Slow Binding Inhibition of *S*-Adenosylmethionine Synthetase by Imidophosphate Analogues of an Intermediate and Product†

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ABSTRACT: *S*-Adenosylmethionine (AdoMet) synthetase catalyzes the only known route of biosynthesis of the primary in vivo alkylating agent. Inhibitors of this enzyme could provide useful modifiers of biological methylation and polyamine biosynthetic processes. The AdoMet synthetase catalyzed reaction converts ATP and L-methionine to AdoMet, PP_i, and P_i, with formation of tripolyphosphate as a tightly bound intermediate. This work describes a nonhydrolyzable analogue of the tripolyphosphate (PPP_i) reaction intermediate, diimidotriphosphate ($O_3P-NH-PO_2-NH-PO_3^{5-}$), as a potent inhibitor. In the presence of AdoMet, PNPNP is a slow-binding inhibitor with an overall inhibition constant (K_i*) of 2 nM and a dissociation rate of 0.6 h^{-1}. In contrast, in the absence of AdoMet PNPNP is a classical competitive inhibitor with a K_i of 0.5 μM, a slightly higher affinity than PPP_i itself ($K_i = 3$ μM). The imido analogue of the product pyrophosphate, imidodiphosphate ($O_3P-NH-PO_3^{4-}$) also displays slow onset inhibition only in the presence of AdoMet, with a K_i* of 0.8 μM, compared to K_i of 250 μM for PP_i. Circular dichroism spectra of the unliganded enzyme and various complexes are indistinguishable indicating that the protein secondary structure is not greatly altered upon complex formation, suggesting local rearrangements at the active site during the slow binding process. A model based on ionization of the bridging −NH− moiety is presented which could account for the potent inhibition by PNP and PNPNP.

S-Adenosylmethionine (AdoMet) occupies a central biological role in all cells, acting as the primary methyl group donor and as a source for the aminopropyl group in polyamine biosynthesis (*1*−*3*). AdoMet is involved in bacterial chemotaxis and DNA restriction, ethylene biosynthesis in plants, and in the gene expression activity in eukaryotes, as well as the regulation of the synthesis of methionine and AdoMet itself (*4*−*8*). Several enzymes have recently been found in which AdoMet is cleaved to form a 5′-deoxyadenosyl free radical as a reaction intermediate, a role until recently thought to be reserved for adenosylcobalamin (*9*−*11*).

S-Adenosylmethionine synthetase (EC 2.5.1.6, ATP:L-methionine S-adenosyl-transferase, AdoMet synthetase) catalyzes the sole known route of synthesis of *S*-adenosylmethionine (AdoMet) from L-methionine and ATP. AdoMet and tripolyphosphate (PPP_i) are initially formed and remain enzyme-bound until PPP_i is hydrolyzed to orthophosphate (P_i) and pyrophosphate (PP_i) before product release as shown[1] (*12*):

$$\text{L-methionine} + \text{ATP} + H_2O \rightarrow [\text{AdoMet} + PPP_i] + H_2O \rightarrow \text{AdoMet} + PP_i + P_i$$

(enzyme bound)

Thus, AdoMet synthetase catalyzes two distinct reactions, a nucleophilic displacement at carbon-5′ of ATP during AdoMet synthesis and the subsequent PPP_i hydrolysis. PPP_i hydrolysis apparently serves to convert a compound with high affinity for the enzyme (PPP_i, $K_i = 3$ μM for the *Escherichia coli* enzyme discussed herein (*13*)) into forms which dissociate more readily (PP_i ($K_i = 0.4$ mM) and P_i ($K_i = 4$ mM)) and to provide thermodynamic driving force for the reaction (*12*).

AdoMet synthetase has been studied from a diverse body of organisms including archaea, bacteria, yeast, plants, and mammals. In many species, there are multiple enzyme forms, suggesting that expression of AdoMet synthetase activity may be modulated by environmental conditions or in a tissue-specific manner, as has been established in humans (*14*, *15*). There is a considerable conservation in the sequences of AdoMet synthetase among various organisms; however, significant differences in physical and kinetic properties indicate evolutionary adjustments in the enzymes from various habitats (*15*).

The variation of properties of AdoMet synthetases among tissues and species have led it to be a target for chemotherapeutic development (*16*−*25*). AdoMet synthetase was one of the earliest cases to which computer-aided inhibitor design

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[1] When formed as an intermediate in AdoMet synthesis, PPP_i does not reorient or dissociate from the enzyme. Thus, the phosphoryl groups of PPP_i remain oriented as in positions occupied by ATP. P_i originates as the P_γ of ATP. AdoMet synthetase requires both Mg^{2+} and K^+ for activity. In all of these studies sufficient amounts of Mg^{2+} and K^+ were added to saturate the enzyme. For simplicity these ions are not included in compositions of the complexes in the text.

