Vol. 138, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1986 Pages 261-267

DIADENOSINE TETRAPHOSPHATE ACTIVATES CYTOSOL 5'-NUCLEOTIDASE

Rosa Mª Pinto, José Canales, María A. Günther Sillero
and Antonio Sillero

Instituto de Investigaciones Biomédicas del C.S.I.C.,
Departamento de Bioquímica, Facultad de Medicina,
Universidad de Extremadura, Badajoz, Spain

Received June 2, 1986

SUMMARY: The rate of hydrolysis of IMP (0.5 mM) by cytosol 5'-nucleotidase from Artemia embryos was increased up to 7-fold by concentrations of around 10 μM diadenosine tetraphosphate (Ap_4A). Half maximal activation of the enzyme was accomplished with 5 μM Ap_4A. The K_m ($S_{0.5}$) values of the nucleotidase for IMP, GMP, AMP, XMP and CMP decreased about 10 fold in the presence of 10 μM Ap_4A. Maximum velocity of the enzyme was not affected by Ap_4A. ATP had been previously described as an activator of the enzyme. However, comparatively with Ap_4A, concentrations of ATP two orders of magnitude higher are needed to elicit similar effects on the enzyme. Preliminary results indicate that Ap_4A is also an activator of the cytosol 5'-nucleotidase from rat liver.

We recently found that Artemia cysts extracts contained a nucleotidase (1), similar to the cytosol 5'-nucleotidase (EC 3.1.3.5) previously reported in rat liver (2), chicken liver (3) and human malignant lymphocytes (4). The enzyme from all these sources cleaves IMP and GMP preferentially, whereas nucleoside 2' or 3'-phosphates are not substrates of the reaction. One relevant property of the enzyme is its activation by ATP (activation constant (Ka) around 0.6 mM). The regulatory meaning of this activation is obscure, as at the usual concentration of ATP in the cell (3 mM), the enzyme would be permanently activated by this nucleotide.

For some time we were looking for other different activators of the nucleotidase. Due both to our previous experience with dinucleoside polyphosphates (5,6,7) and to the fact that diadenosine tetraphosphate (diadenosine 5', 5'''-P^1,P^4-tetraphosphate or Ap_4A) can also be considered as a structural analogous of ATP, the effect of Ap_4A on the nucleotidase was tested and found to be a strong activator of the enzyme.

0006-291X/86 $1.50

Several laboratories are currently investigating the role of Ap_4A in cellular metabolism. This nucleotide has been mainly implicated in the regulation of DNA replication (8,9,10,11), in the onset of cellular stress (12), in platelets function (13,14) and in the control of interconversion of purine nucleotides (6). The activation of Artemia cytosol 5'-nucleotidase here described is ascribable to the last type of effects of Ap_4A.

MATERIALS AND METHODS

Artemia cysts were from Bio-Marine Research, Hawthorne, CA. Ap_4A was from Sigma Chemical Co (Lot 112F-7170). ATP was either from Boehringer (Lot 7294411/1) or from Sigma (Lot 104F-7410). The composition of the ATP samples was analyzed by high pressure liquid chromatography on a Nova Pack C_{18} column (3.9 x 150 mm) from Waters Assoc. and using an HP 1090 chromatograph. Nucleotides were eluted with a linear gradient of sodium phosphate (5-100 mM), pH 7.0 in 5 mM tetrabutylammonium and 20% methanol. The sample from Boehringer contained 97.8% ATP, 1.2% ADP, 0.3% AMP and 0.05% Ap_4A. The sample from Sigma contained 99.0% ATP, 0.7% ADP, 0.06% AMP and less than 0.005% Ap_4A.

The enzyme was prepared from Artemia cysts free of nonspecific phosphatase activity as previously described (1). The method implied ammonium sulphate fractionation (0.25-0.45 saturation), chromatography on Sephacryl S-300, chromatography on DEAE-cellulose and affinity chromatography on Cibacron blue F3G-A Sepharose 4B. The specific activity of the purified enzyme determined at 2.5 mM GMP concentration in the standard assay (see below), lacking effectors, was 0.8 µmol/min/mg protein. The experiments here described were performed with this preparation.

