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# INHIBITION OF ATP:L-METHIONINE *S*-ADENOSYLTRANSFERASE OF BAKERS' YEAST BY STRUCTURAL ANALOGUES OF ATP

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SUMMARY

Highly purified preparations of ATP:L-methionine *S*-adenosyltransferase (EC 2.5.1.6) of bakers' yeast display a virtually completely specific requirement for ATP. Kinetics analyses of the binding of a number of structural analogues and components of ATP have permitted assessment of the importance of various regions of the ATP molecule for "binding" and for "recognition" of the nucleotide. Adenosine or deoxyadenosine have almost no affinity for the enzyme, their mono- and diphosphates are very weak inhibitors competitive with respect to ATP. Various nucleoside triphosphates are more powerful inhibitors competitive with respect to ATP and noncompetitive with respect to L-methionine.

Tripolyphosphate is an excellent inhibitor ($K_i$ = 0.05 mM) and tetraphosphate also inhibits the enzyme ($K_i$ = 0.18 mM). The following phosphonate analogues of ATP are powerful inhibitors: $\alpha,\beta$-methylene-ATP ($K_i$ = 0.55 mM); $\beta,\gamma$-methylene-ATP ($K_i$ = 2.19 mM) and $\alpha,\beta$-methylene-adenosine tetraphosphate ($K_i$ = 0.17 mM). All of these phosphonates compete with ATP and the $K_i$ values refer to this competition when L-methionine approaches saturation. It is concluded from kinetic studies with adenosine and its analogues, that the triphosphate moiety of ATP is of primary importance for the binding of the substrate to the enzyme, while the adenosyl group of ATP is of primary importance for the substrate recognition, but with low affinity for the enzyme. Kinetic patterns obtained with analogues of ATP as inhibitors are consistent with the previous proposition that the ATP:L-methionine *S*-adenosyltransferase reaction involves a random-ordered bi-mechanism. The results of the present studies also suggest that the adenosyl group remains enzyme-bound prior to its binding to the sulfur atom of L-methionine, and that the triphosphate site-directed recognition of the adenosyl moiety enables ATP to gain virtually absolute substrate specificity.

Abbreviations: Ado-Met, (−)-*S*-adenosyl-L-methionine; adenosyltransferase, ATP:L-methionine *S*-adenosyltransferase (EC 2.5.1.6); $PPP_i$, tripolyphosphate; $PPPP_i$, tetrapolyphosphate.

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## INTRODUCTION

This report deals with the nucleotide specificity of the ATP:L-methionine *S*-adenosyltransferase (EC 2.5.1.6) (adenosyltransferase) reaction of bakers' yeast. A kinetic analysis of the behavior of ATP, its analogues, and its structural components led to an understanding of the importance of the various moieties of the ATP molecule in substrate recognition and binding to the enzyme.

Earlier studies on adenosyltransferase from various sources have established that the formation of *S*-adenosyl-L-methionine (Ado-Met) from ATP and L-methionine requires also the presence of mono- and divalent cationic activators[1], preferentially $K^+$ and $Mg^{2+}$, and that the following stoichiometry obtains[2]:

$$\text{ATP} + \text{L-methionine} \xrightarrow{K^+,\, Mg^{2+}} (-)\text{-}S\text{-adenosyl-L-methionine} + PP_i + P_i$$

The complete dephosphorylation of ATP occurs in an asymmetric manner such that pyrophosphate ($PP_i$) and inorganic orthophosphate ($P_i$) are derived from the innermost ($\alpha$ and $\beta$) and the terminal ($\gamma$) phosphate groups of ATP, respectively. The non-random cleavage of the phosphate groups of ATP suggested that enzyme-bound tripolyphosphate ($PPP_i$) was an obligatory intermediate, and this view is supported by binding experiments[3,4] and by kinetic studies[5,6]. Furthermore, Mudd[3] and Mudd and Mann[7] have demonstrated that this enzyme also hydrolyzes exogenous tripolyphosphate and that activity is specifically and profoundly stimulated by low levels of Ado-Met. The hydrolysis of enzyme-bound $PPP_i$ appears to be the rate-limiting step of the overall reaction when endogenous levels of Ado-Met are low[6]. Consequently, the Lineweaver–Burk plots of velocity with respect to substrate concentration exhibit downward inflexions when the concentration of either L-methionine or ATP is varied[6,8,9]. To minimize the deviations from classical steady-state enzyme kinetics, relatively narrow ranges of substrate concentrations were used in the present studies.

