Rat liver nucleotide pyrophosphatase/phosphodiesterase is an efficient adenylyl transferase

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Rat liver nucleotide pyrophosphatase/phosphodiesterase I (NPP/PDE) catalysed efficiently the transfer of adenylate from ATP to alcohols (methanol, ethanol, propanol, ethylene glycol, glycerol, 2,2-dichloroethanol and glycerol 2-phosphate), which acted as adenylate acceptors competing with water with different efficiencies. NPP/PDE kinetics in alcohol/water mixtures were accounted for by rate equations for competitive substrates, modified to include alcohol negative co-operativity and, depending on the nature of the alcohol, enzyme denaturation by high alcohol concentrations or activation by low alcohol concentrations. The correlation of alcohol efficiencies with alcohol

acidities, the comparison of rat liver with snake venom NPP/PDE, and the different effects of ionic additives on the efficiencies of glycerol 2-phosphate and glycerol provided evidence for interaction of the alcohols with a base catalyst, a non-polar and a cationic subsite in the active centre of rat liver NPP/PDE. The enzyme thus appears to be well suited to act as transferase, and we propose that NPP/PDE could be an adenylylating agent in the membrane.

Key words: adenylyl transfer, alcohol, nucleotide ester.

INTRODUCTION

Nucleotide pyrophosphatase/phosphodiesterases (NPP/PDE; EC 3.6.1.9/EC 3.1.4.1) are hydrolases with a broad substrate specificity towards phosphoanhydride or phosphodiester derivatives of 5'-nucleotides, yielding one of these compounds as a product. Much interest is currently devoted to NPP/PDE from mammalian tissues, mainly for two reasons. On the one hand, they are directly related to extracellular dinucleoside polyphosphates, a family of compounds stored in, and releasable from, blood platelets, chromaffin granules and synaptic vesicles, and with many (receptor-mediated) activities in mammals (reviewed in [1–3]). Mammalian NPP/PDEs hydrolyse dinucleoside polyphosphates non-specifically, but with low K_m values [4]. They seem to be responsible for the extracellular turnover of these substrates, since no ecto- or extracellular-enzyme hydrolysing phosphoanhydride linkages of dinucleoside polyphosphates has been demonstrated to be free from the phosphodiesterase activity typical of NPP/PDE enzymes [5]. On the other hand, proteins with NPP/PDE activities are involved in processes important in pathogenesis, such as resistance to insulin [6,7], tumour-cell motility [8,9] and production of pyrophosphate related to bone and cartilage mineralization [10–12]. Not all the possible biological functions of NPP/PDE are necessarily related to their catabolic action on nucleotide pools. For instance, it has been reported that the inhibition of insulin receptor phosphorylation by PC-1, a NPP/PDE ectoenzyme, is not abolished by a single amino acid change that eliminates its NPP/PDE hydrolytic activity [13]. Also, the capability of mammalian NPP/PDE enzymes to act as phosphotransferases (protein kinases) has been indicated previously [14,15] and debated further [16–18].

In this work, we have studied the capability of rat liver NPP/PDE to catalyse the transfer, not of phosphate, but of the AMP moiety of ATP to non-water acceptors. This study was on

the basis of the knowledge that snake venom and mammalian NPP/PDE enzymes catalyse a double nucleophilic displacement on the α -phosphorus atom of the adenylyl moiety of ATP: a threonine lateral chain carries out first an attack that leads to an adenylyl-enzyme intermediate; thereafter, a molecule of water splits the phosphodiester linkage of this intermediate, releasing AMP as a product of hydrolysis [16,19–23]. In this mechanism, one may ask whether, in the active centre of mammalian NPP/PDE, alcohols could substitute for water, as is known to occur in the active centre of snake venom NPP/PDE [24–26]. From the present study, rat NPP/PDE has been identified as a more efficient adenylyl transferase than the snake enzyme, with an active centre capable of establishing multiple interactions with specific alcohol acceptors of the enzyme-bound adenylate.

MATERIALS AND METHODS

Materials

Rat liver NPP/PDE was obtained as described previously [27]. It was obtained in a partially purified form, which generated several protein bands when analysed by SDS/PAGE or by native gel electrophoresis stained with Coomassie Blue, but only one band was produced when native gels were stained for NPP/PDE activity with either 2-naphthyl phenylphosphonate [28] or 1-naphthyl-5'-dTTP [29] (Sigma, Madrid, Spain) as the substrate. Other materials were from sources reported previously [25,26].

Enzyme assays

Enzymic incubations to assay the hydrolysis and alcoholysis of ATP by rat liver NPP/PDE were performed at 37 °C in either 50 mM Tris/HCl, pH 7.5/0.1 mg/ml BSA/1 mM ATP (method 1), or 5 mM sodium phosphate, pH 8/2 mM ATP (method 2)

Abbreviations used: NPP/PDE, nucleotide pyrophosphatase/phosphodiesterase I; TBA, tetrabutylammonium bromide.

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and the indicated concentration of one R-CH₂OH alcohol. Incubations were terminated by heating for 2.5–5 min at 90–100 °C, and the products of ATP hydrolysis (AMP) and alcoholysis (AMP-*O*-alkyl ester) were quantified by HPLC. Reaction rates were measured under conditions of linearity versus incubation length and amount of enzyme.

