THE ENZYMATIC SYNTHESIS OF ATP ANALOGUES

by

Doug Johnson*, Malcolm MacCoss**, and Suree Narindrasorasak*

Department of Biochemistry* and Chemistry**

University of Alberta

Edmonton, Alberta

T6G 2H7, Canada

Received May 6,1976

ABSTRACT: The enzymatic synthesis of selected adenosine 5'-triphosphate analogues from their respective 5'-monosphosphates has been achieved using phosphoenolpyruvate synthetase. Adenosine 5'-monophosphate analogues altered at positions 1, 6, 7, 8 or 9 of the purine ring, or at the ribose 2'- or 3'-positions are substrates with 30% conversion to the nucleoside 5'-triphosphate.

INTRODUCTION: Phosphoenolpyruvate synthetase catalyzes the phosphorylation of pyruvate from the β-phosphate of ATP[1] via an enzyme-phosphate intermediate (Cooper and Kornberg, 1965 and 1967).

$$\text{ATP} + \text{pyruvate} \rightleftharpoons \text{AMP} + P_i + \text{phosphoenolpyruvate}$$

We have used the reverse reaction to synthesize a variety of ATP analogues modified in the sugar or purine ring moieties without any need for the protection of reactive groups. Also radiolabelling of the β-or γ-phosphate positions of these analogues is possible.

[1] NMP refers to any nucleoside 5'-monophosphate, NTP to the 5'-triphosphate. rTu and araA are abbreviations for tubercidin and 9-β-D-arabinofuranosyladenine (structures of respective 5'-monophosphates given in figure 1). Abbreviations for methylated adenosines are taken from the Handbook of Biochemistry, Chemical Rubber Co.

From the variety of analogues tested we can infer which positions on AMP are involved in substrate recognition.

MATERIALS AND METHODS:

Nucleosides and nucleotides were purchased from the following suppliers: Calbiochem (rAMP, rCMP, rCTP, rGTP); P.L. Biochemicals (rATP, dATP, 1-MeAMP, 6-MeAMP, rGTP, rTu); Pfanstiehl, Germany (araA); Raylo, Canada (dAMP); Sigma (rIMP, rITP). Nucleosides were chemically phosphorylated to their 5'-monophosphates by the method of Yoshikawa et al. (1969) and purified by chromatography on Dowex 1x2 (formate) developed with a linear gradient of formic acid (M. J. Robins and M. MacCoss, to be published). Adenosine derivatives methylated at the 2'- or 3'-positions were prepared by methods described by Robins et al. (1974). 6,6-Me_2adenosine was prepared from the 6-chloropurine riboside with anhydrous dimethylamine (see Robins et al. (1976) for analogous preparation). AMP^{OX-RED} was prepared by the method of Smrt et al. (1975) with minor modifications.

Phosphoenolpyruvate synthetase, purified from lactate-grown Escherichia Coli by the method of Berman and Cohn (1970) was judged 90% pure by polyacrylamide gel electrophoresis. The assay conditions for the forward reaction were 0.1 M TrisHCl pH 8.0, 10 mM $MgCl_2$, 2 mM pyruvate, 1 mM nucleoside 5'-triphosphate and 15 µg/ml enzyme (specific activity 19 U/mg in forward direction) and for the reverse reaction 0.1 M KP_i pH 6.8, 20 mM $MgCl_2$, 10 mM phosphoenolpyruvate (Sigma), 10 mM nucleoside 5'-monophosphate and 130 µg/ml enzyme. Both reactions were incubated at 30°C. Synthesis of NTP was monitored for up to 16 hours by thin layer chromatography on cellulose sheets (Eastman #13255) with isobutyric acid/ammonium hydroxide/H_2O (60/1/40 by volume) as solvent and on P.E.I. cellulose sheets (Baker) with 1.6 M LiCl as solvent (K. Randerath and E. Randerath, 1967).

Reaction products were separated by DEAE Cellulose chromatography with a linear gradient 0 - 0.6 M in triethylammonium bicarbonate pH 7.5 (Smith and Khorana, 1963) and concentrated by lyophilization.

RNA polymerase reactions were performed with E. Coli RNA polymerase (Burgess, 1969) in a reaction mixture containing 50 mM TrisHCl pH 8.0, 10 mM $MgCl_2$, 50 µg/ml dTC·dGA, 0.25 mM ^{14}C-GTP (9,200 cpm/nmole), 0.25 mM rATP analogue, 200 µg/ml RNA polymerase. This polyribopurine synthesis can be distinguished from contaminating poly rG synthesis by observation of the kinetics of synthesis.

RESULTS AND DISCUSSION:

Figure 1 gives the structures of the less common AMP analogues and the ability of various analogues to act as phosphoenolpyruvate synthetase substrates for both the forward and reverse reactions is summarized in Table 1. As expected, at no time was ribonucleoside 5'-diphosphate synthesis observed.

