The Mechanism of *S*-Adenosyl-L-methionine Synthesis by Purified Preparations of Bakers' Yeast†

Ting-Chao Chou and Paul Talalay*

ABSTRACT: Highly purified preparations of ATP:L-methionine *S*-adenosyltransferase (EC 2.4.2.13) have been obtained from autolysates of bakers' yeast. Kinetic studies using a sensitive radioactive assay reveal considerable deviation from Michaelis–Menten kinetics characterized by downward inflexions in double-reciprocal plots of initial velocity with respect to either L-methionine or ATP concentration. When product formation is reduced by lowering the temperature or by shortening the incubation time, so that the net accumulation of endogenous *S*-adenosyl-L-methionine (AMet) becomes negligible, a pre-steady-state lag period can be clearly demonstrated. This delay in product formation is shortened by addition of low levels of *S*-adenosyl-L-methionine and prolonged by addition of tripolyphosphate. Initial velocity studies and dead-end inhibition studies suggest a random order of addition of substrates. As the reaction proceeds, the rate-limiting step shifts from hydrolysis of tripolyphosphate to the formation of AMet. *S*-Adenosyl-L-methionine displays stimulatory and inhibitory effects (at low and high concentrations, respectively) on both its own synthesis and tripolyphosphate hydrolysis. Consequently, the enzyme displays biphasic regulatory control by AMet in addition to product inhibition. It is proposed that the release of *S*-adenosyl-L-methionine from the product site results in the inhibition of the overall reaction by virtue of the formation of an enzyme–tripolyphosphate complex in which tripolyphosphate is either very slowly or not at all hydrolyzed. In the presence of endogenous *S*-adenosyl-L-methionine the hydrolysis of tripolyphosphate by the enzyme is activated in an "hysteretic" manner and is followed by the release of all products. The conformational L-methionine analogs, L-2-amino-4-hexynoic acid, DL-2-amino-*trans*-4-hexenoic acid, *S*-trifluoromethyl-L-homocysteine, and 1-aminocyclopentanecarboxylic acid, are competitive inhibitors with respect to L-methionine and noncompetitive with respect to ATP, whereas GTP is a competitive inhibitor with respect to ATP and noncompetitive with respect to L-methionine.

In the course of studies on structural and conformational analogs of L-methionine as inhibitors of the synthesis of *S*-adenosyl-L-methionine (AMet)[1] by purified enzyme preparations of ATP:L-methionine *S*-adenosyltransferase (EC 2.4.2.13), significant deviations from linearity were encountered in double-reciprocal plots of initial velocity with respect to either L-methionine or ATP concentration (Lombardini *et al.*, 1970). With the use of highly purified adenosyltransferase preparations from bakers' yeast and a very sensitive radioactive assay procedure, these kinetic anomalies have been confirmed and a more detailed analysis of the reaction mechanism of this enzyme has been undertaken.

The mechanism of the yeast adenosyltransferase has been studied extensively (Cantoni and Durrell (1957); Mudd and Cantoni (1958); Mudd (1962); Mudd and Mann (1963)). The reaction involves a total removal of the phosphate groups of ATP according to the following stoichiometry:

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

The complete dephosphorylation of ATP occurs in an asymmetric manner resulting in the liberation of the innermost two phosphate groups of ATP as pyrophosphate and of the terminal phosphate as inorganic phosphate. To rationalize the nonrandom splitting of the phosphate groups of ATP, Mudd (1963) proposed that enzyme-bound tripolyphosphate was an obligatory intermediate. This view was supported by experiments showing that the reaction system contained approximately stoichiometric quantities of enzyme-bound tripolyphosphate and that the enzyme possessed tripolyphosphatase activity that was specifically stimulated by low levels of AMet. Detailed kinetic studies of the yeast enzyme (including product inhibition analyses) by Greene (1969) demonstrated an intersecting pattern in initial velocity studies with respect to L-methionine or ATP concentration. PPP_i and PP_i were found to be competitive inhibitors with respect to ATP, and noncompetitive inhibitors with respect to L-methionine, whereas *S*-adenosyl-L-methionine was an uncompetitive inhibitor with respect to either ATP or L-methionine. These results suggested a preferred reaction sequence for addition of substrates and release of products which appeared to be consistent with an ordered bi-ter mechanism.

Further kinetic studies in this laboratory have revealed that the reaction is probably more complex than had been supposed previously. Activation of the synthesis of *S*-adenosyl-L-methionine by low levels of this product is observed under carefully defined experimental conditions. The activation of an enzyme by an immediate reaction product is to our knowledge an unusual feature of enzyme control, and may play an important regulatory role.

Experimental Section

Materials. L-Methionine was purchased from Mann; nucleotides were obtained from P-L Biochemicals. *S*-Adenosyl-L-methionine (hydrogen sulfate salt) was obtained from Boeh-

† From the Department of Pharmacology and Experimental Therapeutics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received October 21, 1971.* These studies were supported by grants from the National Institutes of Health (GM 16492) and from the Gustavus and Louise Pfeiffer Foundation.

[1] Abbreviations used are: AMet or AMe, (−)*S*-adenosyl-L-methionine; adenosyltransferase, ATP:L-methionine *S*-adenosyltransferase (EC 2.4.2.13); PPP_i, tripolyphosphate; PP_i, pyrophosphate.

