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Regulation of AMP nucleosidase in *Azotobacter vinelandii*

AMP nucleosidase (AMP phosphoribohydrolase, EC 3.2.2.4) from *Azotobacter vinelandii*, strain O, catalyzes the hydrolysis of AMP to form adenine and ribose 5-phosphate. It was first described by HURWITZ, HEPPEL AND HORRECKER[1] who demonstrated its absolute dependence on ATP for the catalytic hydrolysis of AMP, and that ATP was recovered unchanged from the reaction mixture. Prior to the investigation of certain aspects of the reaction catalyzed by this enzyme, a new procedure for its purification from *A. vinelandii* was developed. In this report, evidence is presented which characterized inorganic phosphate as allosteric inhibitor of AMP nucleosidase from *A. vinelandii*.

From sonic extracts of *A. vinelandii*, the enzyme was purified by calcium phosphate gel treatment, sodium citrate fractionation, protamine sulfate treatment, heat treatment, ammonium sulfate fractionation and protamine sulfate precipitation and elution. The highest purification obtained was about 600-fold. The enzyme preparation catalyzed the hydrolysis of AMP to form adenine and ribose 5-phosphate and was free from ATPase, 5'-nucleotidase and adenylate kinase. The assay mixture contained AMP, ATP, $MgCl_2$ and enzyme preparation in K_2SO_4. The enzyme activity was measured by the formation of adenine which was extracted by *n*-butanol.

AMP nucleosidase purified by the above methods shows quite different kinetic and stability properties from those reported for previous preparations[1]. The enzyme rapidly loses activity in the absence of several anions, such as sulfate, succinate or citrate. In the presence of 0.25 M K_2SO_4, the enzyme remained stable at 4° or at room temperature, however in the frozen state, half of the activity was lost overnight. These anions appear to be essential for preserving the stability of the more highly purified fractions. The enzyme in solution is inactivated by heating at 60° for 10 min. The inactivation is prevented completely by the presence of 0.25 M K_2SO_4.

As demonstrated by HURWITZ, HEPPEL AND HORECKER[1], it was observed that the enzyme showed an absolute dependence on ATP for the catalytic hydrolysis of AMP. Starting with 0.8 μmole of AMP and ATP, 0.36 μmole of adenine was formed, however ATP was quantitatively recovered, indicating that this compound was not utilized during the course of the reaction.

The enzyme reaction in the presence of various concentrations of Mg^{2+}, AMP and ATP was studied kinetically. When the reaction velocity was plotted as a function of Mg^{2+} or AMP concentrations, a normal hyperbolic curve was obtained. However, plots of reaction velocity as a function of ATP concentrations yielded sigmoidal curves. In the presence of phosphate, the curves were more strongly sigmoidal in shape. The maximum reaction velocity in the presence and absence of various concentrations of phosphate was the same.

The regulation of the enzyme activity by ATP and phosphate was reversible. As shown in Fig. 1, the inhibition by phosphate can be always removed by ATP; the higher the concentration of phosphate, the higher was the concentration of ATP needed to reverse the inhibition and the same maximum velocity was observed. The apparent K_a for ATP varies with the phosphate concentrations, being 1.3 mM at

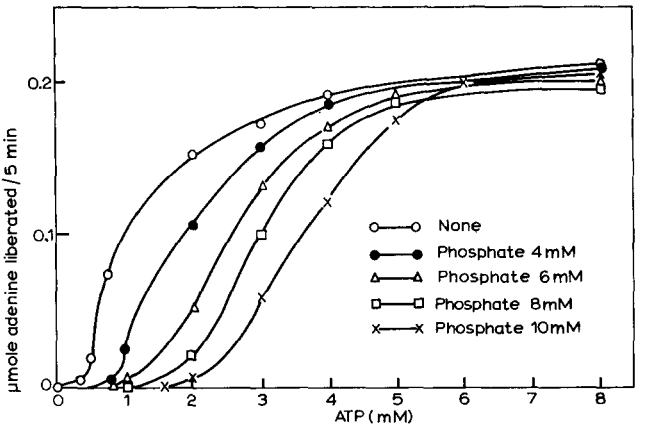


Fig. 1. Effects of inorganic phosphate on initial reaction rates of AMP nucleosidase and its reversal by ATP. The incubation mixture contained 12 µmoles of Tris–HCl (pH 7.95), 2 µmoles of $MgCl_2$, 0.8 µmole of AMP, 7 µmoles of K_2SO_4 and the indicated concentrations of ATP and phosphate in total volumes of 0.2 ml. The mixture was incubated for 5 min at 38°. The reaction was terminated by heating in boiling water for 1 min. After the addition of 0.8 ml of cold water, the adenine formed was extracted with 3 ml of *n*-butanol, and the absorbance at 260 mµ was measured.

