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KINETIC PROPERTIES OF CYTOSOL 5′-NUCLEOTIDASE FROM CHICKEN LIVER

ROICHI ITOH, CHIKAKO USAMI, TOMOKO NISHINO and KEIZO TSUSHIMA

Department of Biochemistry, Yokohama City University School of Medicine, Yokohama (Japan)

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Summary

A highly purified preparation of cytosol 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) from chicken liver was effectively activated by ATP and inhibited by P_i. When AMP was used as substrate, the enzyme displayed sigmoidal kinetics. When either IMP, GMP, UMP or CMP was used as substrate, the substrate saturation curve was slightly sigmoidal or hyperbolic. In the presence of 10 mM ATP, the substrate saturation curves for all substrates tested were hyperbolic, whereas P_i increased the sigmoidicity of the saturation curves. The enzyme had much higher affinities for IMP and GMP than for AMP, UMP and CMP. ATP decreased the $s_{0.5}$, whereas P_i increased the $s_{0.5}$ for all the substrates tested. Inhibition by P_i was removed by ATP, and activation by ATP was removed by P_i. V of the enzyme was determined to be in the same order of the magnitude for all the substrates tested.

The hydrolysis of GMP was inhibited competitively by the other 5′-nucleotides. AMP at low concentration had a stimulatory effect on the hydrolysis of GMP. The enzyme activity was absolutely dependent on the presence of bivalent cations. Increasing concentration of $MgCl_2$ increased the V and affinity of the enzyme for AMP and vice versa.

ATP protected the enzyme against the inactivation by heat or trypsin digestion. It seems likely that this enzyme is an allosteric protein regulated by various ligands through conformational changes.

Introduction

Cytosol 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) which preferentially hydrolyses IMP and GMP was first reported by us [1,2].

h refers to the Hill coefficient, and $s_{0.5}$ is the substrate concentration at half saturation of enzyme.

Correspondence should be sent to: Dr. Roichi Itoh, Department of Biochemistry, Yokohama City University School of Medicine, Urafune-cho, Minami-ku, Yokohama, Japan.

The activity of this enzyme is much higher in chicken liver than in rat liver [3] and increases in response to a high protein diet. Purine nucleoside phosphorylase (EC 1.4.2.1) and xanthine dehydrogenase (EC 1.2.1.37) also exhibit this behavior. These observations suggest the importance of cytosol 5′-nucleotidase in nitrogen elimination in uricotelic animals [4,5].

Using a crude preparation from rat liver, Van den Berghe et al. [6] reported that this enzyme displayed sigmoidal kinetics, with activation by ATP and GTP and inhibition by P_i. This indicates that the activity of cytosol 5′-nucleotidase is regulated by various metabolites at physiological concentrations. Investigation of the regulatory properties of this enzyme in more detail with a highly purified preparation from chicken liver [7] to elucidate the control mechanisms of uric acid production in uricotelic animals is now reported.

Our results suggest the possibility that this enzyme is an allosteric protein regulated by various ligands through the cooperative interaction among subunits.

Materials and Methods

Enzyme. Cytosol 5′-nucleotidase was purified from chicken liver to near-homogeneity as described previously [7].

Chemicals. Nucleotides were purchased from Yamasa Shoyu Co., Ltd., Tokyo, as the free acids or the sodium salts. The Tris salts of the nucleotides were prepared by passing a solution of the sodium salt through a column of Dowex 50 (hydrogen form) at 4° C. The eluates were adjusted to pH 6.5 with Tris base. Concentration of all nucleotide solutions were determined spectrophotometrically.

[8-^{14}C]Adenosine 5′-monophosphate (specific activity, 60 Ci/mol), [U-^{14}C]-guanosine 5′-monophosphate (515 Ci/mol), [U-^{14}C]uridine 5′-monophosphate (516 Ci/mol) and [U-^{14}C]cytidine 5′-monophosphate (516 Ci/mol) were purchased from the Radiochemical Center, Amersham. [8-^{14}C]Inosine 5′-monophosphate (38 Ci/mol) was obtained from Schwarts/Mann, Orangeburg, New York. Trypsin and trypsin inhibitor were from Sigma Chemical Co., St. Louis.

All other chemicals were of reagent grade or the highest quality available.

