A Kinetic Study of the Soluble 5'-Nucleotidase of Rat Liver

By GEORGES VAN DEN BERGHE, CHRISTIANE VAN POTTELSBERGHE*
and HENRI-GÉRY HERS

*Laboratoire de Chimie Physiologique, Université de Louvain and
International Institute of Cellular and Molecular Pathology,
1200 Brussels, Belgium*

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1. The kinetic properties of the 5'-nucleotidase (EC 3.1.3.5) present in the cytosol of rat liver were investigated in relation to the conversion of adenine nucleotides into uric acid, with particular reference to the stimulation of this process by fructose. The enzyme was assayed by the release of P_i and by a new and more sensitive radiochemical procedure. 2. When IMP was used as substrate, the partially purified enzyme displayed almost hyperbolic kinetics ($h = 1.1$) with $s_{0.5} = 1.2$ mM. Similar kinetics were observed with GMP and other nucleoside 5'-monophosphates, except AMP. 3. V_{max} of the enzyme for AMP was about the same as for IMP, but the kinetics were sigmoidal ($h = 1.6$) with $s_{0.5} = 10$ mM. 4. The hydrolysis of IMP was inhibited competitively by GMP. IMP, at concentrations up to 0.5 mM, had a paradoxical stimulatory action on the hydrolysis of 2-5 mM-AMP and was inhibitory at higher concentrations. 5. The activity of the enzyme towards AMP and IMP was stimulated by ATP and GTP, and inhibited by P_i . Activators and inhibitor approximately cancelled each others' effects. At pH 7.4, the enzymic activity with 0.2 mM-AMP was undetectable under physiological conditions. 6. It is concluded that, in the liver cell, AMP is not hydrolysed by the soluble 5'-nucleotidase, but that its degradation requires prior deamination to IMP.

As reviewed by Drummond & Yamamoto (1971), several 5'-nucleotidases (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) have been described in various mammalian tissues, including the liver: the most active one is bound to the cell membrane, another is lysosomal and a third is present in the cytosol. The subcellular localization of the first two 5'-nucleotidases and the complete inhibition of the membrane enzyme by ATP at physiological concentration seem to preclude their participation in the conversion of purine nucleotides into uric acid. This role is therefore presumably played by the cytosolic enzyme, as also indicated by its abundance in the uricotelic avian liver (Itoh *et al.*, 1967).

The precise function of a 5'-nucleotidase in the physiological as well as in the fructose-induced (Mäenpää *et al.*, 1968) catabolism of purine nucleotides remained ill-defined, since, as discussed by Woods *et al.* (1970), this enzyme could dephosphorylate AMP as well as IMP. From the preceding paper (Van den Berghe *et al.*, 1977), it appears that the activity of AMP deaminase (EC 3.5.4.6) is highly regulated and seems to control the catabolism of adenine nucleotides. It is therefore unlikely that any important amount of AMP would be converted into adenosine, but degradation of IMP to inosine is

required. Accordingly, Itoh *et al.* (1968) and Fritzon (1969) have reported that the cytosolic 5'-nucleotidase has a higher affinity for IMP than for AMP. These authors, however, have not studied the kinetics of the enzyme in detail at physiological concentrations of its substrates. In the present paper, we report that the low affinity of the soluble 5'-nucleotidase towards AMP is accompanied by sigmoidal kinetics at low substrate concentrations, making the enzyme totally inactive under physiological conditions. In contrast, the enzyme displays nearly hyperbolic kinetics with IMP and other nucleoside 5'-monophosphates and allows the degradation of these substrates to proceed.

Materials and Methods

Materials

[U- ^{14}C]GMP (specific radioactivity 450 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Neutral chromatographic alumina (type WN-3) was from Sigma Chemical Co., St. Louis, MO, U.S.A. The origin of the other commercial chemicals and enzymes has been given in the preceding paper (Van den Berghe *et al.*, 1977). Radioactive inosine was prepared by incubation of [8- ^{14}C]IMP with 5'-nucleotidase from *Crotalus adamanteus* venom in a medium containing 50 mM-Tris, adjusted to pH 7.4 with 1 M-HCl, and 5 mM-MgCl₂. Partially purified soluble 5'-nucleotidase was

* Present address: National Institute of Neurological and Communicative Disorders and Stroke, N.I.H., Bethesda, MD, U.S.A.

prepared from rat liver as described by Fritzson (1969). Loss of activity occurred after 1 month of storage at -20°C . Three different batches of enzyme, with V_{max} ranging from 10 to 38 nmol of P_i released/min per mg of protein, were used. The enzyme preparation was free of myokinase activity measured by the method of Adam (1963) and did not release P_i from ATP or β -glycerophosphate.