HPLC assays

Reverse-phase HPLC was performed using the equipment described previously [26] on a Hypersyl ODS column [either 150 mm × 3.9 mm internal diameter (i.d.) or 200 mm × 2.1 mm i.d.; Hewlett–Packard, Boise, ID, U.S.A.]. Chromatograms were monitored at A_{250} . Different elution conditions were used, on the basis of whichever AMP-*O*-alkyl ester was expected to be eluted [25,26]. Standard methods were routinely followed, although slight adjustments had to be made occasionally, owing to column aging. When analysing reaction mixtures from experiments with methanol, ethanol, glycerol or glycerol 2-phosphate, the column was equilibrated with 5 mM sodium phosphate, pH 7/20 mM tetrabutylammonium bromide (TBA), and the elution was achieved with a 5-min isocratic wash in the same mobile phase, followed by a 30-min gradient of sodium phosphate (5–300 mM) in 20 mM TBA. For reaction mixtures containing ethylene glycol or dichloroethanol, the column was equilibrated with 5 mM sodium phosphate, pH 7/20 mM TBA/20% (v/v) methanol, and the elution was achieved with a 5-min wash in this mobile phase, followed by a 30-min gradient of sodium phosphate (5–100 mM for reaction mixtures with ethylene glycol; 5–200 mM for reaction mixtures with dichloroethanol) in 20 mM TBA and 20% (v/v) methanol. For reaction mixtures with propanol, the column was equilibrated with 5 mM sodium phosphate, pH 7/20 mM TBA/30% (v/v) methanol, and the elution was performed with a 30-min gradient of sodium phosphate (5–60 mM) in 20 mM TBA and 30% (v/v) methanol.

Equations

Kinetic experiments with rat liver NPP/PDE were performed in mixtures of alcohol and water (designated by A and W in the subsequent equations respectively), where both nucleophilic agents competed as acceptors of the adenylate moiety of ATP. This subsection describes the equations used to account for the kinetic behaviour of the enzyme under such conditions.

When a single enzyme acts simultaneously on two competing substrates, each will act as a competitive inhibitor with respect to the other, with a K_i value equal to its K_m . If both substrates follow Michaelis–Menten kinetics, the predicted rate equations are ([30], pp.105–108):

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

$$v_W = \frac{V_{\max W}[W]}{K_{mW}\left(1 + \frac{[A]}{K_{mA}}\right) + [W]} \quad (2)$$

in which $V_{\max A}$ and $V_{\max W}$ are the limiting rates for alcohol and water respectively, and K_{mA} and K_{mW} are the Michaelis constants of the two reactions in isolation. When these equations are applicable, the ratio $(v_A/v_W)/([A]/[W])$, here named R_{AW} : (i) is constant and independent of both substrate concentration and of any factor, such as enzyme (in)activation, affecting equally the activity of the enzyme on both substrates; and (ii) is equal to

$(k_{\text{cat}}/K_m)_A/(k_{\text{cat}}/K_m)_W$, i.e. the ratio of the specificity constant of the alcohol to that of water. R_{AW} provides a measure of the substrate specificity and, in the present study, is used also as a measure of the efficiency of the alcohol as an adenylate acceptor relative to that of water, since it equals 1 for an alcohol with the same reactivity as water.

In the case that binding of substrate A occurs co-operatively, as the results of the present study will demonstrate for alcohols in rat liver NPP/PDE reactions, this can be represented empirically by a coefficient h , substituting $K_{0.5A}^h$ and $[A]^h$ for K_{mA} and $[A]$ respectively, as in the Hill equation ([30], p. 207). In this case, the predicted rate equations are:

$$v_A = \frac{V_{\max A}[A]^h}{K_{0.5A}^h\left(1 + \frac{[W]}{K_{mW}}\right) + [A]^h} \quad (3)$$

$$v_W = \frac{V_{\max W}[W]}{K_{mW}\left(1 + \frac{[A]^h}{K_{0.5A}^h}\right) + [W]} \quad (4)$$

which are equivalent to eqns. (1) and (2) when $h = 1$. When the binding of substrate A is co-operative, R_{AW} is not independent of $[A]$. From eqns. (3) and (4), it is possible to derive the expression:

$$\log \frac{v_A}{[A]} = \log \left(\frac{k_{\text{cat}}}{K_{0.5A}^h} \right)_A + (h-1)\log[A] \quad (5)$$

which indicates that in this case the plot of $\log R_{AW}$ against $\log [A]$ is linear with a slope equal to $h-1$. However, R_{AW} is still independent of any factor that affects to the same extent both rates, v_A and v_W .