Assay of 5′-nucleotidase. Assay 1: 5′-Nucleotidase activity was assayed in the following incubation mixture: 100 mM imidazole/HCl buffer (pH 6.5), 50 mM $MgCl_2$, 1 mg bovine serum albumin, an appropriate amount of the Tris salt of nucleoside 5′-monophosphate and enzyme preparation in a total volume of 1.0 ml. After incubation at 37° C, the reaction was terminated by addition of 1.0 ml 10% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation and an aliquot of the supernatant fluid was used for P_i determination by the method of Chen et al. [8].

Assay 2: Enzyme activity was assayed in the following incubation mixture: 100 mM imidazole/HCl buffer (pH 6.5), 50 mM $MgCl_2$, 50 μg bovine serum albumin, appropriate amount of [^{14}C]nucleoside 5′-monophosphate and enzyme preparation in a total volume of 50 μl. After incubation at 37° C, the reaction was terminated by addition of 10 μl 300 mM EDTA. A 20-μl aliquot was spotted on Whatman No. 3MM chromatography paper with carrier nucleoside (0.2 μmol). High voltage electrophoresis was performed at 2000 V for 60 min

in 50 mM sodium borate buffer (pH 9.0). The nucleoside spots were located with ultraviolet light, cut out and counted in a Packard Tri-Carb liquid scintillation spectrometer at 55% efficiency.

1 unit of activity corresponds to the release of 1 μmol P_i or adenosine per min with 60 mM AMP as substrate. The specific activity of the enzyme preparation used in the experiments was 14.3 μmol P_i released/min/mg protein.

Results

Effect of nucleoside di- and triphosphates on enzyme activity. The effect of various nucleoside di- and triphosphates at the concentration of 5 mM on the rate of the cytosol 5'-nucleotidase reaction is shown in Table I. Various 5'-mononucleotides were used as substrates at the concentration of 3 mM. ATP, deoxy ATP and ADP were the most effective activators tested with AMP as substrate. The stimulatory effect of GMP is also obvious with UMP and CMP as substrates. The other nucleoside di- and triphosphates were less effective.

With the other 5'-mononucleotides as substrates, ATP was again the most effective among the nucleoside triphosphates tested. 2'- or 3'-mononucleotides also stimulated the enzyme activity to some extent, whereas corresponding nucleosides had almost no effect.

Inhibition by inorganic phosphate. P_i effectively inhibited the enzyme activity. Arsenate was less effective. At a concentration of 5 mM, P_i and arsenate

TABLE I

EFFECT OF PURINE AND PYRIMIDINE COMPOUNDS ON THE CYTOSOL 5'-NUCLEOTIDASE ACTIVITY

The reaction mixture contained 100 mM imidazole/HCl buffer (pH 6.5), 50 mM $MgCl_2$, 0.05 μg bovine serum albumin and 3 mM [^{14}C]nucleoside 5'-monophosphates as substrate, and effectors as indicated in a total volume of 50 μl. The activity in the absence of effectors was taken as the standard for each substrate.

Addition (5 mM)	Relative activity with 3 mM:				
	AMP	GMP	IMP	UMP	CMP
None	1.00	1.00	1.00	1.00	1.00
ATP	7.86	1.50	1.52	13.76	10.80
GTP	3.46	1.33	1.34	7.25	5.78
UTP	2.01	1.14	1.11	2.54	1.84
CTP	1.46	1.15	1.10	2.02	1.68
ITP	2.59				
deoxyATP	6.38				
ADP	4.92				
GDP	1.66				
2'-AMP	1.46				
3'-AMP	2.78				
2'(3')-GMP	3.31				
2'(3')-UMP	1.52				
2'(3')-CMP	1.41				
cyclic AMP	1.11				
Adenosine	0.95				
Inosine	1.19				
Uridine	1.00				
Cytidine	0.97				

inhibited the activity 90% and 25%, respectively, with 15 mM AMP as substrate. Pyrophosphate and sulfate had almost no effect up to the concentration of 5 mM.

Influence of substrate concentration. With AMP as substrate, the saturation curve was clearly sigmoidal (h = 1.8). In the presence of 10 mM ATP, the curve assumed a hyperbolic behavior (h = 1.0). P_i increased the sigmoidicity of the saturation curves (Fig. 1). In the presence of 5 mM P_i, the Hill coefficient was 2.5.