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was applied (*25*); that study focused on methionine analogues as potential inhibitors. A wide variety of inhibitors based on methionine analogues have been synthesized and characterized, the most potent being L-*cis*-2-amino-4-methoxybutenoic acid, which is a competitive inhibitor with respect to methionine with a K_i value generally in the micromolar range, depending on the isozyme (*18*). Bisubstrate analogues which link the methyl group of methionine with the C_5' atom of the nonhydrolyzable ATP analogue AMPPNP, or derivatives thereof, have been prepared in complex syntheses and are competitive inhibitors with K_i values in the 10^{-7} M range (*20*−*22*).

Surprisingly, no studies of the interaction of AdoMet synthetase with analogues of the tripolyphosphate intermediate have been published, although PPP_i is generally one of the most potent inhibitors known with a K_i in the low micromolar range. Since the tripolyphosphate intermediate formed in AdoMet synthesis is hydrolyzed asymmetrically with ∼98% of the P_i formed originating from the γ phosphoryl group of ATP, it must be tightly bound with hindered mobility at the active site (*13, 26*). It has long been established that added PPP_i is hydrolyzed to PP_i and P_i; the k_{cat}/K_m for this reaction is only 10^5 M^{-1} s^{-1} suggesting that there is not totally free access to the active site or that there are enzyme conformational changes accompanying binding (*13, 26*). This observation suggests that nonhydrolyzable analogues of PPP_i might be potent inhibitors. Since few enzymes are likely to have high affinity for these inorganic compounds, such inhibitors promise inherent selectivity.

Previous work has shown that the doubly nonhydrolyzable ATP analogue $\alpha\beta,\beta\gamma$-adenosyldiimidotriphosphate (AMPNPNP) is a substrate but after one turnover the enzyme is trapped as an enzyme·AdoMet·diimidotriphosphate dead end complex which dissociates at a rate of $<10^{-4}$ s^{-1} (*27*). However, it was not clear whether the high affinity of this complex is due to its mechanism of formation or due to chemical properties of the diimidotriphosphate (PNPNP).[2]

In this study we have characterized the inhibition of AdoMet synthetase by pyrophosphate and tripolyphosphate analogues. The characteristics of inhibition by imidodiphosphate and diimidotriphosphate vary enormously depending on whether AdoMet is present. In the presence of AdoMet, PNPNP is the most potent inhibitor of AdoMet synthesis thus far described.

MATERIALS AND METHODS

The pentasodium salt of PNPNP was a gift from Dr. A. Hampton of this Institute or synthesized by the Fox Chase Cancer Center Synthesis Facility according to Ma et al. (*27*). [Methyl-^{14}C]methionine was purchased from Dupont/New England Nuclear. PPP_i, PP_i, P_i, ATP, L-methionine, HEPES, Tris, magnesium chloride, potassium chloride, tetramethylammonium hydroxide, methylene diphosphate, imidodiphosphate, malachite green, ammonium molybdate, HCl, EDTA, and bacterial purine nucleoside phosphorylase were purchased from Sigma. AdoMet was purchased from Research Biochemicals Intl. 2-Amino-6-mercapto-7-methylpurine ribonucleoside (MESG) was either purchased as a component of the EnzCheck Phosphate assay kit from Molecular Probes Inc. or synthesized by the method of Broom and Milne at the FCCC Synthesis Facility (*28*).

AdoMet Synthetase Assays. AdoMet synthetase activity was determined by retention of the [^{14}C]AdoMet formed from L-[methyl-^{14}C]methionine by the cation exchange filter binding method previously described (*13*). The tripolyphosphatase (PPP_iase) activity of AdoMet synthetase was determined in the presence or absence of AdoMet by quantitating orthophosphate production (*29*−*31*). AdoMet activation of PPP_i hydrolysis was evaluated as described previously *(32)*. PPP_iase assays were performed at 25 °C and contained various amounts of tripolyphosphate (Na^+ form) in 50 mM HEPES·$(CH_3)_4N^+$ or 50 mM Tris·HCl at pH 7.8 with 10 mM KCl and 10 mM $MgCl_2$. The 0.2 mM 2-amino-6-mercapto-7-methylpurine ribonucleoside and 0.02 units of bacterial purine nucleoside phosphorylase were included when the coupled spectrophotometric assay (*29*) was used. Neither component interfered with PPP_iase activity. Control experiments performed with the purine nucleoside phosphorylase revealed that neither the PPP_iase assay mix nor the inhibitors compromised the phosphorylase coupling system.

Evaluation of Kinetic Parameters. Inhibition of AdoMet synthetase by compounds that produced rapid, reversible, competitive inhibition were analyzed by computer fitting according to the equation

$$v = \frac{V_M[S]}{K_m(1 + [I]/K_i) + [S]} \quad (1)$$

where K_m is the Michaelis constant, V_M is the maximal catalytic rate at saturating substrate(s) concentration, K_i is the dissociation constant for the enzyme−inhibitor complex, [S] is the substrate concentration, and [I] is the inhibitor concentration (*33*).