Enzyme denaturation, which occurs in certain cases when high concentrations of alcohols are used, is assumed to have a sigmoidal dependency with respect to alcohol concentration [31]. In this situation, $V_{\max}$ must be multiplied by a factor representing the non-denatured enzyme fraction at each alcohol concentration, and eqns. (3) and (4) become modified to give:

$$v_A = \frac{V_{\max A}\left(1 - \frac{[A]^N}{D_{0.5}^N + [A]^N}\right)[A]^h}{K_{0.5A}^h\left(1 + \frac{[W]}{K_{mW}}\right) + [A]^h} \quad (6)$$

$$v_W = \frac{V_{\max W}\left(1 - \frac{[A]^N}{D_{0.5}^N + [A]^N}\right)[W]}{K_{mW}\left(1 + \frac{[A]^h}{K_{0.5A}^h}\right) + [W]} \quad (7)$$

in which $D_{0.5}$ is the concentration of alcohol at which half-denaturation occurs and N is an empirically determined factor that represents the steepness of the denaturation plot (activity against alcohol concentration).

Enzyme activation, which occurs in the presence of glycerol 2-phosphate, was assumed to be due to an effect of the alcohol on the maximal rate of the enzyme, as a result of its binding at an allosteric site. In this scenario, the predicted rate equations are:

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

$$v_w = \frac{V_{\max w} \frac{K_x + \beta[A]}{K_x + [A]} [W]}{K_{mw} \left(1 + \frac{[A]^h}{K_{0.5A}^h} \right) + [W]} \quad (9)$$

in which K_x is the dissociation constant of the enzyme–activator complex and β is the $V_{\max}$ -fold increase obtained at saturating concentrations of activator [32]. It is interesting to note that eqn. (5) holds true not only when rate eqns. (3) and (4) are used, but also when the pairs of eqns. (6 and 7) or (8 and 9) are followed.

Fitting the kinetics of alcoholysis and hydrolysis of ATP catalysed by rat liver NPP/PDE in alcohol/water mixtures

From the linear plot of $\log R_{AW}$ against $\log[A]$, experimental values can be obtained for h ($h = \text{slope} + 1$) and for $(V_{\max A}/K_{0.5A})/(V_{\max W}/K_{mw})$, which equals R_{AW} when $\log[A] = 0$. Using these values of h and $(V_{\max A}/K_{0.5A})/(V_{\max W}/K_{mw})$ as constraints, the experimental v_A and v_W data were fitted by non-linear regression to one of the following pairs of equations: (3) and (4), when no enzyme denaturation or activation was observed; (6) and (7), when the enzyme was denatured by high alcohol concentrations; or (8) and (9), when the enzyme was activated by the alcohol. It is important to note that the actual values of the parameters $V_{\max A}$, $K_{0.5A}$, $V_{\max W}$ and K_{mA} (and $D_{0.5}$, N , K_x and β , if applicable) cannot be determined unambiguously, because several combinations of these parameters give equally good fits of the experimental v_A and v_W values to the rate equations. As K_{mw} must be a constant independent of the particular alcohol assayed, it was arbitrarily fixed as 500 M, and the other parameters were searched using the Macro Solver of the Excel spreadsheet (Microsoft); identical fittings were obtained with K_{mw} values between 0.05 M and 500 M (obviously with quite different values for the other parameters).

RESULTS

Rat liver NPP/PDE-catalysed formation of AMP-*O*-alkyl esters from ATP and alcohols

Rat liver NPP/PDE was incubated with ATP in the presence of different R-CH₂OH alcohols with electrically neutral R groups (methanol, ethanol, propanol, ethylene glycol, 2,2-dichloroethanol and glycerol), as well as an R-CH₂OH alcohol with a negatively charged R group (glycerol 2-phosphate). In each case, the substrate, ATP, and the products typical of its hydrolysis and alcoholysis, AMP and AMP-*O*-alkyl ester, were resolved by HPLC (Figure 1). The AMP-*O*-alkyl ester was identified as a single peak, formed only when both alcohol and enzyme were present. In reaction mixtures with glycerol, two peaks were identified as products of alcoholysis: a major one, corresponding to the AMP-*O*-alkyl ester(s) formed with the primary hydroxy groups, and a minor one, corresponding to the AMP-*O*-alkyl ester formed with the secondary hydroxy group. In addition to their alcohol-dependent formation, AMP-*O*-alkyl esters were identified by the following criteria (results not shown). Their retention times during HPLC coincided with those of the products of snake venom NPP/PDE-catalysed alcoholysis [24–26]. In contrast with AMP, they were resistant to alkaline phosphatase, except for the AMP-*O*-alkyl ester formed in the presence of glycerol 2-phosphate [AMP-*O*-sn-1(3)-glycero-2-phosphate], which was dephosphorylated as expected to yield the same product formed directly from ATP with the primary hydroxy group of glycerol [AMP-*O*-sn-1(3)-glycerol] [26]. Also, all of the AMP-*O*-alkyl esters formed by rat liver NPP/PDE were con-

Figure 1 HPLC chromatograms showing AMP-*O*-alkyl esters formed by alcoholysis of ATP catalysed by rat liver NPP/PDE

The chromatograms illustrate the separation of the substrate (ATP) and reaction products of hydrolysis (AMP) and alcoholysis (AMP-*O*-alkyl ester) in rat liver NPP/PDE reaction mixtures with the indicated alcohol. The presence of ADP in the samples analysed is due mainly to non-enzymic degradation of ATP during the heat inactivation of NPP/PDE at the end of the enzymic incubation (see the Materials and methods section), and to a lesser extent to its occurrence as a contaminant of the commercial preparation of ATP. HPLC conditions are described in the Materials and methods section. Labels: a, AMP; b, ADP; c, ATP; E, AMP-*O*-alkyl ester(s).

verted into AMP by extensive treatment with snake venom NPP/PDE [25].