Assay of 5'-nucleotidase

The activity of the partially purified enzyme was measured by the release of either P_i or the nucleoside, the latter method being used at substrate concentrations below 1 mM. Incubations were performed at 37°C in a medium containing 50 mM-Tris/maleate adjusted to pH 6.3 with 1 M-NaOH, 5 mM-MgCl₂, various concentrations of the substrate and other additions as indicated. When the release of P_i was measured, the reaction was performed in a total volume of 0.5 ml and terminated by the addition of 0.5 ml of 10% (w/v) ice-cold trichloroacetic acid. The amount of P_i liberated was determined by the method of Fiske & SubbaRow (1925) or, when required, by the more sensitive method of Weil-Malherbe & Green (1951).

To measure the release of nucleosides, ^{14}C -labelled IMP, AMP or GMP were used as substrates, and the radioactive product was separated from the remaining substrate by filtration on neutral alumina. This separation is based on the ability of this substance to adsorb P_i and other multivalent anions strongly compared with univalent anions and uncharged compounds (Ramachandran, 1971). The incubation volume was 0.2 ml and the reaction was terminated by heating the tubes for 2 min at 100°C , followed by the addition of 1 ml of 0.1 mM-carrier nucleoside (inosine, adenosine or guanosine) dissolved in 100 mM-Tris adjusted to pH 7.4 with 1 M-HCl. Denatured proteins were eliminated by a brief centrifugation (500 g for 20 min) and the whole supernatant was applied to a small (0.5 cm $\times$ 3 cm) column of neutral alumina, obtained by pouring approx. 600 mg of the dry powder into a Pasteur pipette plugged with glass wool. Each column was washed with 11 ml of 50 mM-Tris/HCl buffer, pH 7.4, and a portion of the eluate was counted for radioactivity with the scintillation mixture of Patterson & Greene (1965). In the absence of enzyme, less than 2% of the radioactivity of the substrate was found in the eluate. The recovery of [^{14}C]inosine was consistently better than 95%. The formation of the radioactive product was linear as a function of time and proportional to the amount of enzyme. The determination of the activity of partially purified 5'-nucleotidase with 1 mM-[^{14}C]IMP gave results that were within 5% of those obtained by the determination of P_i .

Results

Influence of substrate concentration

The various 5'-nucleotidases, including the soluble enzyme, are characterized by their broad substrate specificity (Drummond & Yamamoto, 1971). With our radiochemical method the influence of the concentration of AMP, IMP and GMP could be investigated over a wide range of concentrations, comprising those reported for these nucleotides in the liver: 0.1–0.2 mM for AMP (Start & News-holme, 1968), below 1 mM for IMP (Van den Berghe *et al.*, 1977), 0.02–0.04 mM for GMP (Clifford *et al.*, 1972). Fig. 1 shows that, in accordance with the findings of Fritzson (1969), the enzyme had a markedly higher affinity for IMP ($s_{0.5} = 1.2$ mM) than for AMP ($s_{0.5} = 10$ mM). Further, whereas the saturation curve for IMP was almost hyperbolic ($h = 1.1$), the kinetics for AMP was clearly sigmoidal ($h = 1.6$). The investigation with GMP (Fig. 2) also indicated a slight sigmoidicity, with $h = 1.1$.

Table 1 shows the activity of the enzyme measured by the release of P_i from a larger series of substrates, at two concentrations, 10 and 1 mM. The ratio of activities under these conditions exceeded the value of 10 only with AMP, which is therefore the only substrate for which co-operativity is clearly apparent.

Interaction between substrates

The strikingly different kinetics of the soluble 5'-nucleotidase towards AMP, as compared with IMP and GMP, prompted us to investigate the interaction of these various substrates, to gain information

Fig. 1. Influence of the concentration of IMP and AMP on the rate of their hydrolysis

Fig. 2. Influence of the concentration of GMP on the rate of its hydrolysis

The insert shows a double-reciprocal plot of the data. V_{max} of the preparation used in this experiment was 38 nmol of P_i released/min per mg of protein, compared with 25 nmol/min per mg in the experiment described by Fig. 1.