Inhibitors of AdoMet synthetase which produced reversible, slow binding inhibition were analyzed by the methods of Szedlacsek and Duggleby (*34*). Several models have been proposed to account for slow binding inhibition (*35*−*37*). The models differ primarily in the step of the enzyme−inhibitor interaction where slow onset behavior is observed: either inherently slow association or rapid binding followed by slow isomerization. Characteristic relationships that can be used in choosing an appropriate model exist between the initial onset of inhibition and the concentration of I (*35*−*37*). Since our data show that inhibition is clearly present at the onset of the experiment, Scheme 1 depicts a minimal model consistent with the time dependent inhibition by PNP and PNPNP (*35*). In this case two equilibrium constants describe the inhibition: the initial value for complex formation $K_i = k_4/k_3$ and the final value K_i^* which is $(k_6/(k_5 + k_6))K_I$.

Scheme 1

$$\begin{array}{ccccc} E + S & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & (ES) & \xrightarrow{k_{cat}} & E + P \\ + & & & & \\ I & & & & \\ k_4 \uparrow\downarrow k_3 & & & & \\ EI & \underset{k_6}{\overset{k_5}{\rightleftharpoons}} & EI^* & & \\ \textit{slow} & & & & \end{array}$$

[2] Abbreviations: PNPNP, diimidotriphosphate ($O_3P-NH-P(O)_2-NH-PO_3^{5-}$); PNP, imidodiphosphate ($O_3P-NH-PO_3^{4-}$).

Inhibition kinetics were characterized by progress curve analysis at various inhibitor concentrations. Progress curves consistent with the model in Scheme 1 are described by the equation

$$P = v_s t + (v_o - v_s)(1 - e^{-kt})/k \quad (2)$$

where P represents the product (P_i) formed, v_o and v_s are the initial and final reaction rates, t is time, and k is the apparent first-order rate constant for the establishment of the final steady-state equilibrium.

The relationship between k in eq 2 and the rate and kinetic constants is given by

$$k = k_6 + k_5\left(\frac{[I]/K_i}{1 + [S]/K_m + [I]/K_i}\right) \quad (3)$$

The overall inhibition constant (K_i*) in Scheme 1 is defined as

$$K_i^* = \frac{[E][I]}{[EI] + [EI^*]} = K_i\left(\frac{k_6}{k_5 + k_6}\right) \quad (4)$$

Progress curves were fit to eq 2 by nonlinear least squares to determine the best fit values for v_o, v_s, and k using the SCIENTIST program from MicroMath, Inc.

The value of k_6 was determined by preincubating 0.6 μM AdoMet synthetase, 40 μM AdoMet, and inhibitor (100 μM PNP or 2 μM PNPNP) for 30 min in 50 mM Hepes/KCl, 50 mM KCl, and 10 mM $MgCl_2$ followed by addition of excess of PPP_i (10 mM). Incubations and assays were conducted at 25 °C in 50 mM Hepes/KCl, 50 mM KCl, and 10 mM $MgCl_2$. The progress curve obtained from this experiment reflects the regain in active enzyme following dissociation of the EI* complex in Scheme 1.

The value of K_i was determined by fitting v_o to the equation

$$v_o = \frac{V'}{1 + [I]/K_{i,app}} \quad (5)$$

where

$$V' = \frac{V_{max}}{1 + K_m/[S]} \quad (6)$$

Here $V_{max} = 1.2\ s^{-1}$ and $K_m = 16\ \mu$M.

From this value of $K_{i,app}$, K_i was evaluated using the equation

$$K_i = \frac{K_{i,app}}{1 + [S]/K_m} \quad (7)$$

$K^*_{i,app}$ was determined by fitting v_s to the equation

$$v_s = \frac{V'}{1 + [I]/K_{i,app}^*} \quad (8)$$

where the symbols are defined above. K_i* was determined using the equation

$$K_i^* = \frac{K_{i,app}^*}{1 + [S]/K_m} \quad (9)$$

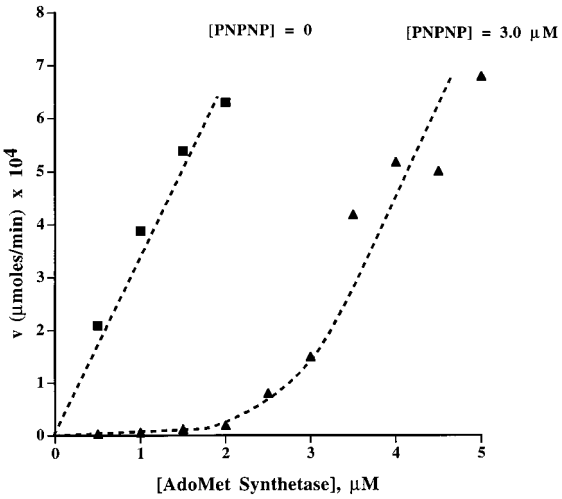

FIGURE 1: Tight binding inhibition of AdoMet synthetase by PNPNP. Enzyme activity in the overall reaction is plotted versus AdoMet synthetase concentration. Samples contained 0.1 mM L-[methyl-^{14}C]methionine and 0.1 mM ATP in 50 mM HEPES·$(CH_3)_4N^+$ with 50 mM KCl and 10 mM $MgCl_2$ at pH 8.0 in the presence or absence of 3.0 μM PNPNP. After incubation for 4 min in the presence of various concentrations of AdoMet synthetase, formation of [^{14}C]AdoMet was quantified as described in Materials and Methods.

