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EFFECT OF ATP ANALOGUES ON T_4 POLYNUCLEOTIDE LIGASE

A.J. Raae and K. Kleppe

Department of Biochemistry, University of Bergen
Bergen, Norway.

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SUMMARY: The influence of three ATP analogues, α, β-methylene adenosine 5'-triphosphate, β, γ-methylene adenosine 5'-triphosphate and β, γ-imidoadenosine 5'-triphosphate on the activity of T_4 polynucleotide ligase has been investigated. Only β, γ-imidoadenosine 5'-triphosphate was active in the joining reaction; the rate of catalysis being slightly less than with ATP. The apparent K_m for this ATP analogue was estimated to be 3.3 μM versus 1.4 μM for ATP. With β, γ-imidoadenosine 5'-triphosphate a sharp pH optimum of pH 8.8 was observed. None of the ATP analogues gave any inhibition of activity when mixed with ATP.

INTRODUCTION - T_4 polynucleotide ligase (EC 6.5.1.1) as well as polynucleotide ligase from mammalian cells use ATP as a cofactor in contrast to the enzyme from E. coli which requires NAD (1). The polynucleotide ligases react according to a ping-pong mechanism where the cofactor binds covalently to the enzyme through a phosphate-oxygen-nitrogen bond between the nucleoside monophosphate and an ε-amino lysine group in the enzyme thus forming an AMP-enzyme complex and PP_i or NMN. This complex then reacts with a 5'-phosphate group in a nick in a double-stranded DNA resulting in a concomitant nick closure and release of enzyme and AMP (1-3).

In the case of T_4 polynucleotide ligase a previous study has shown that of a number of nucleoside 5'-triphosphates tested only ATP could act as a cofactor(4). On the other hand,

Abbreviations: AMP-CPP, α, β-methylene adenosine 5'-triphosphate, AMP-PCP β, γ-methylene adenosine 5'-triphosphate and AMP-PNP, β,γ-imidoadenosine 5'-triphosphate.

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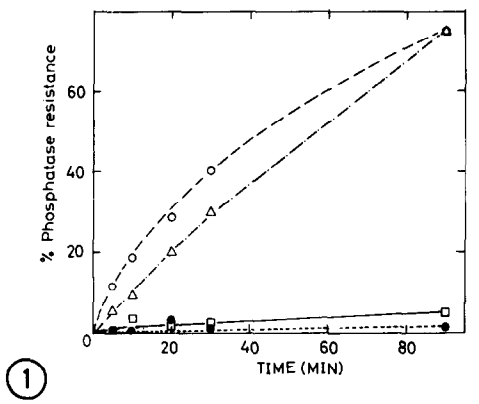
dATP was found to be a competitive inhibitor. In the present work we have examined the cofactor specificity of T_4 polynucleotide ligase using the three ATP analogues α, β-methylene adenosine 5'-triphosphate (AMP-CPP), β, γ-methylene adenosine 5'-triphosphate (AMP-PCP) and β, γ-imidoadenosine 5'-triphosphate (AMP-PNP). These analogues have yielded valuable information concerning the specificity and the nature of the active sites of many enzymes (5).

MATERIALS AND METHODS - Oligo $(dT)_{10}$ and $(dA)_n$ were obtained from P.L. Biochemicals, Inc. The ATP analogues were from Sigma Chemical Company. Radioactive γ-[^{32}P]-ATP, specific activity 2000 Ci/mmole, was purchased from The Radiochemical Centre, Amersham. Radioactive 5'-[^{32}P]-$(dT)_{10}$ was prepared as previously described (6).

T_4 polynucleotide ligase was prepared according to the procedure of Panet et al. (7). Bacterial alkaline phosphatase was a product of Worthington Biochemical Company.

The standard assay contained in a total volume of 0.1 ml: 60 mM Tris-HCl, pH 8.0, 6.6 mM $MgCl_2$, 10 mM dithiothreitol. The concentrations of $(dA)_n \cdot (dT)_{10}$ and ATP or ATP analogues are given in the Legend to each figure. Product analysis was carried out as previously described (8).

RESULTS - Figure 1 shows the time course of joining of 5'-[^{32}P]-$(dT)_{10}$ on the $(dA)_n$ template in the presence of ATP or ATP analogues. Of the ATP analogues tested only AMP-PNP was active. The rate of joining with this cofactor was found to be slightly less than that with ATP. Virtually no joining was observed with the two methylene derivatives, AMP-CPP and AMP-PCP. The apparent K_m for AMP-PNP was determined at a fixed concentration of $(dA)_n \cdot (dT)_{10}$, 1.17 μM (phosphate), and observed to be 3.3 μM versus 1.4 μM for ATP. The apparent V_{max} was found to be identical in the two cases. Neither of the analogues gave any inhibition of activity when mixed with ATP. The size of the products made in the presence of AMP-PNP and ATP was analyzed using the polyacrylamide gel method previously described (8).