Limited information is available on the nucleotide specificity of the adenosyltransferase, although thus far only ATP appears to be able to function as a substrate[2], with the possible exception of very slow reactivity of UTP for the mouse liver enzyme[10]. We[6] have shown that GTP inhibits the yeast adenosyltransferase in competition with ATP and in a noncompetitive manner with respect to L-methionine. The accumulated knowledge of the mechanism of action of adenosyltransferase and its apparent total specificity for ATP raise questions whether the structural integrity of this nucleoside triphosphate is essential for substrate function and whether analogues and components of this molecule can act as inhibitors. We have further addressed ourselves to the question whether structural modifications of one moiety rather than another of ATP is likely to produce more potent inhibitors.

## EXPERIMENTAL PROCEDURE

### *Materials*

The adenosyltransferase was purified from autolysates of bakers' yeast to a final specific activity of about 30 $\mu$moles of Ado-Met formed per mg of protein in 30 min at 37 °C under specified conditions[6]. The sources of all reagents have been

previously described[6,8,9]. L-[*Methyl*-$^{14}$C]methionine (53.6 Ci/mole) and [8-$^{14}$C]ATP (47 Ci/mole) were the products of Amersham/Searle and Schwarz BioResearch. The radioactive substrates were purified by passage through Dowex AG 50 W-X2 ($NH_4^+$) columns, at neutral pH. Nucleotides and nucleosides were obtained from P-L Biochemicals. $\alpha,\beta$-Methylene-ATP, $\beta,\gamma$-methylene-ATP and $\alpha,\beta$-methylene-adenosine tetraphosphate were synthesized according to Myers *et al.*[11] and were obtained from Miles Laboratories. Sodium tripolyphosphate and sodium tetrapolyphosphate were products of Alfa Inorganics, Beverly, Mass. and City Chemical Corp., New York, N.Y., respectively. All inhibitors were neutralized immediately before use.

*Methods*

The assays for the adenosyltransferase activity were performed by a cation-exchange column procedure[9]. The reaction mixture contained, in a final volume of 250 $\mu$l, Tris–histidine buffer, pH 9.0, 90 mM; KCl, 100 mM; $MgCl_2$ (usually 7 mM, but always at 5 mM excess over the ATP concentration); 2-mercaptoethanol, 5 mM; ATP, 0.1–2.0 mM; L-[*methyl*-$^{14}$C]methionine, 0.1–2.0 mM ($5 \cdot 10^5$ cpm); and enzyme, 1–2 $\mu$g. The precise compositions of the reaction mixtures are given with the individual protocols. The reaction was initiated by the addition of the enzyme. The incubations were usually carried out for 3–10 min at 37 °C as indicated in individual protocols. A control value obtained from an incubation in the absence of enzyme, or ATP, was routinely subtracted. When ATP was the variable substrate with L-methionine at constant concentration, [8-$^{14}$C]ATP was used in place of radioactive L-methionine to secure higher accuracies. A cation-exchange paper disc procedure recently devised in this laboratory[12] for the assay of adenosyltransferase was used in some experiments for confirmatory purposes. The observed reaction velocities are all expressed as $\mu$moles of Ado-Met synthesized per mg of protein per 30 min under the conditions specified.

## RESULTS

*Additional observations on the kinetic behavior of the adenosyltransferase of yeast*

The kinetic behavior of the yeast adenosyltransferase has been studied previously[5,6], and sizeable deviations from classical Michaelis–Menten kinetics have been demonstrated[6]. Under assay conditions utilizing highly sensitive radioactive measuring techniques, and low product formation, a clear-cut presteady-state lag period was observed and this could be obliterated by the addition of low levels of Ado-Met[6]. The same phenomenon is vividly displayed in Fig. 1 which demonstrates that the observed velocity (expressed as $\mu$moles of Ado-Met formed per mg of protein in 30 min) is not constant, but increases with the amount of enzyme added (Fig. 1A) and the time of incubation (Fig. 1B). The results shown in Fig. 1 were obtained under conditions of low product formation, which were achieved by using low levels of substrate (ATP, 1 mM; L-methionine, 0.1 mM), brief incubation periods, and low enzyme levels. The lag period leads to considerable deviations from Michaelis–Menten kinetics, characterized by downward inflexions of the Lineweaver–Burk plots of reciprocal velocity with respect to reciprocal concentration of either L-methionine or ATP. Consequently, the kinetic parameters of this enzyme can only be assessed over relatively narrow ranges of substrate concentration and prolonged incubation