Evidence that the alcoholysis and hydrolysis of ATP was catalysed by the same enzyme is extensive. In summary, the alcohololytic and hydrolytic activities, tested with the indicated alcohols, were in agreement (results not shown) in terms of (i) the chromatography profiles on a Sephacryl S-300 column, with methanol and ethanol; (ii) the time courses of product accumulation, with all the alcohols investigated (incubations up to 30 h); (iii) the pH-activity profiles, with methanol (pH 6–10.5) and ethanol (pH 7–9.5); (iv) the temperature–activity profiles, with methanol (linear and parallel Arrhenius plots between

Figure 2 Kinetics of rat liver NPP/PDE-catalysed hydrolysis and alcoholysis of ATP at different concentrations of alcohol

Initial rates are expressed as a percentage of the hydrolytic rate measured in the absence of alcohol, which was in the range 1–5 nmol/min per ml of reaction mixture. The assays were performed by method 1, except in the experiments with glycerol below 0.7 M and with glycerol 2-phosphate, when method 2 was used. The solid lines are (non-)linear fittings performed as described in the Materials and methods section. ATP hydrolysis (**A**) and alcoholysis (**B**) were fitted to eqns. (6) and (7), except for the experiments with glycerol (fitted to eqns. 3 and 4) or glycerol 2-phosphate (fitted to eqns. 8 and 9). Alcohol efficiencies (**B, C**), calculated as $R_{AW} = (v_A/v_W)/([A]/[W])$, were fitted to eqn. (5). In the case of glycerol, only the data for the primary hydroxy groups, and only up to a concentration of 0.7 M, were considered. In the double-logarithmic plot, $h = \text{slope} + 1$ (**C**); h is a Hill-type coefficient measuring the degree of negative co-operativity shown by the alcohol in rat liver NPP/PDE reactions (see equations 3–5). Symbols: $\circ$, initial rates of hydrolysis or alcoholysis by primary hydroxy groups; $\triangle$, initial rates of alcoholysis by the secondary hydroxy group of glycerol; $\bullet$, R_{AW} or $\log R_{AW}$ of primary OH groups; $\blacktriangle$, R_{AW} or $\log R_{AW}$ of the secondary hydroxy group of glycerol.

0 and 40 °C); (v) inactivation between 60 °C and 70 °C, with methanol; and (vi) degree of inhibition by EDTA, with methanol.

Kinetic studies

Kinetic measurements were performed at various alcohol concentrations, measuring the accumulation of AMP-*O*-alkyl ester and AMP by HPLC. Alcoholysis and hydrolysis occurred simultaneously at relative rates that depended on the nature and concentration of the alcohol (Figure 2). In all cases, the rates of alcoholysis (v_A) were typical of those expected for a saturation-like behaviour, with a decrease of activity observed in some cases at the higher concentrations tested, whereas the rates of hydrolysis (v_W) were maximal in the absence of the alcohol and decreased when the concentration of alcohol was increased (except for glycerol 2-phosphate, which activated the hydrolysis of ATP at low concentrations). Taken together, these results suggest that competition occurs between alcohols and water as adenylate acceptors, and that enzyme inactivation takes place at the highest alcohol concentrations tested.

Figure 2(B) shows that, in contrast with what would be normally expected for competing substrates with hyperbolic kinetics (see the 'Equations' subsection of the Materials and methods section), the ratio $R_{AW} = (v_A/v_W)/([A]/[W])$ was not independent of alcohol concentration, but decreased to a differing extent as $[A]$ increased. This can be accounted for by the fact that alcohols adversely affected their own ability to act as adenylate acceptors, a kinetic behaviour typical of substrates showing negative co-operativity. In this case, a plot of $\log R_{AW}$ against $\log[A]$ should be linear with slope $h < -1$ (eqn. 5), h being a Hill-type coefficient that describes the degree of co-operativity.

Figure 2(C) shows that the experimentally derived, double-logarithmic plots were reasonably linear, with negative slopes that yielded values of $h < 1$ for all the alcohols within the concentration ranges studied, except for glycerol. At glycerol concentrations below 1 M, this alcohol did not show appreciable co-operativity ($h = 0.95$), whereas the plot curved steeply downwards at concentrations higher than 2 M, indicating that some other phenomenon, different to the 'negative-co-operativity-like' behaviour represented by the h coefficient for the other alcohols, must be responsible for the marked loss of glycerol efficiency (R_{AW}) at high glycerol concentrations. The other alcohols exhibited h values ranging from 0.61 to 0.82, except for glycerol 2-phosphate ($h = 0.27$).