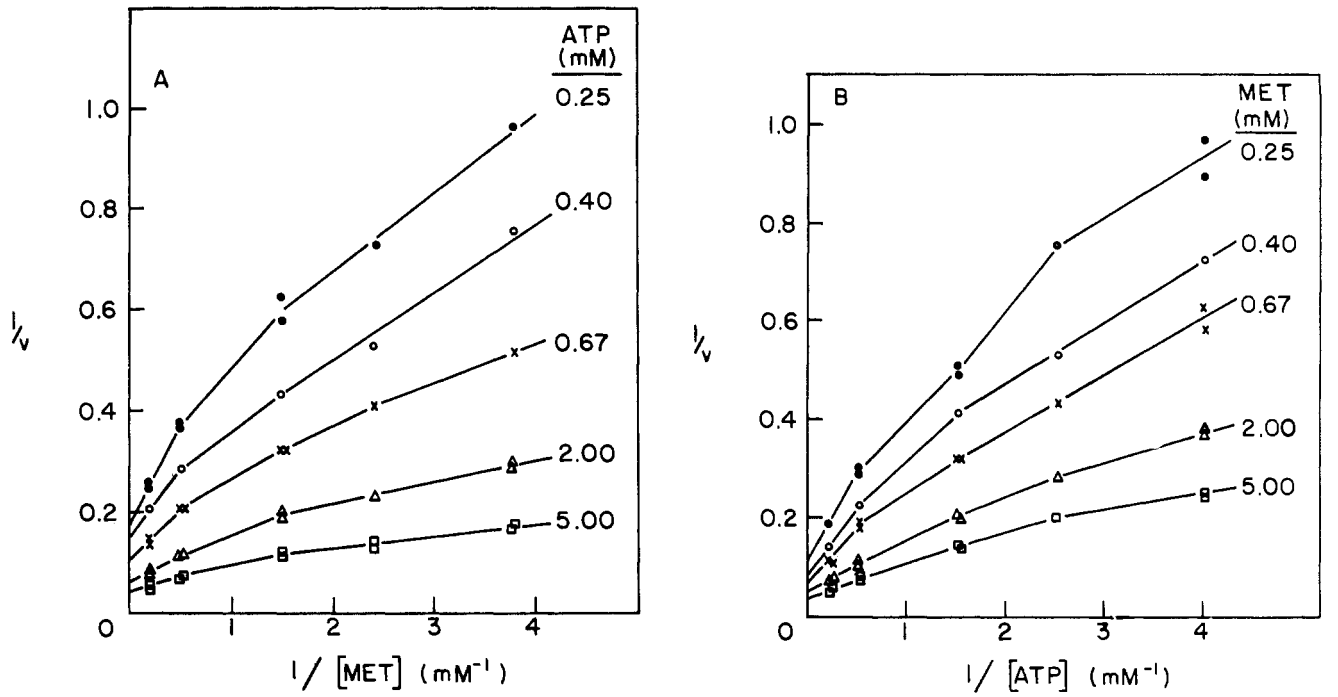


FIGURE 1: Lineweaver–Burk plots of initial velocity patterns for the ATP:L-methionine *S*-adenosyltransferase reaction where one substrate concentration was varied while the other substrate was maintained at several fixed concentrations. (A) Reciprocal velocity as a function of reciprocal L-methionine concentrations at various ATP concentrations (0.25–5.0 mM). (B) Reciprocal velocity as a function of reciprocal ATP concentration at various levels of L-methionine (0.25–5.0 mM). Reaction mixture I with 3 μg of enzyme and 3-min incubation at 37° were used in both expt A and B. All velocities were expressed as μmoles of AMet formed per mg of protein per 30 min.

ringer-Mannheim. The cation-exchange Dowex resin AG 50W-X2 (100–200 mesh) supplied by Bio-Rad was converted to the NH_4^+ form. 2-Mercaptoethanol (Eastman Organic Chemicals) was distilled under reduced pressure (bp 58–60° (23 mm)). L-[^{14}C-*methyl*]Methionine (53.6 mCi/mmole) and [8-^{14}C]ATP (47 mCi/mmole) were the products of Amersham–Searle and Schwarz BioResearch, respectively, and were purified by passage through AG 50W-X2 (NH_4^+) columns, at neutral pH. Radioactivity was determined with a liquid scintillation spectrometer using Bray's solution (Bray, 1960). Fleischmann bakers' yeast was purchased from local food markets. 1-Aminocyclopentanecarboxylic acid and *S*-trifluoromethyl-L-homocysteine were obtained from Cyclo Chemical. Sodium tripolyphosphate was obtained from Alfa Inorganics. L-2-Amino-4-hexynoic acid and DL-2-amino-*trans*-4-hexenoic acid were synthesized by Dr. A. W. Coulter of this laboratory (Coulter and Talalay, 1968). Dr. R. E. Handschumacher kindly supplied 5-diazo-4-oxo-L-norvaline (Handschumacher *et al.*, 1968). All neutralized solutions were kept cold and used on the same day.

Assay of Adenosyltransferase Activity. The assay is based upon the conversion of L-[^{14}C-*methyl*]methionine to *S*-adenosyl-L-[^{14}C-*methyl*]methionine which in contrast to L-methionine is retained by Dowex AG 50W-X2 (NH_4^+) at neutral pH. A modification by Lombardini *et al.* (1970) of the method of Mudd *et al.* (1965) was used.

The kinetic measurements were carried out in two reaction mixtures which differed in that mixture I (pH 7.6) was patterned after Mudd and Cantoni (1958) and contained very high concentrations of Mg^{2+} and K^+ whereas mixture II (pH 9.0) was similar to that of Greene (1969) and had much lower ionic strength. Both reaction systems had a final volume of 0.25 ml and were incubated for the times indicated in individual protocols at 37°, unless otherwise stated. All reaction velocities are expressed in terms of micromoles of product formed by 1 mg of protein in 30 min. Reaction mixture I contained the following: Tris-HCl buffer, pH 7.6, 200 mM; KCl, 200 mM; $MgCl_2$, 300 mM; ATP, 5 mM; L-[^{14}C-*methyl*]methionine, 5 mM (5×10^5 cpm); and enzyme. Reaction mixture II contained the following: Tris–histidine buffer, pH 9.0, 90 mM; KCl, 100 mM; $MgCl_2$ (usually 10 mM, but always at a 5 mM excess over the ATP concentration); ATP, 5 mM; L-[^{14}C-*methyl*]methionine, 5 mM (5×10^5 cpm); and enzyme.

The reaction mixture as well as the enzyme were warmed at 37° for 5 min, prior to initiation of the reaction by addition of enzyme. The contents were mixed immediately and the incubations were carried out with mild agitation in 13-ml glass-stoppered tubes. The enzyme preparations were diluted with bovine serum albumin solution (1 mg/ml). The reaction was arrested by the addition of ice-cold 2 mM Tris-HCl buffer (pH 7.4), containing 0.5 mM sodium tripolyphosphate to an approximate final volume of 13 ml. The solution was passed over a column (0.6 cm × 3 cm) of Dowex AG 50W-X2(NH_4^+). The column was washed with 80 ml of ice-cold water and the radioactive AMet was eluted with two 5-ml aliquots of concentrated NH_4OH. Each eluate was added to 15 ml of scintillation fluid for counting of radioactivity. A blank 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 instead of L-[^{14}C-*methyl*]methionine to secure higher accuracies in the assays.

Assay of Tripolyphosphate Activity. The assay is based upon the hydrolysis of tripolyphosphate to pyrophosphate and orthophosphate, and measurement of the latter by the method of Martin and Doty (1949), as modified by Gibbs *et al.* (1965). Unless otherwise stated, the incubations were carried out for 30 min at 37° in a final volume of 0.5 ml containing Tris–

histidine buffer (pH 9.0, 90 mM), KCl (100 mM), $MgCl_2$ (usually 7 mM, but always at a 5 mM excess over the PPP_i concentration), and sodium tripolyphosphate at concentrations given with individual protocols.

Determination of Protein. Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin (Armour) as a standard.