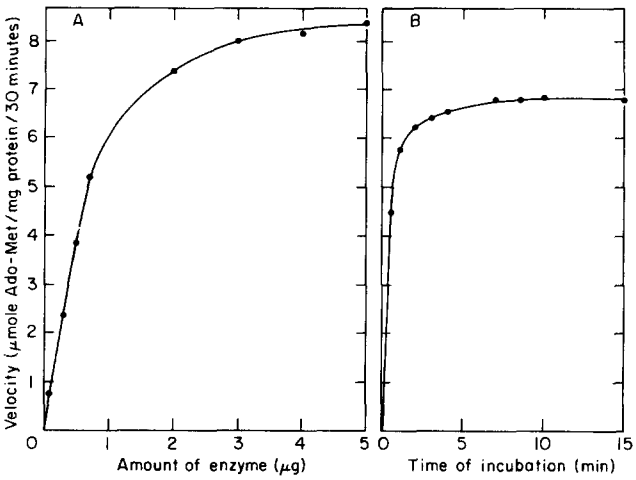


Fig. 1. Observed reaction velocities of purified yeast adenosyltransferase measured as a function of amount of enzyme (A) and time (B). The velocities are normalized in terms of μmoles of Ado-Met formed per mg of protein in 30 min. The reaction mixtures had the composition described under Experimental Procedure, with the ATP concentration at 1 mM and the L-methionine concentration at 0.1 mM. The incubation time for A was 3 min and for B was varied. The amount of enzyme protein used for B was 1.0 μg and was varied for A. The incubations were carried out at 37 °C. Note that the reaction mixture (Experimental Procedure) was modified to enhance the presteady-state properties.

periods. Utilizing limited ranges of L-methionine and ATP concentrations (*i.e.* 0.1–2 mM) the $K_m$ values for these substrates were determined to be 0.36 mM and 0.29 mM, respectively. These values are in reasonable agreement with our earlier corresponding determinations[6] of 0.42 mM and 0.36 mM, respectively.

*Effects of ATP analogues on adenosyltransferase activity*

When the nucleotides listed in Table I were tested in place of ATP in the adenosyltransferase reaction, velocities of less than 1–2 percent of the activity of ATP were observed. However, dATP had higher substrate activity, but contamination by ATP was not excluded. The nucleotides were all tested at concentrations up to 5 mM.

The inhibitory effects of analogues of ATP or portions of this molecule are given in Table I. For all inhibitors with measurable $K_i$ values, competition with ATP was demonstrated. In some instances, noted in Table I, the noncompetitive inhibition with respect to L-methionine has also been established.

Certain generalizations may be drawn from these results: (i) The triphosphates are more powerful inhibitors than the diphosphates or monophosphates or the nucleosides, and the rank order of inhibition correlates with the number of phosphate groups; (ii) With the exception of ITP, the purine-containing compounds are superior inhibitors to the pyrimidine derivatives.

*Effects of inorganic ortho-, pyro- and polyphosphates on adenosyltransferase activity*

Both $P_i$ and $PP_i$ are weak inhibitors of the adenosyltransferase reaction. With the high concentrations of these compounds required to produce significant inhibitions, the formation of insoluble magnesium–phosphate complexes in the reaction

TABLE I

STRUCTURAL ANALOGUES OF ATP AS INHIBITORS OF ADENOSYLTRANSFERASE

The assays were performed as described under Experimental Procedure. Reaction mixtures were incubated with 2 μg of purified yeast enzyme for 10 min at 37 °C. The L-methionine concentration was 2 mM, and the ATP concentration was usually varied from 0.15 mM to 2 mM. The inhibition constants, $K_i$, are expressed with respect to ATP. The $K_m$ for ATP was 0.29 mM (mean of six determinations; range 0.21–0.36 mM). All inhibitors with measurable $K_i$ values were competitive with respect to ATP.

| *Analogue* | *$K_i$ (mM)* | *Analogue* | *$K_i$ (mM)* |
|---|---|---|---|
| Nucleosides | | Nucleotides (cont'd) | |
| Adenosine | Not inhibitory at 5 mM | dGTP | 8.0 |
| Deoxyadenosine | Not inhibitory at 5 mM | ITP | 9.4 |
| Nucleotides | | CTP | 4.0 |
| AMP | Not inhibitory at 5 mM | UTP | 6.4 |
| dAMP | Not inhibitory at 5 mM | TTP | 10.0 |
| ADP | Not inhibitory at 5 mM | Phosphates and polyphosphates | |
| dATP | 2.1 | $P_i$ | 9* |
| GMP | Not inhibitory at 5 mM | $PP_i$ | 2.5* |
| GDP | 4.1 | $PPP_i$ | 0.05** |
| GTP | 3.0 | $PPPP_i$ | 0.18** |

* The $K_i$ values for $P_i$ and $PP_i$ are approximate because of the formation of insoluble magnesium–phosphate complexes at high concentrations.