Using the calculated values for k_6, K_i, and K_i*, the value of k_5 can be determined from eq 3:

$$\frac{k_5}{k_6} = \frac{K_i}{K_i^*} - 1 \quad (10)$$

Circular dichroism spectra were recorded on an Aviv model 62 instrument. Samples were contained in 0.1 mm path length cuvettes. Spectra were recorded from 200 to 260 nm for the enzyme alone and for the enzyme·AdoMet·PNPNP and enzyme·PNPNP complexes. Solutions contained 8 μM enzyme with various combinations of 40 μM AdoMet, 17 μM PNPNP, 12 mM $MgCl_2$ in 25 mM Tris/Cl, and 12 mM KCl at pH 8.0.

RESULTS

Inhibition of the AdoMet Synthetase Reaction by PNPNP. Inhibition of AdoMet formation by PNPNP showed a stoichiometric relationship between PNPNP concentration and enzyme concentration, as illustrated in Figure 1. The dependence of enzyme activity on enzyme concentration indicates that PNPNP is a tight binding inhibitor where free enzyme and the enzyme·inhibitor complex do not rapidly interconvert *(34−37)*. Further analysis of product formation in the presence and absence of PNPNP indicated the slow onset of inhibition. From these data a K_i comparable to the enzyme concentration of <1 μM was estimated. However detailed analysis of PNPNP inhibition of the overall reaction was not pursued because further studies revealed that accumulation of product(s) led to enhanced inhibition and a complex time dependence.

Inhibition of the Tripolyphosphatase Reaction by PNPNP in the Absence of AdoMet. To simplify characterization of inhibition by PNPNP, the inhibition of the tripolyphosphatase enzyme (PPP_iase) activity was characterized. In the absence of AdoMet, slow binding behavior is not observed and PNPNP is a classical competitive inhibitor with a K_i of 0.5

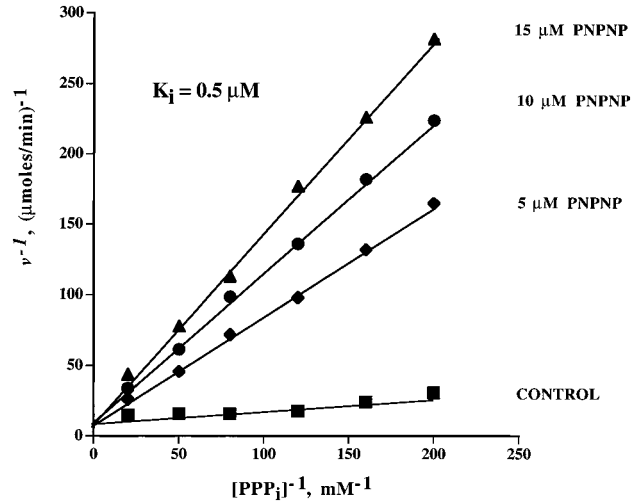

FIGURE 2: Competitive inhibition of the PPP_iase activity by PNPNP in the absence of AdoMet. Samples contained 5−50 μM PPP_i in 50 mM HEPES·$(CH_3)_4N^+$ with 10 mM KCl, 10 mM $MgCl_2$, 0−15 μM PNPNP, and 0.5 μM AdoMet synthetase at pH 7.8.

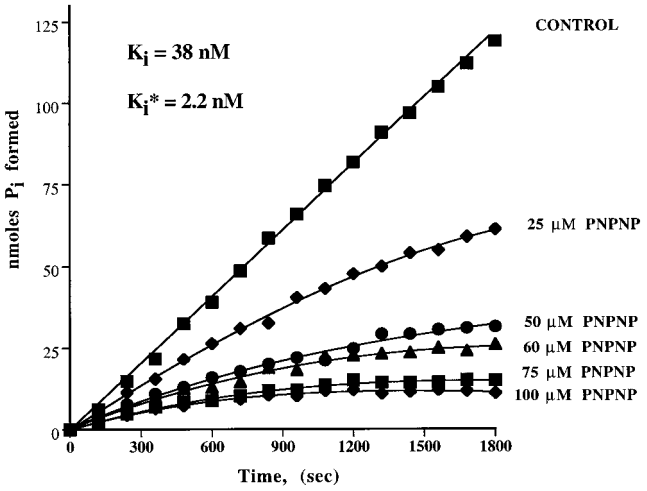

FIGURE 3: Time course for PNPNP inhibition of the PPP_iase activity in the presence of AdoMet. Samples contained 5.0 mM PPP_i in 50 mM HEPES·$(CH_3)_4N^+$ with 10 mM KCl, 10 mM $MgCl_2$, 0−10 μM PNPNP, 40 μM AdoMet, and 0.5 μM AdoMet synthetase at pH 7.8.