Results obtained from the kinetic experiments with various 5'-nucleotides as substrates are summarized in Table II. With GMP or UMP as substrate, the sigmoidicity of the saturation curve was slight. With IMP or CMP as substrate, a hyperbolic saturation curve was obtained. For all the substrates tested, 5 mM P_i increased the sigmoidicity of the substrate saturation curves and $s_{0.5}$, whereas the saturation curves were hyperbolic in the presence of 10 mM ATP. ATP also highly decreased $s_{0.5}$.

The enzyme had markedly higher affinities for IMP and GMP than for the other 5'-nucleotides tested, even though the maximum reaction velocities estimated from the saturation curves in the presence of 10 mM ATP were in the same order of the magnitude for all the substrates tested.

Interaction between substrates. Graphic analysis of Lineweaver-Burk plots with relatively high concentrations of the substrate (1—10 mM) suggested that AMP, IMP, UMP or CMP inhibited the hydrolysis of GMP competitively, indicating that the same active site is responsible for the hydrolysis of all nucleotides. AMP up to 25 mM had a stimulatory effect on the hydrolysis of GMP, whereas CMP, UMP and IMP were inhibitory over the range of the concentrations tested. In the presence of 5 mM P_i, even IMP up to 25 mM showed a stimulatory effect (Fig. 2).

Effect of ATP on the inhibition by P_i. When the enzyme activity was assayed in the presence of increasing concentration of P_i, a slightly sigmoidal inhibition

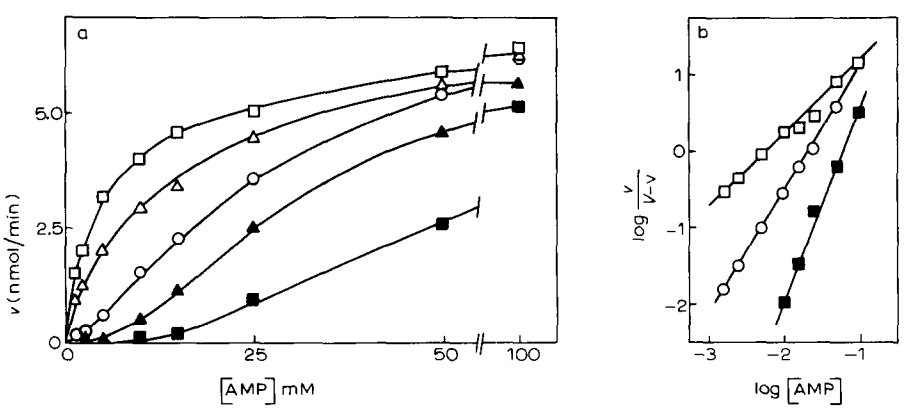

Fig. 1. (a) Effect of ATP and P_i on the cytosol 5'-nucleotidase activity as a function of AMP concentration. Reaction mixture contained 100 mM imidazole/HCl buffer (pH 6.5), 50 mM $MgCl_2$, 0.05 μg bovine serum albumin, $7 \cdot 10^{-3}$ units enzyme, various concentration of [^{14}C]AMP and effectors as indicated in a total volume of 50 μl. Incubation was for 15 min. ○, no addition; ▲, 1 mM P_i; ■, 5 mM P_i; △, 5 mM ATP; □, 10 mM ATP. (b) Hill plots of the data from (a) are presented. ○, no addition (h = 1.8); ■, 5 mM P_i (h = 2.5); □, 10 mM ATP (h = 1.0).

TABLE II

EFFECT OF ATP AND Pi ON THE KINETIC PARAMETERS OF VARIOUS SUBSTRATES

Conditions for the assay were the same as in Fig. 1. Relative values of the maximum reaction velocity for each substrate were estimated from the saturation curves in the presence of 10 mM ATP.