Fig. 3. Influence of GMP on the hydrolysis of IMP
The activity of the enzyme was measured with 1 mM-IMP as substrate.

Table 1. Substrate specificity of the soluble 5'-nucleotidase

Substrate	5'-Nucleotidase activity (nmol/min per mg of protein)		Activity ratio (10mM/1 mM)
	at 10mM- substrate	at 1 mM- substrate	
AMP	22.4	0.5	44.8
IMP	35.9	18.3	2.0
GMP	32.5	15.2	2.1
CMP	10.1	1.3	7.8
TMP	6.4	0.7	9.1
UMP	17.1	3.1	5.5
XMP	29.2	6.4	4.6

Fig. 4. Influence of IMP on the hydrolysis of AMP
The activity of the enzyme was measured with (○) 2 mM- or (●) 5 mM-AMP in the absence and in the presence of various concentrations of IMP.

about the nucleotide-binding site(s). Fig. 3 shows the influence of GMP on the hydrolysis of IMP; the guanine nucleotide readily inhibits the activity of the enzyme towards the other substrate. Calculations showed that the degree of inhibition corresponded closely to the value predicted for a competitive inhibition between both substrates. As expected from the much lower affinity of the enzyme preparation for AMP, the inhibitory effect of the adenine nucleotide on the hydrolysis of IMP was much less pronounced: at 1 mM-IMP, 5 mM-AMP inhibited the enzyme by only 8% (result not shown). Concentrations of IMP up to 0.5 mM had a paradoxical stimulatory effect on the hydrolysis of AMP, whereas

higher concentrations of the inosine nucleotide were inhibitory (Fig. 4).

Effect of metabolites

The parenteral administration of a load of fructose causes important changes in the concentration of several metabolites in the liver, mainly P_i , ATP, GTP and fructose 1-phosphate (Van den Berghe *et al.*, 1977). Accordingly, special attention was paid to the possible influence of these metabolites on the activity of the soluble 5'-nucleotidase.

Fig. 5. Influence of ATP, GTP and P_i . The concentrations of the effectors were those present in the normal liver.

Fig. 5 shows that 3mM-ATP exerted a strong stimulatory effect on the activity of the enzyme with AMP as substrate. From this and other experiments (results not shown), it appeared that the ATP effect was mainly to decrease $s_{0.5}$ about 10-fold. A physiological concentration of GTP had a similar, although less pronounced, influence. In contrast, P_i , when added at its normal concentration, inhibited the enzymic activity. Similar results were obtained with IMP as substrate. Fructose 1-phosphate (10mM) and glucose 6-phosphate (2mM) were without effect; inosine (2mM) inhibited the enzymic activity by 47%, in accordance with previous observations of Itoh *et al.* (1968) and Fritzson (1969).

In view of the pronounced effects of ATP, GTP and P_i on the activity of the soluble 5'-nucleotidase, substrate-saturation curves were obtained in the presence of mixtures of these metabolites at the concentrations known to be present in normal liver (Fig. 6). As is apparent from a comparison with Fig. 1, the activators and inhibitor approximately cancel each others' effects, so that the substrate-saturation curves were similar to those obtained in their absence. When their concentrations were lowered to the values observed in the liver of experimental animals after the administration of a load of fructose, the activity towards AMP decreased by approx. 50% at the concentrations studied, whereas the activity with IMP as substrate was markedly less influenced.

Effect of pH

In accordance with the findings of Itoh *et al.* (1968) and of Fritzson (1969), the partially purified enzyme

Fig. 6. Influence of mixtures of effectors on the substrate-saturation curves.

The enzymic activity was measured in the presence of 3mM-ATP, 0.5mM-GTP and 5mM- P_i (O, ●) and of 1mM-ATP, 0.3mM-GTP and 2.5mM- P_i (Δ, ▲).

displayed a sharp optimum of activity around pH 6.3 with 10mM-IMP (results not shown). A similar pH-activity curve was observed at 0.2mM-IMP when the high-speed supernatant (105000g for 60min) of a crude liver homogenate was used as a source of enzyme; this indicates that the nucleotide is not or only very slowly hydrolysed by the acid and alkaline phosphatases present in that preparation. Fig. 7 shows that, in the presence of a physiological mixture of effectors, a modification of the pH from 6.3 to 7.4 markedly decreased the activity of the enzyme towards both IMP and AMP. With the latter substrate, the enzymic activity became undetectable in physiological conditions.