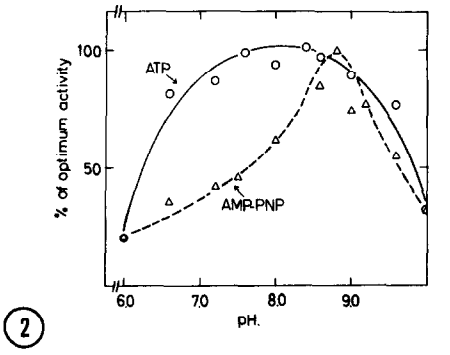


Figure 1. Time course of joining of $(dT)_{10}$ by T_4 polynucleotide ligase in the presence of various cofactors. The concentrations of $(dA)_n \cdot (dT)_{10}$ and cofactors were 2.35 µM (phosphate) and 100 µM, respectively. The reactions were started by additions of 0.6 units of enzyme. The total was volume 200 µl. For other details, see Methods. o—o ATP, △--△ AMP-PNP, □ ····□ AMP-CPP ●·····● AMP-PCP.

Figure 2. Influence of pH on the activity of T_4 polynucleotide ligase in the presence of ATP and AMP-PNP. The concentration of $(dA)_n \cdot (dT)_{10}$ was 0.94 µM (phosphate) and that of the cofactors 100 µM and 0.25 units of enzyme was employed in a total volume of 100 µl. The buffers used were: pH 6.0 and 6.6 Tris-maleate, pH 7.2-9.2 Tris-HCl and pH 9.6-10 glycin-NaOH. For other details, see Methods.

Almost identical molecular weight distributions were seen in the two cases (results not shown).

The influence of pH on the joining reaction using ATP and AMP-PNP was also investigated (Figure 2). With ATP a fairly broad pH optimum was seen, the maximum activity being from pH 7.5 to pH 8.5. AMP-PNP, on the other hand, gave a sharp pH optimum curve, the maximum activity being at pH 8.8.

DISCUSSION - The present work shows that the ATP analogue AMP-PNP can be used by T_4 polynucleotide ligase for joining with almost the same efficiency as ATP. None of the methylene analogues, however, were found to be neither active nor were they inhibitors. This suggests that the introduction of a methylene instead

of an oxygen bridge between the β- and γ-phosphate groups affects the binding of the cofactor to the enzyme. The net charge of the molecule is changed and this certainly might influence the binding of Mg^{2+}. Carbon-phosphorous bonds are also known to be more stable than the normal carbon-oxygen bond (5). In the case of AMP-PNP the nitrogen bridge resembles more the oxygen group found in ATP with regard to bond angles and electrondensity. The finding that the apparent K_m for ATP and AMP-PNP are similar suggests that the introduction of nitrogen only slightly affects the binding of the cofactor. The different pH optimum curves found may be due to the different pK_a values for the -OH group on the terminal phosphate residue of the two cofactors, i.e. pK_a= 7.0 for ATP and 7.7 - 7.9 for AMP-PNP (5). The pH optimum curve for AMP-PNP observed in the present work resembles closely that seen for adenyl cyclase with this ATP analogue (9).

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REFERENCES

1. Lehman, I.R. (1974) Science 186, 791 - 797.
2. Modrich,P. and Lehman, I.R. (1973) J. Biol. Chem., 248, 7502-7511.
3. Raae,A.J., Kleppe,R.K. and Kleppe, K. (1975) Eur. J. Biochem.,60, 437-443.
4. Weiss,B., Jacquemin-Sablon, A., Live,T.R., Fareed, G. and Richardson,C.C. (1968) J. Biol. Chem., 243, 4543-4555.
5. Yount,R.G. (1975) Advances in Enzymology, 43, 1-56.
6. Weiss, B. Live, T.R. and Richardson, C.C. (1968) J. Biol. Chem.,243, 4530-4542.
7. Panet, A. van de Sande, J.H., Loewen, P.C., Khorana, H.G., Raae, A.J., Lillehaug, J.R. and Kleppe, K. (1973) Biochemistry 12,5045-5050.
8. Raae,A.J., Lillehaug, J.R., Kleppe, R.K. and Kleppe, K. (1975) Nucleic Acids Research 2, 423-429.
9. Maguire,M.E. and Gilman,A.G. (1974) Biochim. Biophys. Acta 358, 154-163.