Substrate	No addition			10 mM ATP		5 mM P_i	
	V	$s_{0.5}$ (M)	h	$s_{0.5}$ (M)	h	$s_{0.5}$ (M)	h
AMP	1.00	$2.3 \cdot 10^{-2}$	1.8	$6.3 \cdot 10^{-3}$	1.0	$6.4 \cdot 10^{-2}$	2.5
GMP	1.29	$2.3 \cdot 10^{-3}$	1.1	$1.4 \cdot 10^{-4}$	1.0	$8.9 \cdot 10^{-3}$	1.8
IMP	1.37	$1.4 \cdot 10^{-3}$	1.0	$1.1 \cdot 10^{-4}$	1.0	$5.0 \cdot 10^{-3}$	1.7
UMP	0.79	$5.6 \cdot 10^{-2}$	1.2	$2.5 \cdot 10^{-3}$	1.0	$1.4 \cdot 10^{-1}$	2.1
CMP	0.73	10^{-1}	1.0	$4.5 \cdot 10^{-3}$	1.0	$3.5 \cdot 10^{-1}$	1.1

curve was obtained. ATP not only reversed the P_i inhibition but also increased the sigmoidicity of the inhibition curves (Fig. 3a). As shown in Fig. 3, 10 mM ATP increased the interaction coefficients, n' [9], for P_i from 1.4 to 2.2.

Effect of P_i on the activation by ATP. When the enzyme activity was assayed in the presence of increasing concentration of ATP, a slightly sigmoidal activation curve was obtained. P_i reversed the ATP activation and increased the sigmoidicity of the activation curves (Fig. 4a). As shown in Fig. 4b, 3 mM P_i increased the interaction coefficients, n'', for ATP from 1.4 to 2.2.

Effect of bivalent metal ions. The enzyme was inactive in the absence of bivalent metal ions (Fig. 5). When AMP was used as substrate, the dependence of the initial velocity on $MgCl_2$ or $CoCl_2$ concentration showed a sigmoidal

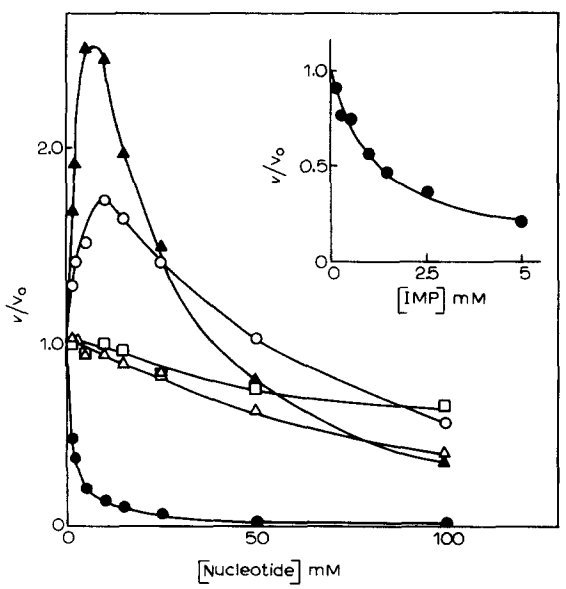

Fig. 2. Influence of various 5'-nucleotides on the hydrolysis of GMP. Reaction mixture contained $1.8 \cdot 10^{-3}$ units of the enzyme, 1 mM [^{14}C]GMP and variius concentration of other nucleotides in a total volume of 50 µl. Incubation was for 15 min. ○, AMP; △, UMP; □, CMP; ●, IMP; ▲, IMP with 5 mM P_i.