μM (Figure 2). Although the enzyme has a greater affinity for PNPNP than for PPP_i (K_m = 3 μM, K_i = 8 μM, with respect to ATP (*13*)), there is no evidence of tight binding behavior.

Inhibition PNPNP in the Presence of AdoMet. In the presence of a saturating concentration of AdoMet, PNPNP inhibition has slow onset behavior (Figure 3). In studies of the time-dependent inhibition by PNPNP the tight binding characteristics of PNPNP were overcome by using a high concentration of PPP_i in activity measurements (*35*). The progress curves reveal a dependence of the initial rates on the concentration of PNPNP showing that formation of the initial enzyme complex with PNPNP is rapid. The model for slow onset inhibition depicted in Scheme 1 best represents the inhibition of AdoMet synthetase by PNPNP (*35*). Values for v_o and v_s, determined by fitting the data in Figure 3 to eq 2 (Materials and Methods), were used to determine values of K_i = 38 nM and K_i* = 2.2 nM. The rate of dissociation of the enzyme·PNPNP·AdoMet complex (k_6) was obtained by preincubating enzyme with AdoMet and PNPNP followed by dilution into a 5000-fold excess of PPP_i and monitoring the recovery of enzyme activity with time; from this experiment a value for k_6 of 0.011 min^{-1} was obtained. The value of k_5 (Scheme 1), the rate of formation of the inhibitory enzyme·AdoMet·PNPNP complex (EI* in Scheme 1), was then calculated to be 0.19 min^{-1}. Table 1 summarizes the inhibition data.

Table 1: Inhibition of the Tripolyphosphatase Reaction by Polyphosphates

inhibitor ± AdoMet	K_i (μM)	slow onset inhibition	K_i* (μM)	k_5 (s^{-1})	k_6 (s^{-1})
PP_i − AdoMet	400	no			
PP_i + AdoMet	250	no			
PNP − AdoMet	19	no			
PNP + AdoMet	5.5	yes	0.8	0.03	5.2×10^{-3}
PPP_i − AdoMet	3.0[a]				
PPP_i + AdoMet	16[b]				
PNPNP − AdoMet	0.5	no			
PNPNP + AdoMet	0.038	yes	0.002	0.003	1.8×10^{-4}

[a] K_m value. In this case the K_m value approximates the dissociation constant (*13*). [b] K_m value. The dissociation constant is probably less than this due to slow dissociation.

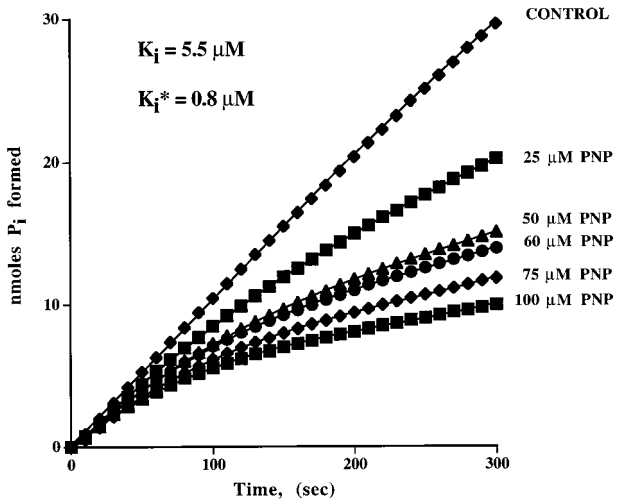

FIGURE 4: Time course for PNP inhibition of the PPP_iase activity in the presence of AdoMet. Samples contained 5 mM PPP_i in 50 mM Tris·HCl with 10 mM KCl, 10 mM $MgCl_2$, 0−100 μM PNP, 40 μM AdoMet, and 0.08 μM AdoMet synthetase at pH 7.8.

Inhibition PPP_i Hydrolysis by Pyrophosphate and the Pyrophosphate Analogue Imidodiphosphate (PNP). The question thus arose as to whether the slow binding behavior of PNPNP was due to its being an intermediate analogue or simply to the presence of the P−N(H)−P linkage. Thus, inhibition by the product PP_i and the analogue PNP was examined. In the absence of AdoMet, PNP and pyrophosphate (PP_i) are linear competitive inhibitors with K_i values of 19 and 400 μM, respectively (Table 1). In the presence of AdoMet, PNP inhibition is characterized by slow onset behavior, similar to that seen with PNPNP (Figure 4). However, unlike PNPNP, a plot of rate vs AdoMet synthetase concentration is linear, showing that tight binding inhibition is not present. Progress curve analysis of the inhibition by PNP reveals a K_i = 5.5 μM and a K_i* = 0.8 μM. Values for the rate of formation and dissociation of the inhibitory enzyme·AdoMet·PNP complex (EI* in Scheme 1) are 1.8 and 0.31 min^{-1}, respectively. Thus the lower affinity of PNP than PNPNP is reflected in the faster rate of the isomerization process with PNP analogue. For comparison, in the presence

of AdoMet PP_i remains a classical competitive inhibitor with a K_i of 250 μM.

