1976) 293–299.
[40] A. Brockhaus, R. Bösing-Schneider, H. Sund, Immunobiology 162 (1982) 186–191.
[41] P. Deterre, L. Gelman, H. Gary-Gouy, C. Arrieumerlou, V. Berthelier, J.-M. Tixier, S. Ktorza, J. Goding, C. Schmitt, G. Bismuth, J. Immunol. 157 (1996) 1381–1388.
[42] M.A. Günther-Sillero, M. Del Valle, E. Zaera, P. Michelena, A.G. García, A. Sillero, Biochimie 76 (1994) 404–409.
[43] J.M. Ribeiro, J. López-Gómez, J.M. Vergeles, M.J. Costas, M. García-íaz, A. Fernández, A. Flores, J.C. Cameselle, Biochem. J. 346 (2000) 25–31.