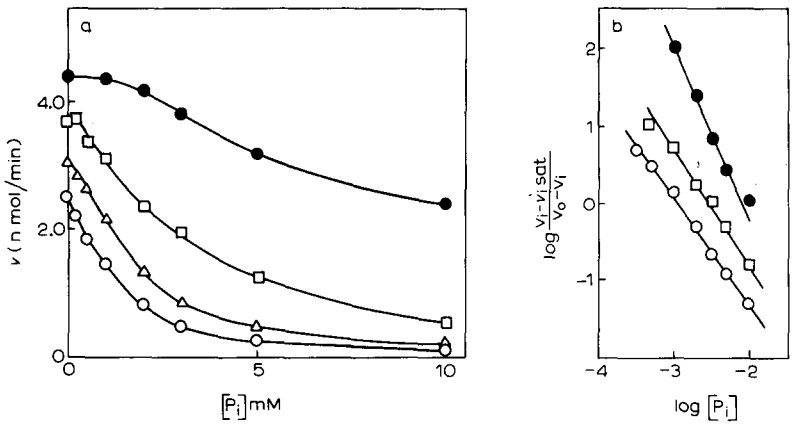

Fig. 3. (a) Reversal of P_i inhibition by ATP. Reaction mixture contained $7 \cdot 10^{-3}$ units of the enzyme, 15 mM [^{14}C]AMP and various concentration of P_i and ATP as indicated in a total volume of 50 µl. Incubation was for 15 min. ○, no addition; △, 1 mM ATP; □, 3 mM ATP; ●, 10 mM ATP. (b) Hill plots of the data from (a) are presented. ○, no addition (n' = 1.4); □, 3 mM ATP (n' = 1.6); ●, 10 mM ATP (n' = 2.2).

characteristic (h = 1.5 for $MgCl_2$). With UMP as substrate, the saturation curve for $MgCl_2$ was also sigmoidal (h = 2.1), whereas with IMP the saturation curve was hyperbolic (h = 1.0).

The sigmoidicity of the saturation curves for AMP (h = 1.8) was independent of the concentration of $MgCl_2$. Increasing concentration of $MgCl_2$ increased the apparent affinity of the enzyme for AMP. V for AMP was limited by the amount of $MgCl_2$ available (Table III). The sigmoidicity of the saturation curves for $MgCl_2$ (h = 1.5) was shown to be independent of the AMP concentration. Higher concentrations of AMP increased the apparent affinity of the enzyme for $MgCl_2$. V for $MgCl_2$ was limited by the amount of AMP available (Table IV).

Inactivation and protection of the enzyme. The addition of 3 mM ATP

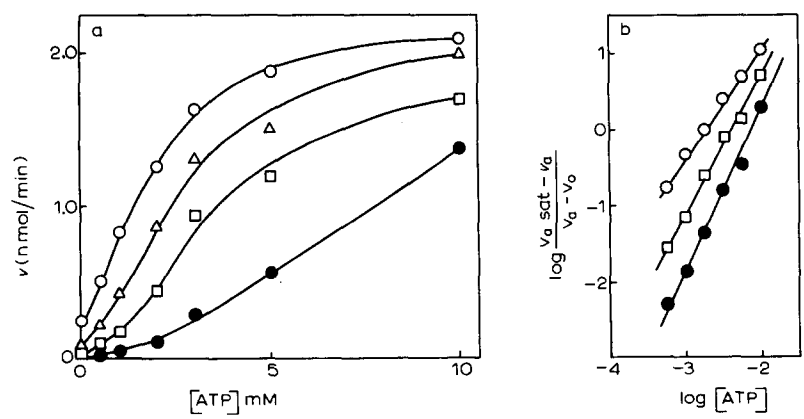

Fig. 4. (a) Reversal of ATP activation by P_i. Reaction mixture contained $7 \cdot 10^{-3}$ units of the enzyme, 3 mM [^{14}C]AMP and various concentration of ATP and P_i as indicated in a total volume of 50 µl. Incubation was for 15 min. ○, no addition; △, 0.4 mM P_i; □, 1 mM P_i; ●, 3 mM P_i. (b) Hill plots of the data from (a) are presented. ○, no addition (n'' = 1.4); □, 1 mM P_i (n'' = 1.8); ●, 3 mM P_i (n'' = 2.2).