Spectroscopic Studies of PNPNP Binding. To investigate the possibility of conformational changes upon formation of the inhibitory complexes, we examined the interaction of PNPNP and PNP with the enzyme by circular dichroism spectroscopy. The CD spectra were indistinguishable for the free enzyme and the enzyme·AdoMet·PNP(NP) complexes (not shown). Apparently gross secondary structural changes do not occur upon complex formation.

Diimidotriphosphate (PNPNP) Inhibition of the R244L AdoMet Synthetase Mutant. We recently characterized a mutant of AdoMet synthetase in which the active site arginine that normally binds the γ-phosphoryl group of ATP was changed to leucine (R244L) (*32*). In this mutant not only k_{cat} for AdoMet synthesis was reduced 1000-fold but most interestingly the PPP_i intermediate became free to reorient end-to-end within the active site, demonstrating weaker binding. When PNPNP inhibition of the AdoMet activated tripolyphosphatase activity of the R244L mutant was examined, the inhibition was competitive with K_i of 10 μM, comparable to the PPP_i K_m value of 16 μM, and the slow binding behavior was absent. The 5000-fold diminished affinity of the R244L mutant for PNPNP is consistent with the role for Arg244 in binding the polyphosphate chain (*32, 38*). The lack of the isomerization step(s) in the mutant and the rapid reorientation of the PPP_i intermediate suggest that the Arg244 mutation has hindered a conformational change that occurs when PPP_i binds to the wild-type enzyme.

DISCUSSION

The present studies have identified the nonhydrolyzable intermediate analogue PNPNP as a slow, tight binding inhibitor of AdoMet synthetase. Plausibly the tight binding behavior of PNPNP is partially due to its mimicry of the tightly bound PPP_i intermediate. Curiously, the PP_i analogue PNP also exhibits slow binding inhibition. Though not nearly as potent an inhibitor as PNPNP, the overall dissociation constant (K_i*) for PNP is 310-fold less than the K_i for PP_i, and the slow binding of PNP is in contrast to PP_i which binds and dissociates rapidly. While the slow binding behavior of PNPNP might have been attributed to a slow association rate as seen with intermediate (analog) binding to other enzymes (*39*), in the absence of AdoMet it is a simple competitive inhibitor. Thus the slow binding behavior of PNPNP probably arises from steps after the initial binding event, steps which depend on the presence of AdoMet. The inhibition of phosphoryl transferases by imidophosphate analogues have been long exploited due to the resistance to cleavage of the P−N bonds (*40*−*51*). Structurally PNP, and presumably other imidopolyphosphates, are essentially isosteric with their oxy analogues (*40, 41*).[3] However, the slow binding behavior of PNP and PNPNP is rather unusual; we found one other report of slow onset inhibition by this class of compounds, AMPPNP inhibition of the F1-ATPase (*43*). This suggests that the slow binding inhibition of AdoMet synthetase by PNP and PNPNP is the result of an unusual enzyme−imidophosphate interaction.

One obvious difference between the PPP_i/PP_i pair and the PNPNP/PNP couple is the chemistry of the −NH− moiety. In solution the Mg^{2+} complexes of PPP_i, PNP, PPP_i, and PNPNP have the same charge and the imido group is in the −NH− form (*42*). However at an active site the imido group might either form a protonated $-NH_2^+-$ bridge linkage or a deprotonated $-N^--$ group, neither of which is available for an −O− linkage. The crystallographically determined active site structure of the $E\cdot 2Mg^{2+}\cdot PP_i\cdot P_i$ complex has the 2 Mg^{2+} ions both coordinated to all 3 phosphoryl groups (*38*); such coordination might favor the deprotonation of the imido nitrogen (Figure 5). Furthermore the positive charge of the sulfonium center of AdoMet could also facilitate ionization of the NH moieties. For comparison, the pK_a of the −NH− group of the tetraethyl ester of PNP is 3.8, showing the acidity of this proton when the oxygens are uncharged (*42*). Formation of a N^- moiety would enhance binding to the metal ions and might provoke movement of positively charged active site residues; candidates for these residues are histidine-14 and lysines-165, -245, and -265 (*38*). Whether the slow inhibition phase is due in part to a proton-transfer reaction as well as active site reorganization is not yet clear.

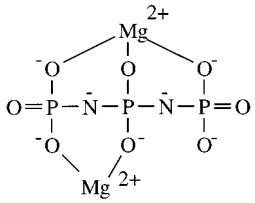

FIGURE 5: Model for ligation and ionization of PNPNP in the tight binding complex.