Figures 2(A) and 2(B) also show that, except for the case of glycerol at high concentrations, experimental v_A and v_W data could be fitted reasonably well to rate equations derived on the assumption that alcohols and water are competing substrates, with alcohols exhibiting negative co-operativity. (i) In the case of 2,2-dichloroethanol, ethylene glycol, methanol, ethanol and propanol, decreases in the rate of alcoholysis were observed at high alcohol concentrations (Figure 2B), and were interpreted as showing evidence for enzyme denaturation, which should affect both the alcoholytic and the hydrolytic reactions. Therefore for these alcohols, v_A and v_W data were fitted to eqns. (6) and (7). It is interesting to note that the marked inhibition of ATP hydrolysis already observed at low alcohol concentrations is not in itself an indication of enzyme denaturation, but of competition of alcohol with water, even when the alcoholytic rate is very low relative to the hydrolytic one. For example, 5 M ethanol inhibited ATP hydrolysis by 50%, with only an assumed inactivation of 10% at this concentration (non-denatured enzyme fraction = 0.91;

Table 1 Effect of P_i and NaCl on the efficiencies of glycerol 2-phosphate and glycerol

Alcohol efficiencies, calculated using the equation $R_{AW} = (v_A/v_W)/([A]/[W])$, are given as means $\pm$ S.D. ($n = 8$). Each number pair shown in parentheses corresponds to the mean reaction rates measured by method 2 (v_A , alcoholysis; v_W , hydrolysis) and are expressed as percentages of controls. P_i stock solution was a mixture of monosodic and bisodic salts that gave the same pH as the standard reaction mixtures.

Additions	R_{AW} values	
	0.11 M glycerol 2-phosphate	0.11 M glycerol
None	10.7 $\pm$ 2.4 (100; 100)	11.3 $\pm$ 1.6 (100; 100)
0.275 M P_i	4.3 $\pm$ 0.5 (34; 84)	9.7 $\pm$ 1.3 (92; 111)
0.75 M NaCl	2.0 $\pm$ 0.8 (7; 42)	10.2 $\pm$ 1.2 (62; 71)

eqn. 7). (ii) In the case of glycerol 2-phosphate, the increase in the rate of hydrolysis observed in the presence of low concentrations of the alcohol (Figure 2A) was taken to indicate that this alcohol is not only a substrate competing with water, but also an enzyme activator, increasing both V_{maxA} and V_{maxW} ; in this case, v_A and v_W data were fitted to eqns. (8) and (9). (iii) In the case of glycerol, the simultaneous formation of two esters presented additional kinetic complications, and only rate data corresponding to the primary ester (the major one) were considered. Reaction rates v_A and v_W obtained in experiments performed with glycerol at concentrations below 0.7 M showed neither an indication of enzyme denaturation nor activation, and could be easily fitted to eqns. (3) and (4). Reaction rates from experiments with high concentrations of glycerol could not be properly fitted to any of the above equations, which is in accordance with the fact that, as indicated above, glycerol at high concentrations did not behave as predicted by eqn. (5).

It is important to note that the fitting of the experimental data to the rate equations cannot be used to estimate the actual kinetic parameters of the enzyme (see the Materials and methods section), and the resulting curves are plotted in Figure 2 only to illustrate that, except for glycerol at high concentrations, the results are compatible with a simple model of alcohol and water being competing substrates, even in the presence of enzyme (in)activation. An alternative model to account for the behaviour of glycerol at high concentrations, which appears to follow a linear plot of R_{AW} measured against $[A]$ (Figure 2B), was not sought after any further, since this was outside the scope of this paper.

Effect of ionic strength on the efficiency of glycerol 2-phosphate

In an independent experiment, the effect of ionic strength on the efficiency of the electrically charged glycerol 2-phosphate was tested at a fixed alcohol concentration of 0.11 M. The R_{AW} ratio for glycerol 2-phosphate was decreased by more than 50% upon addition of 0.275 M sodium phosphate or 0.75 M sodium chloride, whereas the same effect was (almost) negligible on the R_{AW} ratio for glycerol (Table 1).

DISCUSSION

Three structurally related proteins with NPP/PDE activity have been reported to be expressed in rat liver. According to Stefan et al. [33], they have been named as NPP α (autotaxin), NPP β (B10/gp130^{R8013-6}) and NPP γ (PC-1). We do not know to which of these protein(s) the NPP/PDE preparation studied in this

work corresponds; however, the results indicate that the hydrolytic and alcoholytic activities on ATP correspond to the same enzyme(s). Moreover, since all the NPP/PDE enzymes tested so far exhibit alcoholytic activity [24–26,34], and it has been shown previously that the three mammalian NPPs (α , β and γ) may catalyse the hydrolysis of ATP, forming an adenylyl-enzyme intermediate [16,17,21,23], it seems likely that these three enzymes also catalyse the alcoholysis of ATP.