Purification of Adenosyltransferase. The preparation of the enzyme from bakers' yeast reported recently by Lombardini *et al.* (1970), and based on the procedure of Mudd and Cantoni (1958) was followed. The final preparation was further subjected to fractionation between 52 and 64% ethanol (v/v) at 0°. The precipitate was dissolved in 2 mM potassium phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol to give a final protein concentration about 2 mg/ml. The enzyme solution was then precipitated at 42–52% saturation of ammonium sulfate and dissolved in 2 mM potassium phosphate (pH 7.0) containing 5 mM 2-mercaptoethanol. At this stage the enzyme could be stored for at least 6 months at −15°. An approximately 1950-fold purification over the initial autolysate was obtained with a recovery of about 7% of the total enzyme activity. The final specific activity was in the range of 25–32 μmoles of AMet formed per mg of protein in 30 min at 37° (reaction mixture II). No hydrolysis of ATP was detectable in these preparations. For studies of the hydrolysis of tripolyphosphate the enzyme preparation was dialyzed overnight against 2000 volumes of 2 mM Tris-HCl (pH 7.0)–2 mM KCl in order to remove P_i. Gel filtration of the purified enzyme was conducted on a column of Sephadex G-200 (1.0 × 80 cm) in 5 mM Tris-HCl at pH 7.0 at a flow rate of 3 ml/hr at room temperature in conjunction with appropriate standards (Andrews, 1964). In two separate experiments, approximate molecular weights of 43,300 and 44,800 were obtained. These values are much lower than that reported by Mudd (1963) who calculated a molecular weight of 157,000 on the basis of the sedimentation constant of the enzyme from the same source. The reason for this discrepancy is unclear and it remains to be determined whether the enzyme consists of subunits. The enzyme loses 99% of its activity in 8 M urea, and more than one-half of the original activity may be restored by dialysis against 5 mM Tris-HCl buffer (pH 7.0), containing 5 mM dithiothreitol.

Results

Initial Velocity Studies. The effects on the initial velocity of varying L-methionine concentration at different fixed levels of ATP and *vice versa* are shown in Figure 1. The Lineweaver–Burk plots inflect downward when either L-methionine or ATP was the variable substrate. In experiments reported by Greene (1969) at comparable substrate concentrations, no comments on deviations from linearity are made, although the scatter of experimental values is considerable (*cf.* Figure 4 in Greene, 1969). The major discrepancies between our findings and those of Greene (1969) may be due to large differences in the amounts of product formed, which in the light of the findings described below could seriously affect the results. Greene used a spectrophotometric assay which is much less sensitive than the radioactive method, and presumably much higher concentrations of AMet were finally present in his reaction system.

In view of the curved nature of the Lineweaver–Burk plots, it was not possible to estimate the K_m values for substrates, K_i values for inhibitors, or the V_{max}, without approximating the curves to regression lines over limited ranges of substrate concentrations. Such approximate average values obtained from numerous experiments in reaction mixtures I and II at substrate concentrations arbitrarily selected to be below 2 mM are given in Table I. In agreement with the findings of Greene

TABLE I: Summary of Kinetic Patterns for ATP:L-Methionine *S*-Adenosyltransferase.[a]

	Reaction Mixture I	Reaction Mixture II
Initial velocities[b]		
L-Methionine *vs.* ATP	Intersecting (K_m for L-methionine = 0.55)	Intersecting (K_m for L-methionine = 0.42)
ATP *vs.* L-methionine	Intersecting (K_m for ATP = 0.80)	Intersecting (K_m for ATP = 0.36)
PPP_i inhibition		
ATP *vs.* PPP_i	Competitive (K_i = 0.012)	Competitive (K_i = 0.014)
L-Methionine *vs.* PPP_i	Noncompetitive (K_i = 0.025)	Noncompetitive (K_i = 0.060)
Dead-end inhibition		
L-Methionine *vs.* 1-aminocyclopentanecarboxylic acid		Competitive K_i = 6.7 K_i = 2.3[c]
ATP *vs.* 1-aminocyclopentanecarboxylic acid		Noncompetitive (K_i = 18.2)
L-Methionine *vs.* L-2-amino-4-hexynoic acid		Competitive K_i = 4.0 K_i = 1.8[c]
ATP *vs.* L-2-amino-4-hexynoic acid		Noncompetitive (K_i = 16.2)
L-Methionine *vs.* DL-2-amino-*trans*-4-hexenoic acid		Competitive (K_i = 14.3)
ATP *vs.* DL-2-amino-*trans*-4-hexenoic acid		Noncompetitive (K_i = 40.5)
L-Methionine *vs.* L-trifluoromethyl-*S*-homocysteine		Competitive (K_i = 18.9)
ATP *vs.* L-trifluoromethyl-*S*-homocysteine		Noncompetitive (K_i = 43.3)
L-Methionine *vs.* 5-diazo-4-oxo-L-norvaline		Competitive (K_i = 4.2)
ATP *vs.* 5-diazo-4-oxo-L-norvaline		Noncompetitive (K_i = 16.9)
L-Methionine *vs.* GTP		Noncompetitive (K_i = 8.8)
ATP *vs.* GTP		Competitive (K_i = 2.6)

[a] Definitions of kinetic patterns are those of Cleland (1963). All kinetic constants are in mM. Regression lines were drawn from Lineweaver–Burk plots over limited ranges of substrate concentration (below 2 mM). [b] The first mentioned substrate was the varied substrate, the second substrate was the "changing fixed substrate" of Cleland (1963). [c] Assays were carried out at 1 mM ATP instead of 5 mM ATP.