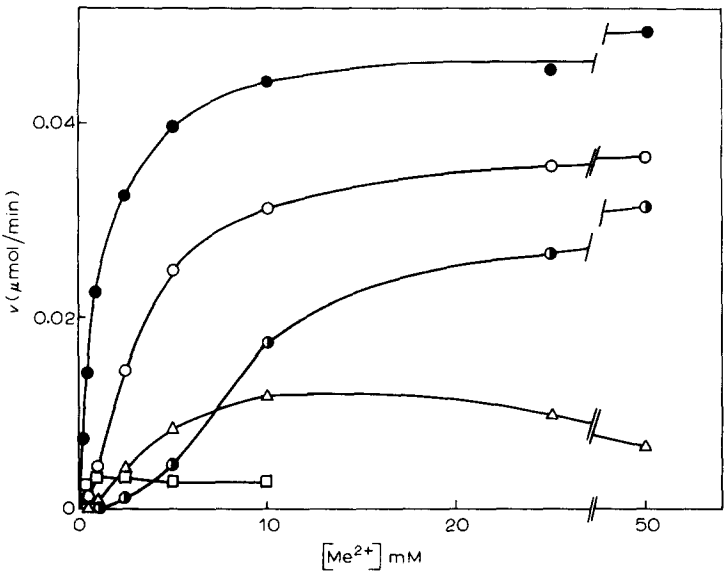

Fig. 5. Effect of bivalent metal cations on the activity of cytosol 5′-nucleotidase. Reaction mixture contained 100 mM imidazole/HCl buffer (pH 6.5), 1 mg bovine serum albumin, 60 mM substrate, $3.6 \cdot 10^{-2}$ units enzyme and various concentration of bivalent metal salts as indicated in a total volume of 1 ml. Incubation was for 5 min. ○, $MgCl_2$ with AMP; ●, $MgCl_2$ with IMP; ◑, $MgCl_2$ with UMP; △, $CoCl_2$ with AMP; □, $MnCl_2$ with AMP.

TABLE III

EFFECT OF $MgCl_2$ CONCENTRATION ON THE SATURATION CURVES FOR AMP

Conditions for the assay were the same as Fig. 5.

Kinetic parameters	$MgCl_2$ concentration (mM)		
	2.5	5.0	50.0
$[AMP]_{0.5}$ * (M)	$35 \cdot 10^{-3}$	$30 \cdot 10^{-3}$	$15 \cdot 10^{-3}$
V	0.39	0.67	1.00
h	1.8	1.8	1.8

* The AMP concentration at half-enzyme saturation.

TABLE IV

EFFECT OF AMP CONCENTRATION ON THE SATURATION CURVES FOR $MgCl_2$

Conditions for the assay were the same as Fig. 5.

Kinetic parameters	AMP concentration (mM)		
	12	20	60
$[MgCl_2]_{0.5}$ * (M)	$10 \cdot 10^{-3}$	$7 \cdot 10^{-3}$	$3 \cdot 10^{-3}$
V	0.47	0.62	1.00
h	1.5	1.5	1.5

* The $MgCl_2$ concentration at half-enzyme saturation.

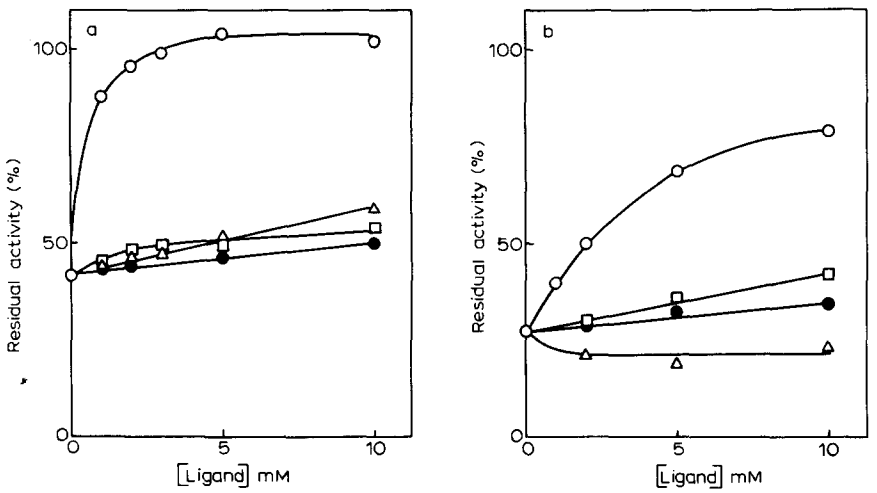

Fig. 6. Effect of ATP and other substances on inactivation of cytosol 5'-nucleotidase. (a) Highly purified cytosol 5'-nucleotidase ($9 \cdot 10^{-2}$ units) were incubated in 0.05 M Tris/HCl buffer (pH 7.4), 0.2 M NaCl at 50°C for 5 min with either ATP (○), P_i (△), GMP (□) or AMP (●) at the concentration indicated on the abscissa. (b) Highly purified cytosol 5'-nucleotidase ($9 \cdot 10^{-2}$ units) were incubated with 0.25 μg trypsin and either ATP (○), P_i (△), GMP (□) or AMP (●) at the concentration indicated on the abscissa in 0.05 M Tris/HCl buffer (pH 7.4), 0.2 M NaCl at 37°C for 5 min. Digestion was terminated by the addition of trypsin inhibitor. In each case, the enzyme activity was assayed with 60 mM [^{14}C]AMP as substrate using a 2-μl aliquot of the treated enzyme solution in a total volume of 50 μl.