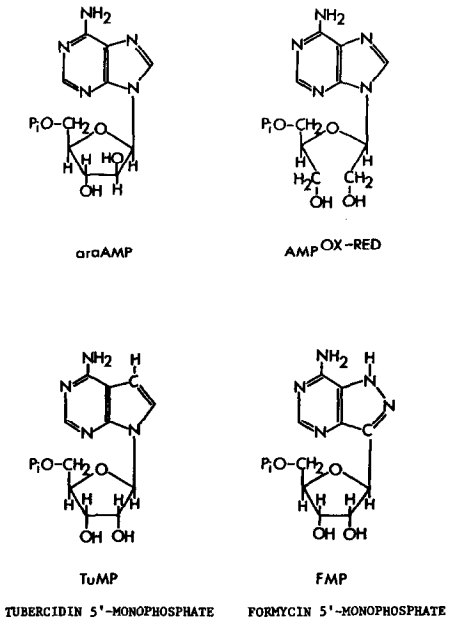

Figure 1. Structures of various AMP analogues.

In the reverse direction dAMP, araAMP, 3'-MeAMP, 6-MeAMP, TuMP, FMP and AMP can be phosphorylated with ≈ 30% recovery of the NTP from a DEAE Cellulose column. 2'-MeAMP is converted to the triphosphate with about 15% recovery and 1-MeAMP is phosphorylated with 5-17% recovery. This variability is probably due to the insolubility of this analogue under the reaction conditions. 6,6-Me_2AMP, AMP^{OX-RED} and αAMP (the 1'-epimer of 5'-AMP) are not substrates and 6,2'-Me_2AMP is phosphorylated to a very low extent. The 6-keto nucleotides IMP and GMP and the 4-amino pyrimidine nucleotide CMP cannot substitute for AMP in the reverse reaction nor can the corresponding 5'-triphosphates substitute for ATP in the forward direction.

Although dATP, 6-MeATP, araATP, TuTUP and FTP are ultimately synthesized to the same extent as ATP, their initial rates for the forward reaction are very different. Compared to ATP these range

from 0.23 for 6-MeATP to 0.71 for TuTP. These NTPs were also checked as substrates for E. Coli RNA polymerase and each was incorporated

TABLE 1. Substrate Recognition by Phosphoenolpyruvate Synthetase

COMPOUND	REVERSE REACTION: Isolation of NTP by DEAE Cellulose Chromatography[1,2]	FORWARD REACTION: Initial Reaction Rate of Analogue Compared to ATP[1]
AMP/ATP	34	1.00
dAMP/dATP	33	0.33
2'-MeAMP	15	ND[4]
3'-MeAMP	24	ND
1-MeAMP	5-17[3]	ND
6-MeAMP/6-MeATP	34	0.23
6,6-Me_2AMP	0	ND
6,2'-Me_2AMP	1	ND
araAMP/araATP	28	0.31
αAMP	0	ND
AMP^{OX-RED}	0	ND
TuMP/TuTP	29	0.71
FMP/FTP	28	0.42
GMP/GTP	0	0
IMP/ITP	0	0
CMP/CTP	0	0

1. as described in Materials and Methods
2. percent of ultraviolet absorbing material (λ=260nm) eluting as NTP
3. variable, see text
4. ND = not determined

into polyribopurine (data not shown). As expected araATP showed less than 3% synthesis compared to ATP (Cohen 1966).

Several conclusions can be drawn from an evaluation of the substrate specificity. Firstly, the 2'- or 3'-positions appear unimportant for recognition since AMP, dAMP, araAMP, 2'-MeAMP and 3'-MeAMP are all substrates. The lower yield of 2'-MeATP may reflect a steric problem and this would preculude the phosphorylation of AMP analogues by this method if the 2' substituent is even bulkier. Removal of the structural rigidity of the ribose ring as in AMP^{OX-RED} results in a loss of substrate recognition. As expected, the configuration at the anomeric centre is also important since αAMP is not a substrate. With regard to the heterocyclic base, variations in the imidazole ring are tolerated since both TuMP and FMP are substrates. Methylation of the 1-position does not result in a loss of recognition. If the 6-amino is monomethylated, recognition is not affected but dimethylation at the 6-position results in a complete lack of synthesis. Replacement of the 6-amino by oxygen, as in IMP or GMP, results in no synthesis of the corresponding triphosphate. These last results indicate that of the various positions tested only the 6-amino is directly involved in substrate recognition, presumably forming a hydrogen bond to the enzyme. Since the 4-amino pyrimidine CMP is not phosphorylated the purine ring moiety is necessary.

In comparison to chemical methods of NTP synthesis (Smith and Khorana, 1963) this enzymatic synthesis is limited both by substrate recognition and reaction scale since it is not suitable for the routine synthesis of mmoles of NTP without a large enzyme supply (yield from 1 kilogram of cells is about 200 mgs.). However it is convenient for the synthesis of 25-50 μmoles of a particular NTP without the need for blocking agents and requires no "seed" NTP as

does a previously described enzymatic synthesis of FTP (Ward, Cerami and Reich, 1969). Since the β-phosphate is derived from phosphoenolpyruvate and the γ-phosphate from buffer, specific radiolabelling should also be possible.

ACKNOWLEDGEMENTS:

The authors wish to thank Mr. G. G. Cross for the preparation of AMP^{OX-RED} and Drs. W. A. Bridger, A. R. Morgan and M. J. Robins for discussions. This work was supported by Medical Research Council of Canada grants to W.A.B. and A.R.M. and grants from the National Cancer Institute of Canada and National Research Council (A5890) to M.J.R.

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