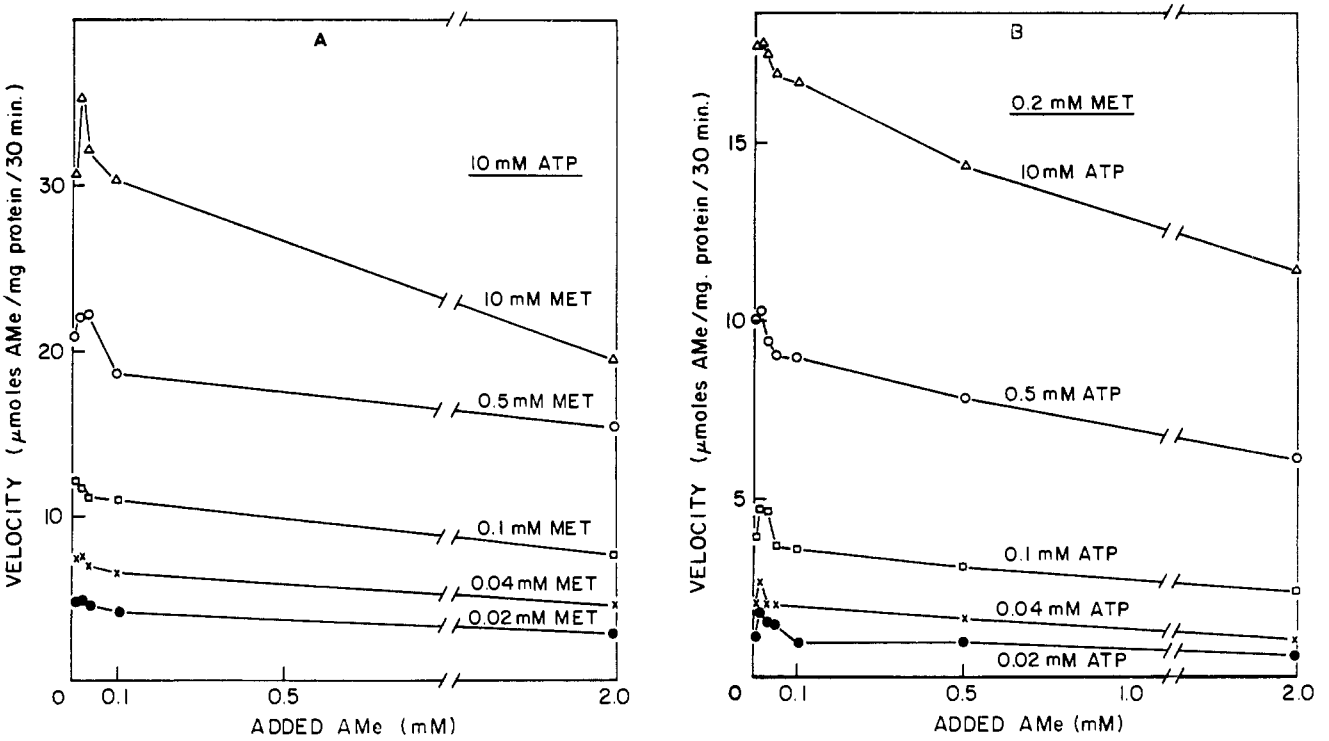


FIGURE 2: Biphasic effects of exogenous AMet on the velocity of its own synthesis: (A) L-methionine concentration varied with [ATP] = 10 mM; (B) ATP concentration varied with [L-methionine] = 0.2 mM. Reaction mixture II with 5 μg of enzyme was incubated at 37° for 5 min. The velocities are expressed in terms of μmoles of AMet formed per mg of protein per 30 min. AMe = *S*-adenosyl-L-methionine.

(1969), the curves have a common intercept to the left of the vertical axis suggestive of a sequential addition of substrates to the enzyme, and not a Ping-Pong mechanism (Cleland, 1963). The Hill plots (Hill, 1913; Atkinson *et al.*, 1965) of the same experiments as shown in Figure 1 give n values equal 0.5–0.6 at low substrate concentrations, and these n values increase to 1.4–1.5 when either substrate concentration is raised. Thus the reaction system appears to be shifting from a negative to a positive cooperative pattern when the substrate concentrations are increased (Koshland, 1969). The deviations from Michaelis–Menten kinetics are even more clearly displayed when initial velocities are plotted against the initial velocity divided by substrate concentration (Eadie–Hofstee plots).

Kinetic studies of the adenosyltransferase reaction were carried out in both reaction mixtures I and II. Glutathione, dithiothreitol, or *p*-chloromercuribenzoate had no significant effect on the velocity. Despite the marked differences in pH, ionic strength, and cofactors concentrations in the two reaction mixtures, both systems have almost the same maximum velocity. However, in all cases, the Lineweaver–Burk plots were bent in the fashion described. These deviations from Michaelis–Menten kinetics could not be attributed to a variety of technical factors. The possibility of the retention on the ion-exchange columns of the small quantities of AMet synthesized was excluded by demonstrating the quantitative recovery of small quantities of radioactive AMet in the presence of various quantities of unlabeled AMet. Moreover, the observed curvature of the Lineweaver–Burk plots is in the opposite direction to that expected for the nonquantitative recovery of low levels of AMet. Entirely comparable results were obtained whether [^{14}C]ATP or L-[^{14}C]methionine served as the radioactive tracer. Since no adenosine triphosphatase activity was detectable in the enzyme preparations, the kinetic anomalies could not be ascribed to this possibility.

Effect of AMet on Adenosyltransferase Activity. Since AMet is a reaction product that inhibits the overall reaction yet profoundly activates the hydrolysis of tripolyphosphate, the possibility was entertained that AMet might be an activator of its own formation, and that this phenomenon might be demonstrable under special conditions. At high concentrations of either ATP or L-methionine, a greater acceleration of reaction velocity is observed than would be expected from Michaelis–Menten kinetics (Figure 1). One possible explanation for this phenomenon is that at high substrate concentrations the formation of larger quantities of endogenous AMet accelerates the recycling of the enzyme by increasing the rate of hydrolysis of enzyme-bound tripolyphosphate. To test this assumption directly, small amounts of exogenous AMet (comparable to the endogenous amounts formed) were added to the reaction system. The reaction rate was slightly but consistently increased by AMet when the added AMet concentration was less than 0.05 mM (Figure 2), but inhibition was observed as the concentration of AMet was increased above this level. Similar phenomena were observed whether the L-methionine (Figure 2A) or the ATP (Figure 2B) concentration was varied. However, it should be noted that the activation resulted from the presence of both exogenously added and endogenously formed AMet. Whereas it is not certain whether the inhibition observed at high AMet levels is of a regulatory nature or represents product inhibition, it seems likely that the enzyme displays truly biphasic regulatory behavior, since activation and inhibition occur at levels of AMet (Figure 2A,B) far below those required for product inhibition. Moreover, as will be shown later, AMet exerts also a biphasic effect on the hydrolysis of tripolyphosphate, a reaction in which AMet neither serves as a substrate nor as product, suggesting that there is a regulatory site for AMet on the enzyme in addition to a product inhibition site. When the data in Figure 2 are replotted

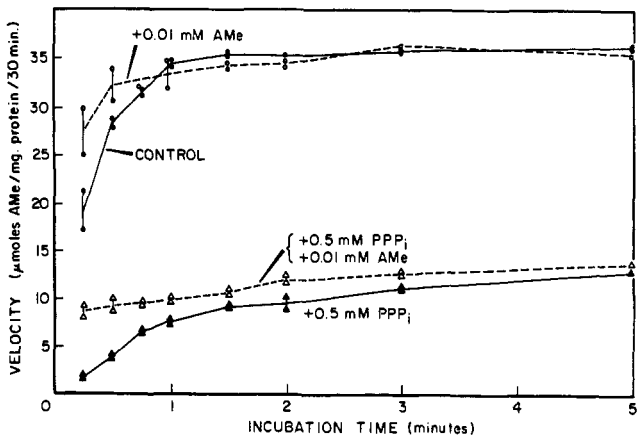


FIGURE 3: Time course of the change in rate of AMet synthesis in the presence and absence of exogenous PPP_i (0.5 mM) and/or AMet (0.01 mM). Reaction mixture II with 5 μg of enzyme, 5 mM L-methionine, and 10 mM ATP. Incubated at 37°. The concentrations of endogenous AMet at the end of 0.5 and 5.0 min were 9.40 and 120 μM, respectively, in control experiment (*i.e.*, in the absence of exogenous PPP_i and AMet). AMe = *S*-adenosyl-L-methionine.