The 5'-nucleotidase activity was followed through evaluation of the free inorganic phosphate (Pi) liberated from the corresponding substrate. The reaction mixture contained, in a final volume of 0.2 ml, the following components: 100 mM Imidazole-HCl buffer, pH 7.0, 7.5 mM $MgCl_2$, 0.1% bovine serum albumin, 0.2 M NaCl, 1 µg enzyme protein, substrate, and effector as indicated. Controls without enzyme or substrate were run in parallel. After incubation at 37ºC for 30 min the reaction was stopped by addition of 1.45 ml of a solution prepared shortly before use by mixing 6 vol of 3.4 mM ammonium molybdate in 1 N H_2SO_4, 1 vol of 10% (wt/vol) ascorbic acid and 1 vol of 3.7% (wt/vol) sodium dodecyl sulphate. The samples were incubated for 20 min at 45ºC and A_{820} was determined after cooling at room temperature. The amount of Pi formed was calculated from standard curves. In our conditions, 1 nmole of Pi rendered a net reading of 0.015-0.020 A_{820} units, which was stable for at least 2 h.

RESULTS AND DISCUSSION

For the reasons shown above, we tested the effect of Ap_4A on the cytosol 5'-nucleotidase from Artemia, using IMP as substrate of the reaction. The concentration of Ap_4A was raised from 0 to 200 µM. The activation of the enzyme by Ap_4A followed a rather hyperbolic activation curve (interaction coefficient (n")=0.85) (Fig.1). In the same experimental conditions, and as

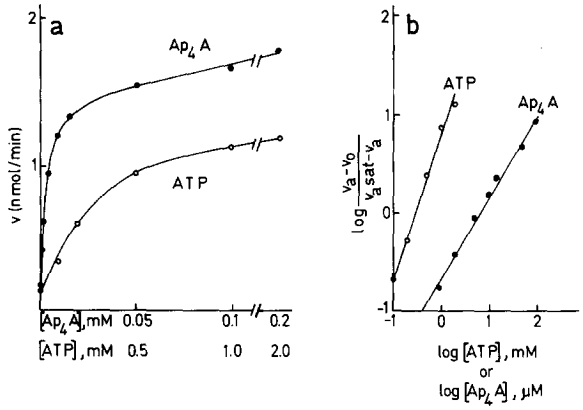

Fig. 1 Activation of cytosol 5'-nucleotidase by Ap_4A and ATP. a) Reaction mixture as described in the text containing 0.5 mM IMP as substrate and activator (Ap_4A or ATP) as indicated. b) Hill plots and interaction coefficients (n") of the data from (a) were determined as described (3).

a comparison, ATP was also tested as activator of the enzyme in a range of concentrations from 0 to 2mM. In this case, the activation curve was slightly sigmoidal (n"=1.4). Half maximal activation of the enzyme, by Ap_4A and ATP, was accomplished, as calculated from Fig. 1b, at concentrations of 5 and 300 µM, respectively. This figure for ATP is similar to that previously described for the enzyme from Artemia (1).

The effect on the nucleotidase of fixed amounts of either Ap_4A (10µM) or ATP (1mM), in the presence of variable concentrations of IMP is presented in Fig. 2. Both Ap_4A and ATP activated the hydrolysis of IMP by decreasing

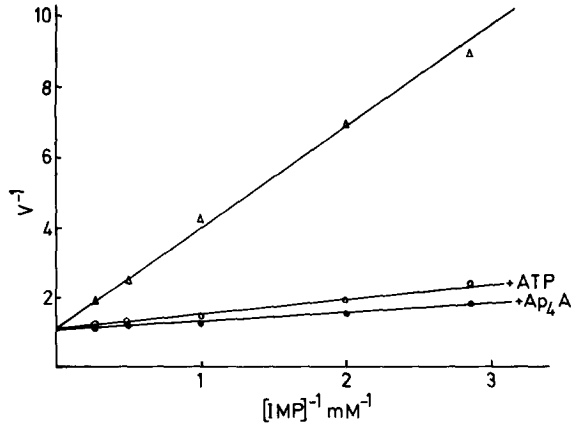

Fig. 2 Effect of Ap_4A and ATP on the cytosol 5'-nucleotidase activity as a function of IMP concentration. Reaction mixture as described in the text. Δ , no addition; o, 1mM ATP; •, 10 µM Ap_4A.