Discussion

Kinetic properties of the soluble 5'-nucleotidase

In contrast with the hyperbolic kinetics reported by Fritzson (1969) for all substrates tested, the saturation curves were sigmoidal for IMP, GMP and AMP. This property of the enzyme was especially marked towards AMP, making the enzyme almost totally inactive at physiological concentrations of this nucleotide and can be explained by the fact that, thanks to our radioactive assay, we could include lower concentrations of the substrates. The enzyme was further characterized by its inhibition by P_i and its stimulation by ATP. These data help to distinguish the cytoplasmic 5'-nucleotidase from its membrane counterpart, which is much more active on AMP than

Fig. 7. Influence of pH on the substrate-saturation curves. The activity was measured in the presence of 3 mM-ATP, 0.5 mM-GTP and 5 mM- P_i .

on IMP and GMP (Widnell & Unkeless, 1968) and is completely inhibited by ATP at physiological concentration in the liver (Evans & Gurd, 1973) as well as in other tissues (Baer *et al.*, 1966; Burger & Lowenstein, 1967, 1975).

From the study of the interaction between IMP and GMP, it appears that both nucleotides compete for the same catalytic site. The inhibitory effect of high concentrations of IMP on the hydrolysis of AMP also supports the hypothesis that all the substrates bind to the same active site. The stimulatory influence of low concentrations of IMP on the hydrolysis of the adenine nucleotide suggests that the former substrate induces a conformation which favours the hydrolysis of the latter. The effect of the inosine nucleotide on the hydrolysis of AMP resembles the effect on native aspartate transcarbamoylase of maleate, an analogue of its substrate (Gerhart & Pardee, 1964): low concentrations of maleate mimic aspartate in terms of increasing the affinity of the remaining sites for the substrate. In our case, it is, however, surprising to observe this activating effect with a substrate displaying little co-operativity by itself.

Physiological role of the 5'-nucleotidases

From the kinetic properties and the substrate specificity of the soluble 5'-nucleotidase, it appears that the enzyme is designed to degrade the various 5'-mononucleotides with the apparently unique exception of 5'-AMP. Indeed, AMP is not hydrolysed at its concentration in the liver cell in the presence of physiological concentrations of its known effectors and at pH 7.4. This is explained by the low

affinity of the adenine nucleotide for the enzyme and by the sigmoidal shape of the substrate-saturation curve. Further, the finding that the activity of the enzyme towards AMP decreased in the conditions prevailing in the liver of experimental animals after the administration of a load of fructose, and the observation that the concentration of AMP barely increases in these conditions (Mäenpää *et al.*, 1968; Van den Berghe *et al.*, 1977), rules out the participation of 5'-nucleotidase in the hyperuricaemic effect of fructose. As discussed in the preceding paper (Van den Berghe *et al.*, 1977), AMP can be converted into IMP by AMP deaminase, which is highly regulated and nearly completely inhibited in physiological conditions. This tight control of the AMP-degrading pathway is required in order to preserve the adenine nucleotide pool within the cell, since the high activity of adenylate kinase in the liver (Adelman *et al.*, 1968) results in a free interconversion of ATP, ADP and AMP. The high-energy phosphoryl compounds corresponding to the other substrates of the cytoplasmic 5'-nucleotidase are not subjected to the same danger.

At the concentrations of IMP observed after the administration of fructose (Van den Berghe *et al.*, 1977) one can estimate a rate of degradation of 0.1 $\mu\text{mol/min per g}$ of liver. This is still far below the rate of degradation of ATP, exceeding 1 $\mu\text{mol/min per g}$ of liver, observed during the first minutes after a fructose load, and explains the accumulation of IMP under these circumstances as well as the delayed formation of uric acid and allantoin.

The subcellular localization of both the lysosomal and the membrane 5'-nucleotidase and the complete inhibition of the latter enzyme by physiological concentrations of ATP (Evans & Gurd, 1973) precludes their participation in the conversion of purine nucleotides into uric acid. Data demonstrating that intact adipocytes (Newby *et al.*, 1975) or the external surface of skeletal muscle (Woo & Manery, 1975) can degrade 5'-nucleotides suggest that the membranous enzyme may be an ectoenzyme, whose primary function may be the uptake of adenosine from AMP, as documented in human lymphocytes by Fleit *et al.* (1975).

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