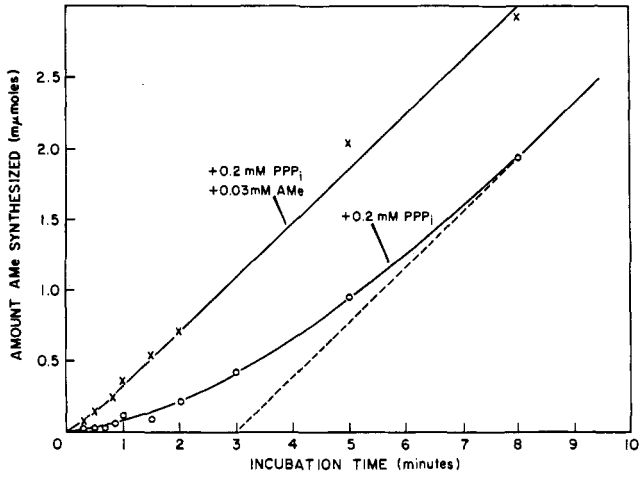


FIGURE 4: Effect of exogenous PPP_i and (PPP_i plus AMet) on the time course of AMet production at reduced temperature. Experiments were carried out in reaction mixture II with 5 mM L-methionine, 10 mM ATP, and 5 μg of enzyme at 20°. The concentrations of endogenous AMet at the end of 1 and 5 min were 0.36 μM and 3.68 μM, respectively, in the experiments with added PPP_i (0.2 mM) and without added AMet (*i.e.*, the lower curve). AMe = *S*-adenosyl-L-methionine.

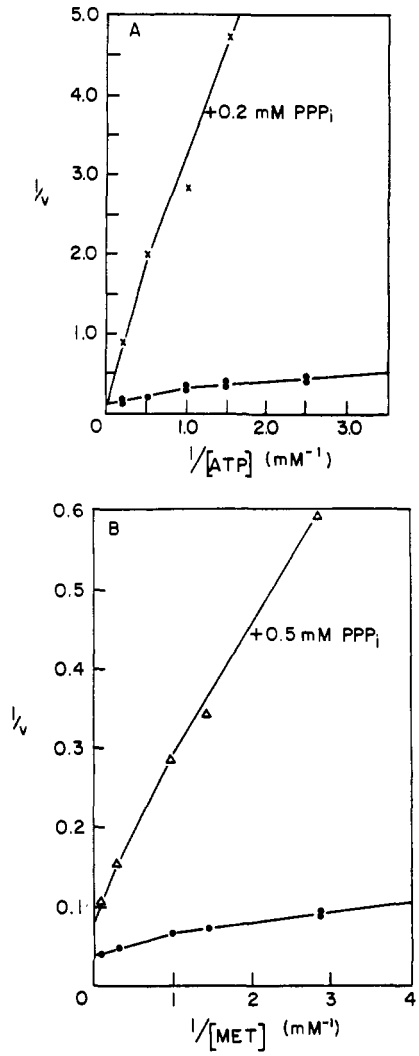


FIGURE 5: Inhibition of AMet synthesis by PPP_i. Lineweaver–Burk plots of the velocity of reaction with (A) ATP and (B) L-methionine as varied substrate. Experiment A was carried out in reaction mixture I with 1 mM L-methionine and 2.9 μg of enzyme. Incubation was 3 min at 37°. Experiment B was carried out in reaction mixture II with 10 mM ATP and 2.9 μg of enzyme. Incubation was 10 min at 37°. Entirely similar inhibition patterns are obtained in reaction mixtures I and II. Note that experiments with both reaction mixtures show downward curvatures.

according to the Lineweaver–Burk method, it is not possible to analyze the pattern of inhibition, since the intercepts of the lines may lie either on, or to the left, or to the right of the vertical axis, depending on the AMet concentration added.

A more effective approach to the study of the influence of AMet on the transferase activity was the use of reaction conditions under which the amount of AMet that is synthesized endogenously is reduced to very low levels, by either shortening the incubation time or by lowering the incubation temperature. With very brief incubations a well-defined lag period was observed before steady-state velocity was attained (Figure 3). The lag period was considerably shortened by addition of 0.01 mM AMet to the reaction mixture, and this effect was particularly prominent when the enzyme was partially inhibited by tripolyphosphate. Considerable prolongation of the lag period was observed in the presence of exogenous PPP_i. When the temperature was reduced from 37 to 20° and the amount of AMet synthesis was determined at various time periods (Figure 4), as much as a 3-min lag period in partially inhibited enzyme preparations could be observed; however, the addition of 0.03 mM AMet eliminated almost completely this lag period.

Effect of Tripolyphosphate on AMet Synthesis. Tripolyphosphate is the most potent known inhibitor of the adenosyltransferase. It behaved as a competitive inhibitor with respect to ATP ($K_i \approx 12$ μM in reaction mixture I and $K_i \approx 14$ μM in reaction mixture II), and as a noncompetitive inhibitor with respect to L-methionine ($K_i \approx 25$ μM in reaction mixture I and $K_i \approx 60$ μM in reaction mixture II) (Figure 5). These results are comparable to those obtained by Greene (1969). When the reaction velocity was reduced by lowering the temperature and lower concentration of substrates were used, the PPP_i inhibition gave semititrative types of Ackermann–

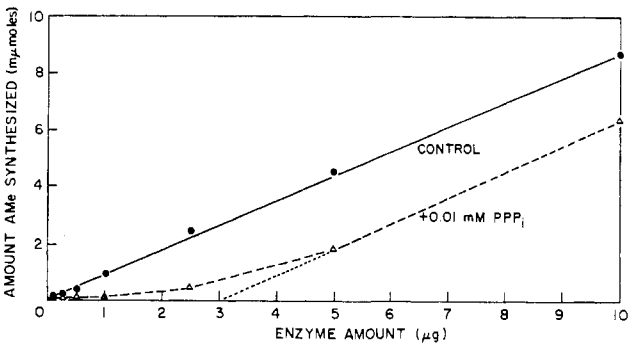


FIGURE 6: Ackermann–Potter plots of AMet synthesis in the presence and absence of PPP_i as inhibitor. The experiments were carried out at 16° with 0.5 mM L-methionine and 0.5 mM ATP. Reaction mixture II was used. Incubation time was 5 min. AMe = *S*-adenosyl-L-methionine.