** These inhibitors were shown to be noncompetitive with respect to L-methionine ($K_m$ for L-methionine was 0.36 mM).

mixture precludes detailed studies and unequivocal interpretations of product inhibition studies[5,6]. On the other hand, the proposed enzyme-bound intermediate, $PPP_i$, has been shown to be a very powerful inhibitor of the reaction ($K_i$ = 0.05 mM) in competition with ATP[6]. Further studies with inorganic tetraphosphate ($PPPP_i$) have revealed that it is also a relatively powerful inhibitor ($K_i$ = 0.18 mM) which competes with ATP (Fig. 2), and is noncompetitive with respect to L-methionine (not shown).

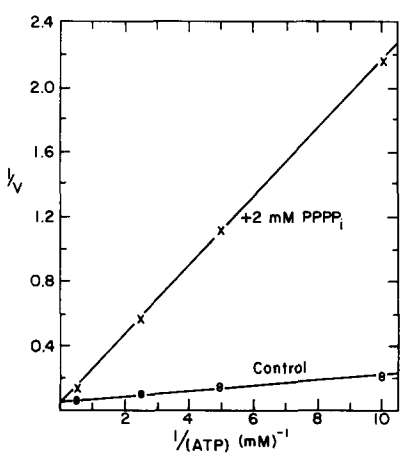


Fig. 2. Lineweaver–Burk plots showing the competitive inhibition of the formation of Ado-Met by 2 mM sodium $PPPP_i$. The reaction system described under Experimental Procedure was used, with L-methionine at 0.4 mM and ATP varied from 0.1 to 2.0 mM. The amount of enzyme was 2 μg and the incubation was for 10 min at 37 °C. The $K_i$ for $PPPP_i$ is 0.18 mM and the $K_m$ for ATP is 0.33 mM.

TABLE II

METHYLENE PHOSPHONATE ANALOGUES OF ATP AS INHIBITORS OF ADENOSYLTRANSFERASE

The assays were performed as described under Experimental Procedure. Reaction mixtures were incubated with 2 $\mu$g of purified yeast enzyme for 5 min at 37 °C. The L-methionine concentration was held constant at 2 mM or varied from 0.15 to 2 mM. The ATP concentration was varied from 0.15 to 2 mM or held constant at 2 mM. All inhibitors were competitive with respect to ATP, and noncompetitive with respect to L-methionine. The $K_m$ values are 0.23 mM and 0.4 mM for ATP and L-methionine, respectively.

| *Analogue* | *$K_i$ with respect to ATP (mM)* | *$K_i$ with respect to L-methionine (mM)* |
|---|---|---|
| $\alpha,\beta$-Methylene-ATP | 0.55 | 4.05 |
| $\beta,\gamma$-Methylene-ATP | 2.19 | 3.04 |
| $\alpha,\beta$-Methylene-Adenosine tetraphosphate | 0.17 | 0.40 |

*Effects of methylene phosphonate analogues of ATP on adenosyltransferase activity*

Since the adenosyltransferase releases $P_i$ nonrandomly from the terminal phosphate group of ATP[4], it appeared of interest to determine whether the methylene phosphonate analogues of ATP[11], in which the bridging oxygen atoms of the phosphate groups have been replaced by methylene groups, might serve as inhibitors of the reaction.

Of the three methylene phosphonate analogues of ATP examined in the present studies, the $\alpha,\beta$-methylene-adenosine tetraphosphate was the most potent inhibitor. It was competitive with respect to ATP ($K_i$ = 0.17 mM) and noncompetitive with respect to L-methionine ($K_i$ = 0.40 mM) (Table II and Fig. 3). The next most potent inhibitor in this series was the $\alpha,\beta$-methylene analogue of ATP which was competitive