AdoMet is required for both PNPNP and PNP to obtain maximally tight binding to the enzyme. This parallels the ~20-fold AdoMet activation of the PPP_iase activity (*13, 32*). Presumably, AdoMet binding provokes active site residues to reorient to provide better juxtaposition for PPP_i hydrolysis. Perhaps in the absence of AdoMet the group(s) that interact with the imido nitrogen(s) of PNPNP is (are) not in an optimal position. Thus, in the presence of AdoMet the initial complexes formed by PNP and PNPNP have 7- and 19-fold less affinity than the final complexes, respectively. However, even the initial complexes formed in the presence of AdoMet have 3.5- and 13-fold higher affinity than the complexes formed in the absence of AdoMet.

In light of these results it is interesting that α,β-imido-ATP (AMPNPP) has a K_i of ~2 μM, nearly 50 times less than the K_m for either ATP or the β,γ-imido analogue AMPPNP (*27*). This suggests that the origin of tight binding is in the α,β-imido moiety, which is the anticipated binding position of PNP.

The notable difference between wild-type AdoMet synthetase and the R244L mutant in the inhibition by PNPNP is rather tantalizing. In contrast to the wild-type enzyme, the inhibition of the R244L mutant is rapid with a 10 μM K_i,

[3] AdoMet synthetase has poor affinity for the methylene-bridged ATP analogues, α,β-methylene-ATP and β,γ-methylene-ATP, and does not detectably bind α,β:β,γ-dimethylene-ATP (*27*). This behavior is in contrast with the good affinity for the corresponding imido-ATP analogues (*27*). These observations reinforce the preferred use of imido rather than methylene polyphosphates due to greater steric fidelity to the natural compounds. In addition the imido-bridged compounds have available a unique ionization state and can potentially form novel species as proposed herein.

comparable to the 16 μM K_m for PPP_i for the mutant. The 5000-fold decrease in affinity for PNPNP by the R244L mutant relative to wild-type enzyme parallels the 1200-fold decrease in specific activity for AdoMet synthesis of the R244L mutant, suggesting that the free energy of PNPNP binding correlates with the catalytic activity. The tripolyphosphatase V_{max} in the mutant is only decreased 10-fold, showing that the R244 side chain and PNPNP affinity most closely reflect the factors influential in AdoMet forming step. This observation follows Wolfenden's analysis of the behavior of the interactions of enzymes with transition state analogues (*52*) as extended to mutant enzymes (*53*, *54*) and suggests that PNPNP acts at least in part as a transition state analogue. Since the K_m value for PPP_i is comparable in both mutant and wild-type enzymes, events after binding which yield the high affinity for PNPNP seen with the wild-type enzyme appear to be impaired in the mutant. Although the detailed rationale for the potent inhibition by imidophosphates is not yet ascertained, ongoing spectroscopic and crystallographic studies may elucidate the structural basis for these interactions.