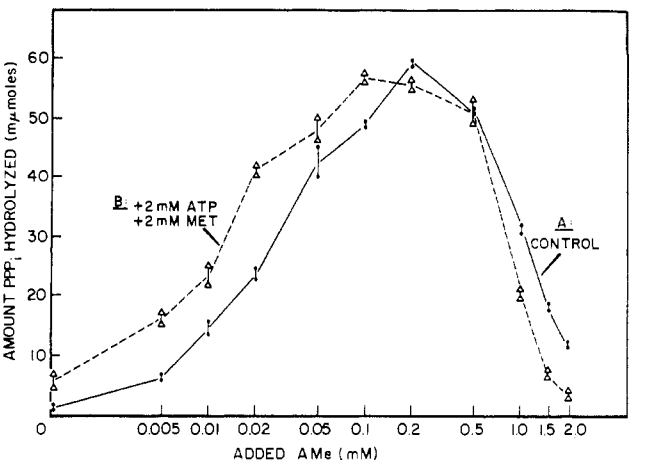


FIGURE 7: Biphasic effect of AMet on tripolyphosphatase activity. Experiment A shows the effects of varying concentrations of exogenous AMet on the reaction. Experiment B is similar, but the reaction systems also contain enzymatically generated AMet by virtue of the addition of L-methionine and ATP. The assay was carried out in a reaction mixture containing: Tris–histidine buffer (pH 9.0), 90 mM; KCl, 100 mM; PPP_i, 2 mM; $MgCl_2$, 7 mM (expt A) or 9 mM (expt B); and enzyme 3 μg. For expt B, ATP, 2 mM and L-methionine, 2 mM. Incubation was 15 min at 37°. The final concentration of endogenously generated AMet in expt B was 0.004 mM. AMe = *S*-adenosyl-L-methionine.

Potter plots at low enzyme concentrations (Figure 6). This behavior is a reflection of very tight binding in a pseudo-irreversible manner (Ackermann and Potter, 1949).

Comparison of Maximal Velocities of AMet Synthesis and Tripolyphosphatase Activities. The activation of tripolyphosphatase activities by AMet has been described by Mudd (1962, 1963) and by Greene (1969). The fact that it has not been possible to separate AMet synthesis from tripolyphosphatase activity by a variety of purification procedures led to the suggestion that both of these reactions are catalyzed by a single enzyme or enzyme complex. The finding that tripolyphosphatase activity is stimulated by low concentration of AMet and has similar cation requirements as the synthesis of AMet provides further support for this view (Mudd, 1962, 1963; Mudd and Mann, 1963). Attempts were made in this laboratory to compare the maximal velocities of AMet synthesis and tripolyphosphate hydrolysis with the same amounts of enzyme under identical incubation conditions, and in the presence and absence of AMet. The V_{max} for tripolyphosphate hydrolysis in the absence and in the presence of AMet (0.1 mM) was 6.45 and 66.6 μmoles per mg of protein per 30 min, respectively, for purified preparations. The K_m for tripolyphosphate in the absence and in the presence of AMet (0.1 mM) was 0.11 and 0.23 mM, respectively. These V_{max} and K_m values suggest that the activation of tripolyphosphatase activity by AMet was due to an increase in the turnover number of the enzyme rather than an increase in affinity for PPP_i. When the same reaction conditions in the absence of added PPP_i were used for the assay of adenosyltransferase activity, the V_{max} for AMet formation was 26.3 μmoles per mg of protein per 30 min and the K_m was 0.29 mM for L-methionine. These results suggest that tripolyphosphate hydrolysis is the rate-limiting step in the initial phases of the reaction when endogenous AMet levels are very low, and that as the reaction progresses, AMet formation may become the rate-limiting step. Since the initial velocity is a function of AMet activator concentration and the AMet concentration is a function of time, it is not unexpected that there is a presteady-state lag period and that the Lineweaver–Burk plots bend downward at higher substrate concentrations because more AMet is formed. The deviations from linearity in the Lineweaver–Burk plots appear therefore to be an intrinsic property of the enzyme attributable to the biphasic effect of AMet and are not susceptible to modification. Addition of L-methionine analog inhibitors, did not linearize the Lineweaver–Burk plots.

Effects of AMet on Tripolyphosphatase Activity. The dialyzed enzyme preparation had very little tripolyphosphatase activity in the absence of AMet, but the activity was increased markedly by addition of AMet with an optimum concentration near 0.2 mM. The effect of AMet on this activity was biphasic and a bell-shaped curve is obtained when velocity is plotted against AMet concentration on a logarithmic scale (Figure 7). The curve was shifted to the left by the addition of both 2 mM L-methionine and 2 mM ATP, but this shift was not observed when L-methionine or ATP alone was added.

Under the experimental conditions used in Figure 7, the addition of L-methionine and ATP led to the final formation of 0.004 mM AMet during the incubation period, and the presence of endogenous AMet is probably the major factor causing the shift of the curve. This biphasic effect of AMet on polyphosphatase activity is likely to be regulatory in nature since AMet is not a product of this reaction in contrast to the overall ATP:L-methionine *S*-adenosyltransferase reaction in which AMet is synthesized.

Inhibition of Methionine Adenosyltransferase by Substrate Analogs. Earlier studies in this laboratory have shown that certain analogs of L-methionine inhibited adenosyltransferase preparations from various sources. Analysis of the structural, electronic, and conformational features of these inhibitors has been employed to deduce the conformation of L-methionine at the active site of the enzyme (Lombardini *et al.*, 1970; Lombardini and Talalay, 1971). For the yeast enzyme, when regression lines were drawn over the curved Lineweaver–Burk plots at low substrate concentrations, it appeared that the following analogs of L-methionine were competitive with respect to L-methionine and noncompetitive with respect to ATP: 1-aminocyclopentanecarboxylic acid, L-2-amino-4-hexynoic acid, DL-2-amino-*trans*-4-hexenoic acid, L-trifluoromethyl-*S*-homocysteine, and 5-diazo-4-oxo-L-norvaline. Prior incubation of the enzyme with L-2-amino-4-hexynoic acid for time periods up to 50 hr did not alter the degree of inhibition. The kinetics of the inhibition patterns observed for 1-aminocyclopentanecarboxylic acid (Figure 8) is virtually identical