Comparison of alcohols and water as adenylate acceptors

The results support the hypothesis that alcohols and water are competing substrates of rat liver NPP/PDE, with alcohols displaying negative co-operativity, and some of them being much more efficient than water as adenylate acceptors in the enzyme's active centre. To quantify the relative efficiencies of alcohols and water, the ratio R_{AW} can be employed. The efficiency of water can be taken as unity, since this is the R_{AW} value that would correspond to an alcohol with the same intrinsic reactivity as water. R_{AW} values higher or lower than 1 correspond to alcohols that are respectively more or less efficient than water as adenylate acceptors. However, since R_{AW} was not independent of $[A]$, to allow comparisons to be made among alcohols, data at a fixed alcohol concentration have to be considered, and in order to minimize possible effects of the alcohols on the structure of the enzyme, it is better to rely upon R_{AW} data obtained at low alcohol concentrations. For instance, at 0.3 M alcohol the following R_{AW} values were obtained (Figure 2C): dichloroethanol, 22.2; glycerol (primary alcohol), 9.3; glycerol (secondary alcohol), 2.4; ethylene glycol, 9.2; methanol, 9.9; ethanol, 1.4; propanol, 0.6; glycerol 2-phosphate, 3.4.

Comparison of the rat liver enzyme with other NPP/PDEs showing alcoholytic activity

Snake venom and potato tuber NPP/PDE also display alcoholytic activity. In methanol/water mixtures, the methanolysis/hydrolysis ratio of the potato enzyme, but not of the snake one, changes with pH and temperature, being optimal at high pH and low temperature [34]. In this respect, rat liver NPP/PDE behaved like the enzyme from snake venom, i.e. the R_{AW} ratio was independent of both pH and temperature (results not shown). The snake venom NPP/PDE catalyses alcoholytic reactions of ATP with the same set of alcohols used in the present study; however, compared with rat liver NPP/PDE, the efficiencies of uncharged alcohols are lower (Figure 3), and change very little with alcohol concentration in most cases. When results from previous snake venom NPP/PDE studies [25,26] were treated as in Figure 2(C) in the present paper, the calculated h values ranged from 0.9–1.0 for all the uncharged R-CH₂OH alcohols, indicating that 'negative co-operativity-like' behaviour was almost negligible, except for the case of glycerol 2-phosphate, with $h = 0.4$ (results not shown).

Interactions of alcohols in the active centre of rat liver NPP/PDE

The results of the present study taken both in isolation and in comparison with results obtained with snake venom NPP/PDE provide evidence for up to three kinds of interaction of R-CH₂OH alcohols in the enzyme active centre of rat liver NPP/PDE, with only two of them in common with the snake enzyme. All of these interactions facilitate the efficiency of alcohols as adenylate acceptors.

Figure 3 Relationship between the efficiencies and the acidities of R-CH₂OH alcohols with electrically neutral R groups: comparison between rat liver and snake venom NPP/PDE

All the data correspond to 0.3 M alcohol. Results are shown only for alcohols for which a pK_a value was available [25]. Water is shown with a $R_{AW} = 1$, which is the theoretical efficiency of an alcohol with the same reactivity as water. Rat liver data were obtained from Figure 2(C). Those for snake venom NPP/PDE were obtained in the same way from published rate data [25].

Rate-limiting general base catalysis

García-Díaz et al. [25] reported that the efficiencies of alcohols as adenylate acceptors in snake venom NPP/PDE reactions increased with the acidity of the hydroxy group in a biphasic manner. These results, expressed in the same manner as those in our study in the form of R_{AW} ratios, are represented in Figure 3 for purposes of comparison with the rat liver enzyme. For alcohols with $pK_a \geq 15.4$, the slope of the logarithmic plot was 1.5, whereas for alcohols with $pK_a \leq 15.4$, it was only 0.15. From these data, it was concluded that, in snake NPP/PDE reactions: (i) the efficiency of alcohols as acceptors of adenylate is related to the same electron-withdrawing factors that determine acidity, opposite to the known increase of in-water alcohol nucleophilicity with OH basicity, and that (ii) this contrast points to a rate-limiting proton transfer from the hydroxyl nucleophile to a general-base catalyst in the active centre. It was reasoned that, since the rate constant of this transfer should increase in correlation with an increase in the OH acidity, proton transfer would control the rate of the alcoholic reactions with the less acidic alcohols ($pK_a \geq 15.4$), but as acidity increases ($pK_a \leq 15.4$) this step would exert less of an influence on the overall reaction rate [25]. This argument accounts in part for the high efficiency of some alcohol adenylate acceptors with pK_a values lower than water. Figure 3 also shows the results obtained studying rat liver NPP/PDE reactions, which also gave a biphasic plot with slopes similar to the snake enzyme. The remarkable similitude of the two plots indicates that the above reasoning concerning the occurrence of rate-limiting general base catalysis in the active centre of the snake enzyme can also be applied to the rat one. The significance of the plots of Figure 3 being parallel, rather than exactly coincident, is discussed below.