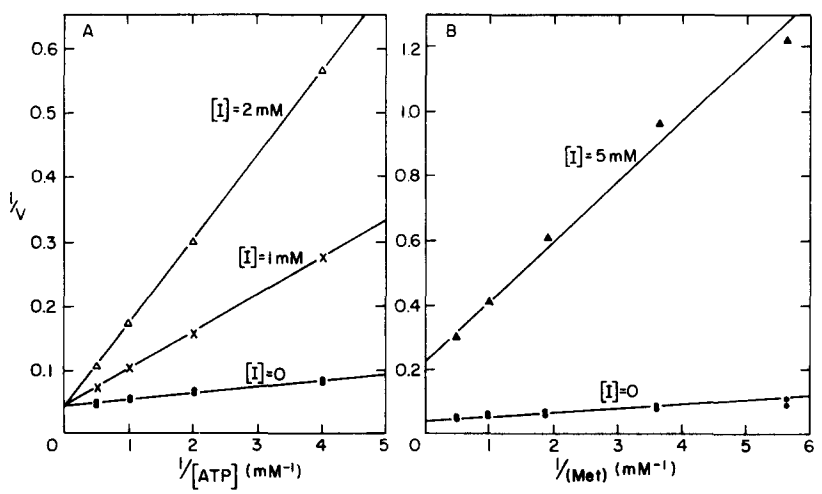


Fig. 3. Lineweaver–Burk plots showing the inhibition of the formation of Ado-Met by the $\alpha,\beta$-methylene phosphonate analogue of ATP. The inhibitor concentrations (0–5 mM) are indicated. For Expt A, the ATP concentration was varied from 0.25 to 2.0 mM, while the L-methionine concentration was 2 mM, whereas for Expt B, the L-methionine concentration was varied from 0.15 to 2.0 mM and ATP was maintained at 2 mM. The incubation was carried out with 2 $\mu$g of enzyme for 5 min at 37 °C. The kinetic parameters for this inhibitor are given in Table II.

with respect to ATP ($K_i$ = 0.55 mM) and noncompetitive with respect to L-methionine ($K_i$ = 4.05 mM). The $\beta,\gamma$-methylene analogue of ATP, which contains the structural modification at the position corresponding to the site of cleavage of ATP, was the least powerful inhibitor, competitive with respect to ATP ($K_i$ = 2.19 mM) and noncompetitive with respect to L-methionine ($K_i$ = 3.04 mM). None of the phosphonate analogues could replace ATP as a substrate in the adenosyltransferase reaction.

## DISCUSSION

Although substrates and inhibitors for an enzyme exert their effects as molecular entities, different moieties of these structures may be concerned with particular aspects of these functions[13]. In the present studies, advantage was taken of the unique fact that the relatively complex nature of one substrate (ATP) permitted its dissection into structural moieties and individual modification of each part. Thus, the relative contribution of structural parts of a complex molecule toward binding and specificity could be assessed. The results indicate that $PPP_i$ alone has a high affinity for the enzyme either as a substrate for the tripolyphosphatase activity ($K_m$ = 0.11 or 0.23 mM, in the absence or presence of 0.1 mM Ado-Met, respectively), or as an inhibitor of the overall adenosyltransferase activity ($K_i$ = 0.05 mM), whereas adenosine has little affinity for the enzyme. The slightest modification of any portion of the ATP molecule destroys its ability to serve as a substrate. However, the $PPP_i$ region of the nucleotide molecule tolerates considerable structural alterations without producing great changes in affinity, as may be deduced for instance from the potent inhibitory effects of the methylene phosphonate analogues of ATP and $PPPP_i$.

It may therefore be proposed that there is a "specificity site" on the enzyme for the adenosyl moiety of the ATP molecule and an "affinity site" which is complementary and juxtaposed to the $PPP_i$ region of the ATP molecule. Since exogenous adenosine is only very poorly bound to the enzyme, it is reasonable to assume that in the adenosyltransferase reaction, the cleavage of the ribose–phosphate bond of ATP and the transfer of the adenosyl group to the sulfur of L-methionine are concerted reactions in which the adenosyl group never leaves the enzyme surface. The very high degree of specificity of the reaction for ATP, suggests that the triphosphate site-directed recognition of the adenosyl group imparts virtually absolute specificity for ATP.

Our earlier initial velocity studies[6] with the yeast adenosyltransferase gave intersecting patterns suggesting an ordered addition of substrates. Dead-end inhibitor experiments showed that all the L-methionine analogues studied were competitive with respect to L-methionine and noncompetitive with respect to ATP. In addition, it was shown that GTP was noncompetitive with respect to L-methionine and competitive with respect to ATP[10]. Since the dead-end inhibitors cannot back up the reaction sequence and thus do not affect the slopes of Lineweaver–Burk plots when the inhibitor adds after the variable substrate[14], a plausible interpretation of the kinetic patterns would be a random-ordered mechanism in which no product release occurs prior to the addition of both substrates to the enzyme. The present studies with ATP analogues such as the methylene phosphonate analogues of ATP (Table II) indicate a competitive inhibition with respect to ATP and noncompetitive (but not

uncompetitive) inhibition with respect to L-methionine. These results are consistent with the previous proposition that the reaction sequence involves a random-ordered addition of substrates.

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