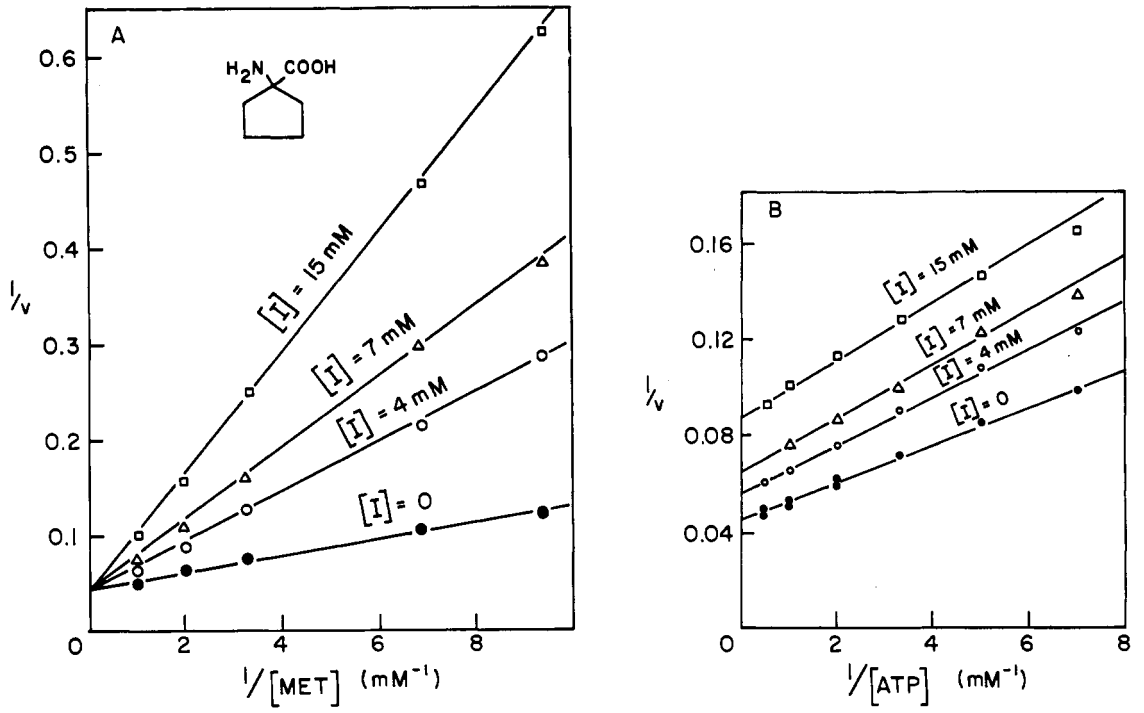


FIGURE 8: Inhibition of adenosyltransferase by 1-aminocyclopentanecarboxylic acid. Lineweaver–Burk plot for AMet synthesis with (A) L-methionine and (B) ATP as variable substrate. The inhibitor concentrations [I] are indicated. Reaction mixture II with 2 μg of enzyme was used except 1 mM ATP was used in expt A and 1 mM L-methionine was used in expt B. Incubation was 3 min at 37° in both experiments.

with that for L-2-amino-4-hexynoic acid. The latter is however a slightly more potent inhibitor (Table I).

The results obtained for the other inhibitors are also summarized in Table I. It may be noted that the K_i values of all of the L-methionine analogs were lower with respect to L-methionine than for ATP. Similarly the K_i value for GTP was lower with respect to ATP than for L-methionine. We conclude that the inhibitors are specific (and competitive) analogs of their respective substrates, and that the amino acids are specific conformational analogs of L-methionine.

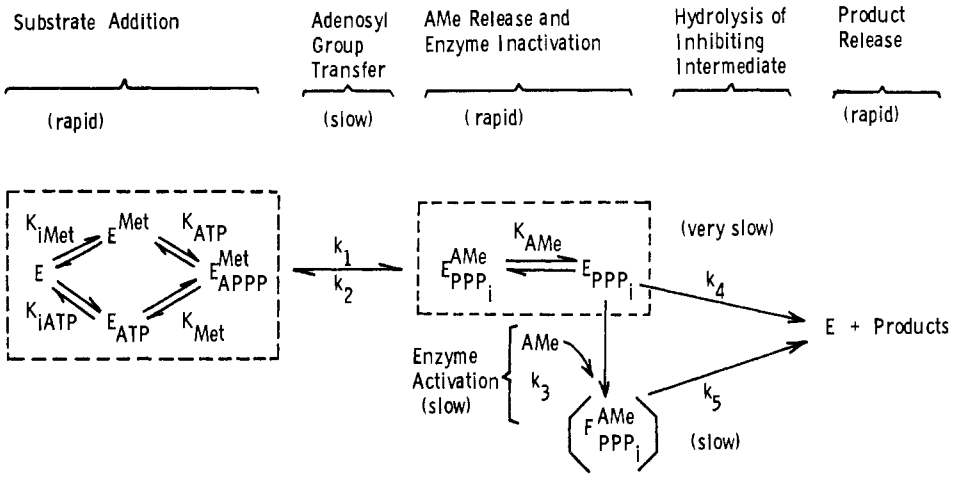
The intersecting initial velocity patterns (Figure 1) suggest a mechanism in which no irreversible step occurs between the binding of substrates to the enzyme (thus excluding a Ping-Pong mechanism). Since the dead-end inhibitors cannot backup the reaction sequence and thus do not affect the slopes of Lineweaver–Burk plots when the inhibitor adds after the variable substrate (Cleland, 1963), a plausible interpretation of the kinetic patterns would be a sequential addition of substrates with L-methionine as the first substrate. This conclusion is not in agreement with the previous report of Greene (1969) who suggested ordered addition of substrate with ATP as the first substrate as the major mechanism. Further information on this point was obtained from a study of the inhibition pattern of GTP which is structurally related to ATP but cannot serve as a substrate. When GTP was used as an inhibitor, it was competitive with respect to ATP and noncompetitive with respect to L-methionine. The latter finding suggests that ATP was the first substrate added. Thus, a random-ordered sequence in which either L-methionine or ATP may be added as the first substrate appears to be most closely consistent with our data.

Inhibition of Adenosyltransferase by Products. Although AMet stimulated the enzyme at very low concentrations, the other products (PP_i and P_i) appear to be inhibitors at sufficiently high concentrations (*i.e.*, 2 mM). However, such inhibitor studies with phosphates are complicated by the formation of insoluble magnesium phosphate complexes (*cf.* Greene, 1969). Product inhibition studies have shown irregular results depending on the reaction mixture used, and on the concentrations of inhibitors. The results could not be reconciled with any simple-ordered reaction sequence. As already described, in the case of AMet, the biphasic effects complicated the analysis.

Discussion

Many lines of evidence support the view that the capacity for hydrolysis of tripolyphosphate is an intrinsic property of the enzyme which promotes the formation of *S*-adenosyl-L-methionine from L-methionine and ATP: (1) the absolute requirement for both monovalent and divalent cations and the similarity in efficacy of different ions in each group for both reactions (Mudd, 1963). (2) Highly purified enzyme preparations used in these studies contain similar ratios of tripolyphosphatase to adenosyltransferase activities as reported previously (Mudd, 1962; Mudd and Mann, 1963). (3) Further evidence in support of this view is now provided by the finding that AMet, an extremely specific and powerful activator of tripolyphosphatase activity, also accelerates the formation of AMet under carefully experimental conditions. The failure of other investigators to observe the direct stimulatory effect of AMet on its own synthesis is probably attributable to the use of less sensitive assay methods or the use of assay systems in which the quantities of AMet formed approach levels at which inhibition is observed.

The direct demonstration of the stimulatory effect of AMet on its own synthesis was an outgrowth of efforts to explain the sizable deviations from linearity of Lineweaver–Burk plots of reciprocal initial velocity with respect to reciprocal concentrations of either L-methionine or ATP. The deviations of these



SCHEME I

plots (downward inflections) suggested that the velocities became abnormally high as the concentrations of either substrate was raised. The anomalous behavior was observed under a variety of experimental conditions and could not be attributed to methodological problems arising from the assay technique. It has been shown in these studies that the initial reaction velocity of the adenosyltransferase reaction is not alone a function of the concentrations of the two substrates (L-methionine and ATP), but is also under regulatory control of AMet, a product of the reaction. Since the formation of AMet is a time-dependent process and the effect of AMet is biphasic with respect to concentration, a steady-state kinetic analysis of the enzymatic reaction is not possible without imposing approximations. The stimulating effect of AMet on its own synthesis provides a reasonable explanation for the types of deviations from Michaelis–Menten kinetics shown in Figure 1.