almost completely protected the enzyme from heat inactivation at 50°C. P_i, AMP or GMP was much less effective, even if the concentrations were increased up to 10 mM (Fig. 6a). The addition of ATP also protected the enzyme from the inactivation by trypsin digestion. P_i slightly stimulated the inactivation. AMP or GMP was much less effective (Fig. 6b). In both cases, addition of 10 mM $MgCl_2$ did not influence the protective effect of ATP. Both ADP and GTP were as effective as ATP.The partially inactivated preparations were still sensitive to the stimulatory effect of ATP.

Discussion

Our results obtained with an highly purified chicken liver cytosol 5'-nucleotidase preparation confirmed the regulatory properties suggested by Van den Berghe et al. [6], who used partially purified rat liver enzyme. Higher values of $s_{0.5}$ for each substrate reported in the present paper are due primarily to the difference in the buffer system used for the assay of the enzyme activity. We used the imidazole/HCl buffer instead of the Tris/maleate system used by Van den Berghe et al. A high concentration of maleic acid was found to activate the enzyme by decreasing the $s_{0.5}$ for the substrates.

Considering the oligomeric configuration of the chicken liver cytosol 5'-nucleotidase [7], some properties of this enzyme reported in the present paper can be interpreted on the basis of the symmetry model for an allosteric enzyme proposed by Monod et al. [10], in which the enzyme exists as an equilibrium of two states: an active form, R, which has active site freely accessible to the substrates and an inactive form, T, in which the active site is inaccessible to the

substrates. The active form, R, can be stabilized in two ways: by substrates and activators. The inactive form, T, can be stabilized by inhibitors. When the protein goes from one state to another state, its molecular symmetry is conserved.

As predicted by the model, in the presence of an inhibitor, P_i, the substrate saturation curve of this enzyme is S-shaped, while in the presence of an activator, ATP, it is hyperbolic (Fig. 1 and Table I). Results presented in Figs. 3 and 4 are also predicted by the model. Sigmoidicity of the saturation curves of the inhibitor, P_i, increases with the concentration of the activator, ATP, whereas sigmoidicity of the saturation curves of the activator, ATP, increases with the concentration of the inhibitor, P_i.

Another prediction, concerning the effect of one substrate on another, is that low concentration of one substrate will stimulate binding of the other but high level of one will compete with and so inhibit binding of the other, if two substrates bind to the same form of the protein. This was actually observed in the experiments on the interaction between substrates. As expected, AMP at low concentrations stimulates the hydrolysis of GMP. Even IMP at low concentrations has a stimulatory effect when the enzyme is inhibited with P_i. At high concentrations, both inhibit the hydrolysis of GMP competitively (Fig. 2).

ATP, ADP and GTP were shown to protect the enzyme from inactivation by heat or trypsin digestion. It seems likely that a stable conformation can be induced by the binding of activators to the enzyme. Conversely, AMP and GMP have almost no effect on the stabilization of the enzyme. To establish a more precise model of this enzyme, detailed studies on binding properties and ligand induced conformational changes will be essential.

The cytosol 5′-nucleotidase is clearly distinguishable from the other 5′-nucleotidase by absolute dependence on bivalent metal ions. $MgCl_2$ was the most effective of the bivalent metal salts tested [1,2]. One possible interpretation of the kinetic data is that the nucleotide · metal complex is the true substrate of the enzymic reaction. ATP effectively stabilized the enzyme in the absence of $MgCl_2$, suggesting that the free activator makes a complex with the enzyme.

In uricotelic animals, uric acid production through IMP is possibly regulated not only by the induction of the cytosol 5′-nucleotidase [4,5], but also allosterically by the regulation of the enzyme through the changes in the concentration of metabolites, mainly ATP and P_i. The rate of the degradation of IMP by the cytosol 5′-nucleotidase in liver cells of uricotelic animals in the presence of physiological concentration of various effectors remains to be determined.

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