Methylene group interaction

In Figure 3 regarding the plot of the snake venom NPP/PDE (○), water fits quite well into the linear segment of the plot defined by high- pK_a alcohols. This indicates that neutral R-CH₂OH alcohols competed with water for the snake enzyme-bound adenylate mainly as a function of the electronic factors of the R group governing OH acidity (see previous subheading). In

contrast, the linear segment corresponding to rat NPP/PDE reactions with high- pK_a alcohols was well below the co-ordinates of water (Figure 3), which points towards another factor participating, in this case, in the competition between alcohol and water, in favour of the former. The parallelism between the 'efficiency-versus-acidity' plots of the two enzymes indicates that such factor is almost independent of the acidity of the alcohols. On average, the R_{AW} ratio of each alcohol (at 0.3 M) in rat NPP/PDE reactions is twice that in snake NPP/PDE reactions. This may suggest that the affinity of the rat enzyme for the alcohol relative to water is twice that of the snake enzyme. Considering the structure of alcohols and water, this must be related to a favourable hydrophobic interaction of the alcohol's proximal methylene group in the active centre of rat liver NPP/PDE. Such a contact could contribute to the free energy of binding of the alcohol with up to about -3.4 kJ/mol (-0.8 kcal/mol), estimated from the average contribution that one methylene group adds to the free energy of transfer of a linear hydrocarbon chain from water to a non-polar solvent [35]. A 2-fold difference in the binding constant would represent only a difference of 1.7 kJ/mol (0.4 kcal/mol). Perhaps when alcohol binds in the active centre of rat liver NPP/PDE, one side of the methylene group remains exposed to water [35]. Since the strength of such a hydrophobic interaction would depend on the polarity of the solvent, one may expect that it weakens when alcohol concentration increases, inasmuch as the alcohol contributes as a co-solvent to increase the hydrophobicity of the medium. This could explain a concentration-dependent decrease of alcohol efficiency, i.e. the 'negative co-operativity-like' response of alcoholysis (Figure 2), with alcohols being significantly less polar than water. Interestingly, glycerol displayed an almost constant efficiency up to 2 M concentration (Figure 2), which is in agreement with the fact that the polarities of water and glycerol are not very different [36]. The same could be true for ethylene glycol. Furthermore, it is noteworthy that, whereas at low alcohol concentrations the rat enzyme showed larger R_{AW} ratios than the snake one (Figure 3), at higher concentrations (e.g. ≈ 10 M alcohol; results not shown) the rat R_{AW} ratios approached those of the snake enzyme.

Phosphate group interaction

Glycerol 2-phosphate, at low concentrations, was the most efficient adenylate acceptor among those tested. Its R_{AW} value at a concentration of 5 mM was 64, i.e. it was 64-fold more efficient than water under these conditions. In contrast, the efficiency of glycerol under the same conditions was much lower ($R_{AW} = 12$). A pK_a value for the primary alcohol groups of glycerol 2-phosphate is not available, therefore this alcohol could not be included in Figure 3. However, as discussed earlier, the presence of the phosphate group is likely to make that pK_a value higher than the corresponding one of glycerol [26]. Therefore the strikingly high efficiency of glycerol 2-phosphate as an adenylate acceptor at low concentrations cannot be justified in terms of hydroxy group acidity and methylene group interactions. In addition, the efficiency of glycerol 2-phosphate decreased steeply as concentration increased (Figure 2), and it was also diminished by addition of sodium phosphate or sodium chloride, which did not affect the efficiency of glycerol in the same way (Table 1). These results point strongly towards a favourable electrostatic interaction of the phosphate group of glycerol 2-phosphate, which disappeared when ionic strength was increased. This feature of rat liver NPP/PDE is shared with the snake enzyme [26].

Concluding remarks: could rat liver NPP/PDE be a protein-adenylylating agent?

Rat liver NPP/PDE, more so than the snake enzyme, is well suited to act as a true transferase of adenylyl from ATP to alcohols. These adenylyl acceptors can be recognized by the active centre through at least one base catalyst, one hydrophobic and one cationic subsite. Glycerol 2-phosphate is the only alcohol among those tested with the potential to establish the three types of interaction and, depending on concentration, it exhibits the highest efficiency among the adenylyl acceptors tested. It can serve as a model to define the geometry of the active subsites that recognize the nucleophile acceptor of the enzyme-bound adenylyl, and to devise new hypotheses to search for a physiological acceptor in the environment of the plasma membrane where rat liver NPP/PDEs are located. Interestingly, rat liver membranes contain several proteins which are adenylylated upon incubation with [α - 32 P]ATP. Some of them may correspond to catalytic intermediates of the NPP/PDE enzymes themselves, but with respect to others, it has been suggested that they could be products of enzyme-catalysed adenylylation [37]. Given their ability to transfer efficiently the adenylyl moiety to a large variety of R-CH₂OH alcohols, we propose that NPP/PDE enzymes could act as adenylylating agents of other proteins in their natural locations.