A lag in the formation of AMet was alluded to by Mudd and Mann (1963). In the experiments here described, the lag period was clearly demonstrated by reducing the substrate and enzyme concentrations and by lowering the temperature. This lag period in product formation was dramatically shortened by the addition of appropriate levels of exogenous AMet. The lag period could also be prolonged by the addition of exogenous PPP_i. These results provide further evidence that PPP_i is an obligatory intermediate in the reaction and that its hydrolysis is the rate-limiting step in the initial phases of the reaction. It has not been established whether the enzyme has an absolute requirement for AMet with respect to its tripolyphosphatase activity. Since the concentrations of AMet required for activation of tripolyphosphatase activity are very low, residual amounts of AMet may remain bound to the activating site of the enzyme during the purification procedure.

The activation of an enzymatic reaction by low levels of a direct product of that reaction appears to be an uncommon phenomenon. Long and Pardee (1967) have described the activating effect of CTP on its own formation by cytidine triphosphate synthetase (EC 6.3.4.2) of *Escherichia coli*. This complex enzyme displays strongly sigmoidal kinetics at low concentrations of its reactants and modifiers (UTP, glutamine, ATP, and GTP), and in this case the activation by CTP could probably be ascribed to the ability of CTP to substitute for one of the activating nucleoside triphosphates.

The possibility that the biphasic controlling influence of AMet on its own synthesis is of regulatory importance requires serious consideration. Although the levels of AMet in yeast are profoundly influenced by culture conditions, the concentrations of AMet prevailing in various biological systems are approximately 0.02–0.10 mM (Baldessarini and Kopin, 1966; Lombardini and Talalay, 1971) which brackets the range at which AMet displays maximum stimulation in the case of the yeast adenosyltransferase (Figure 2).

These kinetic studies and the effects of AMet and tripolyphosphate on the reaction may be rationalized by a mechanism such as is diagrammed in Scheme I. Initial velocity studies and dead-end inhibition studies suggest that the addition of substrates is a sequential mechanism in which either substrate can be added first. The adenosyl group transfer is assumed to be a slow step as suggested by Mudd and Mann (1963). The release of AMet interrupts the cycling of the enzyme by forming a firmly bound enzyme–PPP_i complex. The dissociation of AMet from the product site appears to be rather facile as manifested by a high K_i value. The hydrolysis of PPP_i in the absence of AMet was a rate-limiting step (k_4) as indicated by the studies on the lag period and on the maximal velocities. However, the released AMet in turn activates the enzyme to hydrolyze PPP_i at a more rapid rate (k_5). When this occurs, the rate-limiting step may be shifted from tripolyphosphate hydrolysis to the adenosyl group transfer reaction as suggested by comparing V_{max} values of adenosyltransferase and tripolyphosphatase in the presence and absence of a small amount of exogenous AMet. Since the affinity of products for the enzyme was low, it is expected that they are readily dissociable from the enzyme.

Examination of several enzymes with time-dependent changes in reaction velocity led Frieden (1970) to propose the hysteretic enzyme concept for metabolic regulatory control in which rapid changes in the concentration of a metabolite in one pathway would not cause rapid changes in substrate concentrations affecting other pathways. Two mechanisms which may be primarily responsible for slow responses have been proposed: (a) isomerization processes involving the enzyme itself, and (b) displacement of a tightly bound ligand by another with a different effect. Both models may apply to the case of ATP: L-methionine *S*-adenosyltransferase in which the tightly bound PPP_i is gradually hydrolyzed by the intrinsic enzyme activity

which is activated by another endogenous ligand, AMet. There is suggestive evidence that AMet may activate the enzyme in a hysteretic manner (Figure 3).

There are two main factors which may contribute to the time-dependent change of reaction velocity in the synthesis of AMet. (a) Time-dependent formation of endogenous AMet which is required for activation of tripolyphosphatase activity. The significance of this effect on the overall enzymatic reaction has been shown by adding small amounts of exogenous AMet to the reaction system (Figure 2). (b) Time-dependent activation of tripolyphosphatase by AMet. The finding that externally added AMet did not eliminate the lag period completely (Figure 3) is in favor of this possibility.

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Studies of Flavin–Protein Interaction in Flavoproteins Using Protein Fluorescence and Circular Dichroism†

Joseph A. D'Anna, Jr.,‡ and Gordon Tollin*

ABSTRACT: Preparation of the apoproteins of *Desulfovibrio vulgaris* and *Rhodospirillum rubrum* flavodoxins and the equilibrium constants for riboflavin binding to these proteins are reported. Far-ultraviolet circular dichroism (CD) spectra of the apo- and holoproteins of *Clostridium pasteurianum*, *Peptostreptococcus elsdenii*, *D. vulgaris*, and *R. rubrum* flavodoxins indicate that significant changes in protein secondary structure accompany FMN binding. Protein fluorescence maxima and excitation spectra have also been measured for these materials and found to correlate with riboflavin binding ability and visible CD spectra of the holoproteins. The kinetics of FMN and protein fluorescence quenching upon coenzyme binding to apoprotein have been found to be second order (first order in flavin and first order in protein) and rate constants have been determined. The visible CD spectra of *C. pasteurianum* and *D. vulgaris* flavodoxins indicate that these proteins may be classified into two previously distinguished subclasses of dehydrogenases (Edmondson, D. E., and Tollin, G. (1971a,c) *Biochemistry 10*, 113, 133). These subclasses are shown to differ in their ability to bind riboflavin. Trends among these various types of measurements are tabulated and are shown to be consistent with the classification based upon CD spectroscopy and to correlate with enzymic activity. Fluorescence quenching studies of glucose oxidase apoprotein during binding of FAD have shown that protein fluorescence quenching is a faster process than is flavin fluorescence quenching. Protein fluorescence is not affected by ADP which binds to the apoprotein and which competes for FAD binding sites. Possible quenching mechanisms are discussed.

Flavin absorption and fluorescence properties have been widely utilized in studying coenzyme environment in flavoproteins (Swoboda, 1969a,b; Edmondson and Tollin, 1971a–c; Mayhew, 1971). Protein fluorescence (Massey and Curti, 1966; D'Anna and Tollin, 1971) and circular dichroism (CD) spectroscopy (Edmondson and Tollin, 1971a,b) have been less frequently employed. The use of these latter

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‡ Present address: Department of Biochemistry and Biophysics, Oregon State University.

* To whom correspondence should be addressed.