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REFERENCES

1. Cantoni, G. L. (1975) *Annu. Rev. Biochem. 44*, 435−451.
2. *The Biochemistry of Adenosylmethionine* (1977) (Salvatore, F., Borek, E., Zappia, V., Williams-Ashman, H. G., and Schlenk, F., Eds.), Columbia University Press, New York.
3. Tabor, C. W., and Tabor, H. (1984) *Annu. Rev. Biochem. 53*, 749−790.
4. Aletta, J. M., Cimato, T. R., and Ettinger, M. J. (1998) *Trends Biochem. Sci. 23*, 89−91.
5. Eden, S., Hashimshony, T., Keshet, I., and Cedar, H. (1998) *Nature 394*, 842.
6. Ravanel, S., Gakiere, B., Job, D., and Douce, R. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 7805−7812.
7. Chiang, P. K., Gordon, R. D., Tal, J., Zeng, G. C., Doctor, B. P., Pardhasaradhi, K., and McCann, P. P. (1996) *FASEB J. 10*, 471−480.
8. Zingg, J.-M., and Jones, P. A. (1997) *Carcinogenesis 5*, 869−882.
9. Frey, P. A. (1990) *Chem. Rev. 90*, 1343−1357.
10. Reichard, P. (1997) *Trends Biochem. Sci. 22*, 81−85.
11. Johnson, M. K. (1998) *Curr. Opin. Chem. Biol. 2*, 173−181.
12. Mudd, S. H. (1973) in *The Enzymes* (Boyer, P. D., Ed.) Vol. 8, pp 121−154, Academic Press, New York.
13. Markham, G. D., Hafner, E. W., Tabor, C. W., and Tabor, H. (1980) *J. Biol. Chem. 255*, 9082−9092.
14. Kotb, M., and Geller, A. M. (1993) *Pharmacol. Ther. 59*, 125−143.
15. Mato, J. M., Alvarez, L., Ortiz, P., and Pajares, M. A. (1997) *Pharmacol. Ther. 73*, 265−280.
16. Lombardini, J. B., and Talalay, P. (1973) *Mol. Pharmacol. 9*, 542−560.
17. Sufrin, J. R., and Lombardini, J. B. (1982) *Mol. Pharmacol. 22*, 752−759.
18. Sufrin, J. R., Coulter, A. W., and Talalay, P. (1979) *Mol. Pharmacol. 15*, 661−677.
19. Sufrin, J. R., Lombardini, J. B., and Alks, V. (1993) *Biochim. Biophys. Acta 1202*, 87−91.
20. Kappler, F., Vrudhula, V. M., and Hampton, A. (1987) *J. Med. Chem. 30*, 1599−1603.
21. Vrudhula, V. M., Kappler, F., Afshar, C., Ginell, S. L., Lessinger, L., and Hampton, A. (1989) *J. Med. Chem. 32*, 885−890.
22. Kappler, F., and Hampton, A. (1990) *J. Med. Chem. 33*, 2545−2551.
23. Lavrador, K., Guillerm, A. D., and Guillerm, G. (1998) *J. Enzyme Inhib. 13*, 361−367.
24. Goldberg, B., Rattendi, D., Lloyd, D., Sufrin, J. R. and Bacchi, C. J. (1998) *Biochemical Pharmacol. 56*, 95−103.
25. Sufrin, J. R., Dunn, D. A., and Marshall, G. R. (1980) *Mol. Pharmacol. 19*, 307−313.
26. Mudd, S. H. (1963) *J. Biol. Chem. 238*, 2156−2163.
27. Ma, Q.-F., Kenyon, G. L., and Markham, G. D. (1990) *Biochemistry 29*, 1412−1416.
28. Broom, A. D., and Milne, G. H. (1975) *J. Heterocycl. Chem. 12*, 171−174.
29. Webb, M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4884−4887.
30. Chifflet, S., Torriglia, A., Chiesa, R., and Tolosa, S. (1986) *Anal. Biochem. 168*, 1−4.
31. Baykov, A. A., Evtushenko, O. A., and Avaeva, S. M. (1988) *Anal. Biochem. 171*, 266−270.
32. Reczkowski, R. S., Taylor, J. C., and Markham, G. D. (1998) *Biochemistry 39*, 13499−13506.
33. Cleland, W. W. (1979) *Methods Enzymol. 63*, 103−138.
34. Szedlacsek, S. E., and Duggleby, R. G. (1995) *Methods Enzymol. 249*, 144−180.
35. Morrison, J. F., and Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 201−301.
36. Morrison, J. F., and Stone, S. R. (1985) *Comments Mol. Cell. Biophys. 2*, 347−368.
37. Williams, J. W., and Morrison, J. F. (1979) *Methods Enzymol. 63*, 437−467.
38. Takusagawa, F., Kamitori, S., and Markham, G. D. (1996) *Biochemistry 35*, 2586−2596.
39. Cleland, W. W. (1990) *Biochemistry 29*, 3194−3197.
40. Larsen, M., Willet, R., and Yount, R. G. (1969) *Science 166*, 1510−1511.
41. Yount, R. G., Babcock, D., Ballantyne, W., and Ojala, D. (1971) *Biochemistry 10*, 2484−2489.
42. Reynolds, M. A., Gerlt, J. A., Demou, P. C., Oppenheimer, N. J., and Kenyon, G. L. (1983) *J. Am. Chem. Soc. 105*, 6475−6481.
43. Philo, R. D., and Selwyn, M. J. (1974) *Biochem. J. 143*, 745−749.
44. Lillehaug, J. R. (1978) *Biochim. Biophys. Acta 525*, 357−363.
45. Cross, R. L., and Nalin, C. M. (1982) *J. Biol. Chem. 257*, 2874−2881.
46. Belda, F. J. F., Carmona, F. G., Cánovas, J. C., Gómez-Fernández, J. C., and Lozano, J. A. (1983) *Biochem. J. 210*, 727−735.
47. Gresser, M. J., Beharry, S., and Moennich, D. M. (1984) *Curr. Top. Cell. Reg. 24*, 365−378.
48. Batra, J. K., Lin, C. M., and Hamel, E. (1987) *Biochemistry 26*, 5925−5931.
49. Leventhal, P. S., and Bertics, P. J. (1991) *Biochemistry 30*, 1385−1390.
50. Fraichard, A., Trossat, C., Perotti, E., and Pugin, A. (1996) *Biochemie 78*, 259−266.
51. Li, R. S., Muscate, A., and Kenyon, G. L. (1996) *Bioorg. Chem. 24*, 251−261.
52. Wolfenden, R. (1969) *Nature 223*, 704−705.
53. Philips, M. A., Kaplan, A. P., Rutter, W. J., and Bartlett, P. A. (1992) *Biochemistry 31*, 959−963.
54. Kerr, K. M., and Hedstrom, L. (1997) *Biochemistry 36*, 13365−13373.

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