TABLE I

Effect of Ap_4A and ATP on the kinetic parameters of various substrates of cytosol 5'-nucleotidase

Substrate	No addition			10 μM Ap_4A		1mM ATP	
	V *	K_m ($S_{0.5}$) * mM	n_H *	K_m ($S_{0.5}$) mM	n_H	K_m ($S_{0.5}$) mM	n_H
IMP	1.0	2.8	1.0	0.3	1.0	0.4	1.0
GMP	1.2	3.5	1.0	0.3	1.0	0.3	1.0
XMP	1.1	9	1.6	1.3	1.2	2	1.3
AMP	1.0	21	1.9	9	1.1	10	1.2
CMP	0.8	175	1.1	10	2.3	10	1.9

*Relative values of the maximum reaction velocity (V) for each substrate were estimated from Lineweaver-Burk or Hill plots; n_H refers to the Hill coefficient; $S_{0.5}$ is the substrate concentration at half saturation of the enzyme.

the K_m value (2.8 mM) of the enzyme towards its substrate. This value decreased to 0.3 and 0.4 mM in the presence of Ap_4A and ATP, respectively.

We also tested the effect of both Ap_4A (10 μM) and ATP (1 mM) on the hydrolysis of several nucleoside 5'-monophosphates (Table 1). In every case, both effectors decreased the apparent K_m values of the enzyme, without affecting maximum velocity. Sigmoidal kinetics were observed with XMP and AMP, which in the presence of the effectors, became rather hyperbolic. On the contrary, CMP was hydrolyzed following an hyperbolic kinetics which was transformed in sigmoidal following activation by either Ap_4A or ATP. The significance of these changes are not known at present. It is worthy to recall that in the presence of 10 μM Ap_4A, the apparent K_m values of the enzyme for the different nucleotides, and in particularly for IMP and GMP, approaches the range of their physiological concentrations.

As the presence of Ap_4A had been described in some preparations of ATP (15), we investigate whether the Ap_4A present in the samples of ATP, rather than ATP itself, could be responsible for the activation of the nucleotidase. In this regard, 6.8 fold activation of the enzyme was obtained with 1 mM ATP (from Boehringer). In those conditions, the contaminant Ap_4A is present in the reaction mixture at a concentration of around 0.5 μM ,

which would produce only 1.7 fold activation of the nucleotidase (Fig. 1). Additionally the experiment of Fig. 1 was repeated with identical results, using ATP (from Sigma) containing less than 0.005% Ap_4A. From here, we conclude that both Ap_4A and ATP are activators of the reaction but with Ka values differing in two orders of magnitude. Probably, ATP is acting as a structural analogous of the true activator Ap_4A.

Diadenosine tetraphosphate is present in Artemia cysts at a concentration of around 2 μM; it decreases to 25 nM after decapsulation and storage of the embryos in saturated NaCl during 12 days. When development is reiniciated the Ap_4A content of the decapsulated embryos undergoes a rapid increase, reaching a concentration of around 3.3 μM after 10 hours of incubation, at a time when the synthesis of DNA is initiated (16). These peculiar experimental conditions were chosen to better follow a possible correlation between the amount of Ap_4A and the rate of synthesis of DNA during development of the cysts. To our knowledge, there is no information on the level of Ap_4A attained at the time of emergence when normal (capsulated cysts) are incubated. From the result presented in (16) it may be assumed that the concentration of Ap_4A either remains unchanged (at around 2-3 μM) or increases during the first 12 hours of incubation of the cysts. During this period there is a rapid use of the stored Gp_4G to serve as a source of both adenine and guanine nucleotides (17,18).

Referring to the role of Ap_4A in the interconversion of purine nucleotides, the following points could be raised. a) Ap_4A is an activator (Ka = 5 μM) of AMP deaminase from rat muscle (6); b) maximum velocity of this enzyme in Artemia cysts is very low (around 0.25 mU/g (18), compared to that of the following enzymes acting in the interconversion of purine nucleotides, with the following V values: adenylosuccinate synthetase (EC 6.3.4.4) 50 mU/g (19); adenylosuccinate lyase (EC 4.3.2.2) 180 mU/g (20); IMP dehydrogenase (EC 1.2.1.14) 5 mU/g (21), and adenosine deaminase (EC 3.5.4.4) 140 mU/g (22). Nevertheless, the activation of AMP deaminase by Ap_4A would favor the synthesis of IMP (and probably also of GMP) from AMP. c) The nucleotidase activated by Ap_4A, here reported, cleaves IMP and GMP preferentially and, to a much lesser extent, AMP. The degree of activation of the enzyme by Ap_4A seems to be directly related to the amount of Ap_4A present in the cysts ((16) and Fig. 1). The following fac-

tors, among others, can also influence the actual velocity of the enzyme *in vivo*: concentration of ATP (around 1-2 mM in *Artemia* (23); concentration of inorganic phosphate (a known inhibitor of the enzyme (2,3,4)) ; concentration of Mg^{2+} (1,2,3,4), and the possibility of compartmentation and/or binding of substrates and effectors to cellular macromolecules (24). For us it is difficult to further speculate on that, given the complex array of reactions and effectors comprising the purine interconversion pathway.