0 mM phosphate, 2.2 mM at 4 mM phosphate, 2.7 mM at 6 mM phosphate, 3.3 mM at 8 mM phosphate and 3.9 mM at 10 mM phosphate. In the double reciprocal plot the curve approximates a parabola indicating kinetics of an order higher than one with respect to ATP. Phosphate increases Hill coefficients, which are employed for the quantitative representation of the cooperativity and calculated for ATP according to MONOD, CHANGEUX AND JACOB[2].

TABLE I

EFFECTS OF SALTS AND ACIDS ON ACTIVITY OF AMP NUCLEOSIDASE

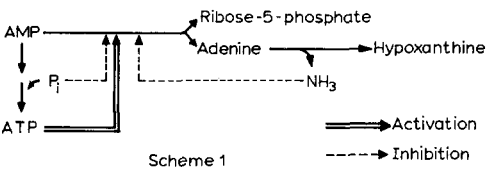
The reaction mixture contained 12 µmoles of Tris–HCl (pH 7.95), 2 µmoles of $MgCl_2$, 0.8 µmole of AMP, 7 µmoles of K_2SO_4, enzyme preparation and the indicated concentrations of ATP and various salts and acids. The enzyme assays were the same as those in Fig. 1.

Additions	*Concn. (mM)*	*Enzyme activity (µmole/5 min)*		*Concn. (mM)*	*Enzyme activity (µmole/5 min)*	
		ATP: 1 mM	*ATP: 4 mM*		*ATP: 0.75 mM*	*ATP: 4 mM*
None	—	0.107	0.193	—	0.087	0.201
Phosphate	10	0.000	0.092	7.5	0.000	0.170
Pyrophosphate	10	0.101	0.200	17.5	0.031	0.200
Sulfate	10*	0.099	0.192	70**	0.003	0.192
Sulfite	10	0.091	0.184	70	0.000	0.127
Nitrate	10	0.109	0.186	70	0.038	0.194
Nitrite	10	0.109	0.179	70	0.062	0.191
Chloride				70	0.073	0.225
Carbonate	10	0.099	0.179	70	0.007	0.140
Acetate				70	0.081	0.201
Succinate				70	0.104	0.197
Citrate	10	0.125	0.194	70	0.003	0.203

* The final concentration; 45 mM.
** The final concentration; 105 mM.

Table I represents that 10 mM phosphate ions most markedly inhibited the reaction at low concentrations of ATP, while other ions at equal concentrations did not inhibit AMP nucleosidase. At 70 mM, sulfate, pyrophosphate, sulfite, nitrate, nitrite, carbonate and citrate inhibited the reaction at low concentrations of ATP, and the inhibitory patterns were same as phosphate. The effects of several cations were also studied, and only the ammonium ion showed inhibitory effects. Other cations had no pronounced effect on the reaction.

A number of recent observations *in vivo* and *in vitro* indicate that AMP and ATP levels play an important role in the regulation of metabolism[3,4]. Crude extracts of *A. vinelandii* showed quite high AMP nucleosidase activity. It seems reasonable to assume that the physiological significance of our findings is connected with a homeostatic mechanism responsible for a balanced supply of AMP for the synthesis of ATP.



Scheme I

A possible mechanism is summarized in Scheme I. We can start with AMP which is hydrolyzed to adenine and ribose 5-phosphate. Adenine is further deaminated to hypoxanthine. The ammonia formed can inhibit the hydrolysis of AMP. On the other hand, ATP which is formed from AMP and inorganic phosphate activates the hydrolysis of AMP. Whereas phosphate inhibits this hydrolysis by changing the apparent K_a for ATP.

The activation–inhibition control of AMP nucleosidase shown in Scheme I, indicates a system in which the levels of AMP may be self-regulating. When an external stimulus is applied to the system, such as an increase in ammonia concentration, the inhibition of AMP nucleosidase is increased and subsequently the rate of formation of ATP is increased, and the levels of phosphate concentration are decreased. The increase in ATP concentration and the decrease in phosphate concentration then lead to a renewed activation of AMP nucleosidase.

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1 J. HURWITZ, L. A. HEPPEL AND B. L. HORECKER, *J. Biol. Chem.*, 226 (1957) 525.
2 J. MONOD, J-P. CHANGEUX AND F. JACOB, *J. Mol. Biol.*, 6 (1963) 306.
3 D. E. ATKINSON, *Science*, 150 (1965) 851.
4 W. GEVERS AND H. A. KREBS, *Biochem. J.*, 98 (1966) 720.

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