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REFERENCES

- McLennan, A. G. (ed.) (1992) *Ap₄A and Other Dinucleoside Polyphosphates*, CRC Press, Boca Raton
- Kisselev, L. L., Justesen, J., Wolfson, A. D. and Frolova, L. Yu. (1998) *FEBS Lett.* **427**, 157–163
- Miras-Portugal, M. T., Gualix, J. and Pintor, J. (1998) *FEBS Lett.* **430**, 78–82
- Guranowski, A. and Sillero, A. (1992) in *Ap₄A and Other Dinucleoside Polyphosphates* (McLennan, A. G., ed.), pp. 81–133, CRC Press, Boca Raton
- Gasmi, L., Cartwright, J. L. and McLennan, A. G. (1998) *Biochim. Biophys. Acta* **1405**, 121–127
- Maddux, B. A., Sbraccia, P., Kumakura, S., Sasson, S., Youngren, J., Fisher, A., Spencer, S., Grupe, A., Henzel, W., Stewart, T. A., Reaven, G. M. and Goldfine, I. D. (1995) *Nature (London)* **373**, 448–451
- Frittitta, L., Youngren, J. F., Sbraccia, P., D'Adamo, M., Buongiorno, A., Vigneri, R., Goldfine, I. D. and Trischitta, V. (1997) *Diabetologia* **40**, 282–289
- Lee, H. Y., Clair, T., Mulvaney, P. T., Woodhouse, E. C., Aznavoorian, S., Liotta, L. A. and Stracke, M. L. (1996) *J. Biol. Chem.* **271**, 24408–24412
- Kawagoe, H., Stracke, M. L., Nakamura, H. and Sano, K. (1997) *Cancer Res.* **57**, 2516–2521
- Oyajobi, B. O., Caswell, A. M. and Russell, R. G. G. (1994) *J. Bone Miner. Res.* **9**, 99–109
- Lotz, M., Rosen, F., McCabe, G., Quach, J., Blanco, F., Dudler, F., Solan, J., Goding, J., Seegmiller, J. E. and Terkeltaub, R. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10364–10368
- Cardenal, A., Masuda, I., Haas, A. L. and McCarty, D. J. (1996) *Arthritis Rheum.* **39**, 245–251
- Grupe, A., Alleman, J., Goldfine, I. D., Sadick, M. and Stewart, T. A. (1995) *J. Biol. Chem.* **270**, 22085–22088
- Oda, Y., Kuo, M.-D., Huang, S. S. and Huang, J. S. (1991) *J. Biol. Chem.* **266**, 16791–16795
- Uriarte, M., Stalmans, W., Hickman, S. and Bollen, M. (1995) *Biochem. J.* **306**, 271–277
- Belli, S. I., Mercuri, F. A., Sali, A. and Goding, J. W. (1995) *Eur. J. Biochem.* **228**, 669–676
- Stefan, C., Stalmans, W. and Bollen, M. (1996) *Eur. J. Biochem.* **241**, 338–342
- Goding, J. W., Terkeltaub, R., Maurice, M., Deterre, P., Sali, A. and Belli, S. (1998) *Immunol. Rev.* **161**, 11–26
- Bryant, F. R. and Benkovic, S. J. (1979) *Biochemistry* **18**, 2825–2828
- Burgers, P. M. J., Eckstein, F. and Hunneman, D. H. (1979) *J. Biol. Chem.* **254**, 7476–7478
- Culp, J. S., Blytt, H. J., Hermodson, M. and Butler, L. G. (1985) *J. Biol. Chem.* **260**, 8320–8324
- Culp, J. S. and Butler, L. G. (1986) *Arch. Biochem. Biophys.* **246**, 245–249
- Clair, T., Lee, H. Y., Liotta, L. A. and Stracke, M. L. (1997) *J. Biol. Chem.* **272**, 996–1001
- García-Díaz, M., Avalos, M. and Cameselle, J. C. (1991) *Eur. J. Biochem.* **196**, 451–457
- García-Díaz, M., Avalos, M. and Cameselle, J. C. (1993) *Eur. J. Biochem.* **213**, 1139–1148
- Vergeles, J. M., García-Díaz, M. and Cameselle, J. C. (1995) *Eur. J. Biochem.* **233**, 442–447
- López-Gómez, J., Costas, M. J., Ribeiro, J. M., Fernández, A., Romero, A., Ávalos, M. and Cameselle, J. C. (1998) *FEBS Lett.* **421**, 77–79
- Kelly, S. J., Dardinger, D. E. and Butler, L. G. (1975) *Biochemistry* **14**, 4983–4988
- Manchenko, G. P. (1994) *Handbook of Detection of Enzymes on Electrophoretic Gels*, CRC Press, Boca Raton
- Cornish-Bowden, A. (1995) *Fundamentals of Enzyme Kinetics*, Portland Press, London
- Mozhaev, V. V., Khmelitsky, Y. L., Sergeeva, M. V., Belova, A. B., Klyachko, N. L., Levashov, A. V. and Martinek, M. (1989) *Eur. J. Biochem.* **184**, 597–602
- Segel, I. H. (1975) *Enzyme Kinetics*, p. 228, John Wiley & Sons, New York (reprinted in 1993)
- Stefan, C., Gijsbergs, R., Stalmans, W. and Bollen, M. (1999) *Biochim. Biophys. Acta* **1450**, 45–52
- Agudo, A., Ribeiro, J. M., Canales, J. and Cameselle, J. C. (1998) *Biotechnol. Bioeng.* **59**, 62–67
- Jencks, J. P. (1969) *Catalysis in Chemistry and Enzymology*, pp. 400–403, McGraw-Hill, New York
- Tanford, C. (1980) *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, 2nd edn., p. 12, John Wiley & Sons, New York
- San José, E., Benguría, A. and Villalobo, A. (1990) *J. Biol. Chem.* **265**, 20653–20661

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