Finally, preliminary experiments with cytosol 5'-nucleotidase from rat liver, obtained and assayed as described in (2), showed that half maximal activation of the hydrolysis of IMP (0.25 mM) was accomplished in the presence of 20 μM Ap_4A or 300 μM ATP.

ACKNOWLEDGEMENT

This investigation was supported by the Comisión Asesora de Investigación Científica y Técnica (grant 1159/84). The authors acknowledge Pablo Lozano for typing the manuscript.

REFERENCES

1. Pinto, R.M., Canales, J., Faraldo, A., Sillero, A., and Sillero, M.A.G. Comp. Biochem. Physiol. In press.
2. Van den Berghe, G., Van Pottelsberghe, C., and Hers, H-G. (1977) Biochem. J. 162, 611-616.
3. Itoh, R., Usami, C., Nishino, T., and Tsushima, K. (1978) Biochim. Biophys. Acta 526, 154-162.
4. Carson, D.A., and Wasson, D.B. (1982) Cancer Res. 42, 4321-4324.
5. Renart, M.F., Renart, J., Sillero, M.A.G., and Sillero, A. (1976) Biochemistry 15, 4962-4966.
6. Fernández, A., Costas, M.J., Sillero, M.A.G., and Sillero, A. (1984) Biochem. Biophys. Res. Commun. 121, 155-161.
7. Costas, M.J., Cameselle, J.C., and Sillero, A. (1986) J. Biol. Chem. 261, 2064-2067.
8. Rapaport, E., and Zamecnik, P.C. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3984-3988.
9. Weinmann-Dorsch, C., Hedl, A., Grummt, I., Albert, W., Ferdinand, F.J., Friis, R.R., Pierron, G., Moll, W., and Grummt, F. (1984) Eur. J. Biochem. 138, 179-185.
10. Grummt, F., Waltl, G., Jantzen, H.M., Hamprecht, K., Huebscher, U., and Kuenzle, C.C. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 6081-6085.
11. Baril, E., Bonin, P., Burstein, D., Mara, K., and Zamecnik, P. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 4931-4935.
12. Bochner, B.R., Lee, P.C., Wilson, S.W., Cutler, C.W., and Ames, B.N. (1984) Cell 37, 225-232.

13. Flodgaard, H., and Klenow, H. (1982) Biochem. J. 208, 737-742.
14. Lüthje, J., and Ogilvie, A. (1983) Biochem. Biophys. Res. Commun. 115, 253-260.
15. Zamecnik, P. (1984) Anal. Biochem. 134, 1-10.
16. Mc Lennan, A.G., and Prescott, M. (1984) Nucleic Acids Research 12, 1609-1619.
17. Finamore, F.J., and Clegg, J.S. (1969) in The Cell Cycle (Padilla, G.M., Whitson, G.L., and Cameron, I.L. eds.) pp. 249-278, Academic Press, New York.
18. Sillero, A., and Sillero, M.A.G. (1986) Proceedings of the II International Symposium on the Brine Shrimp Artemia. Antwerpen, Belgium. In press.
19. Faraldo, A., Pinto, R.M., Canales, J., Fernández, A., Sillero, A., and Sillero, M.A.G. (1983) Comp. Biochem. Physiol. 75B, 221-226.
20. Pinto, R.M., Faraldo, A., Fernández, A., Canales, J., Sillero, A., and Sillero, M.A.G. (1983) J. Biol. Chem. 258, 12513-12519.
21. Canales, J., Fernández, A., Faraldo, A., Pinto, R.M., Sillero, A. and Sillero, M.A.G. (1985) Comp. Biochem. Physiol. 81B, 837-844.
22. Fernández, A., Canales, J., Pinto, R.M., Faraldo, A., Sillero, M.A.G., and Sillero, A. (1984) Comp. Biochem. Physiol. 78B, 481-484.
23. Warner, A.H., and Finamore, F.J. (1967) J. Biol. Chem. 242, 1933-1937.
24. Sols, A., and Marco, R. (1970) in Current Topics in Cellular Regulation (Horecker, B.L., and Stadtman, E.R., eds.) pp 227-273, Vol. 2, Academic Press, New York.