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Specificity of *S*-Adenosylmethionine Synthetase for ATP Analogues Mono- and Disubstituted in Bridging Positions of the Polyphosphate Chain†

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ABSTRACT: The entire family of ATP analogues that are either mono- or disubstituted with imido and methylene bridges in the polyphosphate chain of ATP have been investigated as substrates and inhibitors of *S*-adenosylmethionine synthetase (ATP:L-methionine *S*-adenosyltransferase). The disubstituted analogues adenosine 5′-(α,β:β,γ-diimidotriphosphate) (AMPNPNP) and adenosine 5′-(α,β:α,β'-diimidotriphosphate) [AMP(NP)$_2$] have been synthesized for the first time, and a new route to adenosine 5′-(α,β:β,γ-dimethylenetriphosphate) (AMPCPCP) has been developed. *S*-Adenosylmethionine synthetase catalyzes a two-step reaction: the intact polyphosphate chain is displaced from ATP, yielding AdoMet and tripolyphosphate, followed normally, but not obligatorily, by the hydrolysis of the tripolyphosphate to pyrophosphate and orthophosphate. Uniformly, the imido mono- or disubstituted derivatives are both better substrates and better inhibitors than their methylene counterparts. AMPNPNP reacts rapidly to give a single equivalent of product per active site, but subsequent turnovers are at least 1000-fold slower, enabling it to be used to quantify enzyme active site concentrations. In contrast, AMPCPCP is not detectably a substrate ($<10^{-5}$% of ATP). AMP(NP)$_2$, a branched isomer of linear AMPNPNP, was not a substrate but was a linear competitive inhibitor, >100 fold more potent than ADP, indicating a reasonable degree of bulk tolerance at the α-phosphoryl group binding site. Adenosine 5′-(α,β-imidotriphosphate) (AMPNPP) is a surprisingly potent inhibitor as well as substrate, with an inhibition constant that is ~60-fold less than the K_m for ATP, and is an ~1000-fold better inhibitor than adenosine 5′-(α,β-methylenetriphosphate) (AMPCPP). These findings reinforce the notion that imido analogues of ATP are more suitable analogues of ATP than their corresponding methylene compounds.

The only known biosynthetic route to *S*-adenosylmethionine arises from a reaction catalyzed by *S*-adenosylmethionine synthetase. *S*-Adenosylmethionine (AdoMet) synthetase (ATP:L-methionine *S*-adensoyltransferase) catalyzes a two-step reaction in which ATP and methionine first combine to form AdoMet and tripolyphosphate; the tripolyphosphate is then hydrolyzed to PP_i and P_i (which originates as the γ-phosphoryl group of ATP) before product release (Mudd, 1973; Tabor & Tabor, 1984). In the presence of the ATP analogue AMPPNP[1] multiple turnovers of AdoMet formation can occur without polyphosphate bond cleavage (Markham et al., 1980); therefore, AdoMet synthetase is a rare enzyme for which the influence of substitution in both the α,β- and the β,γ-bridging positions of the polyphosphate chain of ATP on enzyme affinity and activity can be assessed. Furthermore, there is the possibility that an ATP analogue containing nonhydrolyzable bonds in both the α,β- and β,γ-bridges would

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[1] Abbreviations: AMPNPNP, adenosine 5′-(α,β:β,γ-diimidotriphosphate); AMP(NP)$_2$, adenosine 5′-(α,β:α,β'-diimidotriphosphate); PNPNP, diimidotriphosphate; AMPNP, adenosine 5′-(α,β-imidodiphosphate); AMPNPP, adenosine 5′-(α,β-imidotriphosphate); AMPPNP, adenosine 5′-(β,γ-imidotriphosphate); PCPCP, bis[(dihydroxyphosphinyl)methyl]phosphinate; AMPCPCP, adenosine 5′-(α,β:β,γ-dimethylenetriphosphate); AMPCPP, adenosine 5′-(α,β-methylenetriphosphate); AMPPCP, adenosine 5′-(β,γ-methylenetriphosphate); AMP(CP)$_2$, adenosine 5′-(α,β:α,β'-dimethylenetriphosphate); AMP(OP)$_2$, adenosine 5′-(α,β:α,β'-triphosphate); PEI, poly(ethylenimine); LSIMS, liquid secondary ion mass spectrometry; SP, sulfopropyl; TEA, triethylammonium; QAE, quaternary aminoethyl; DEAE, diethylaminoethyl.

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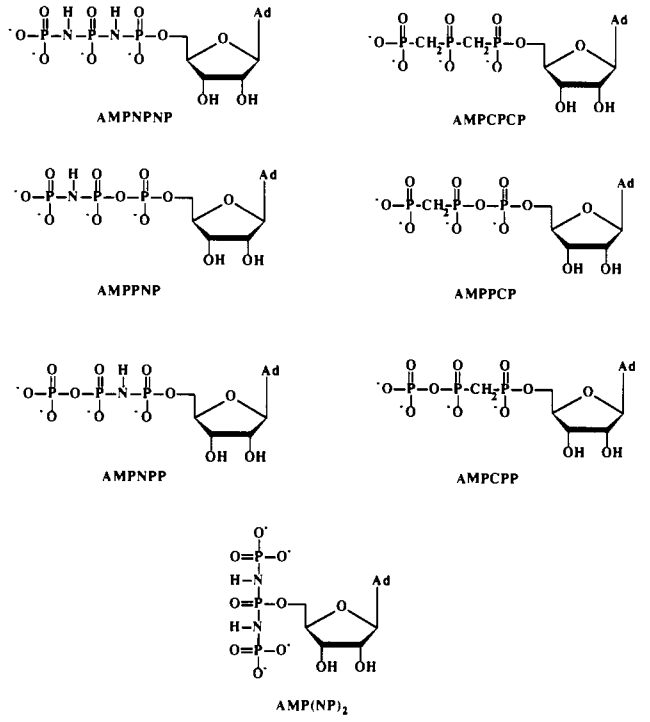

FIGURE 1: Structures of the ATP analogues used in this study.

allow direct measurement of the equilibrium constant for the formation of AdoMet and tripolyphosphate (analogue) from ATP (analogue) and methionine, a chemical reaction for which the thermodynamics have not been characterized. In reactions of monosubstituted nonhydrolyzable ATP analogues such as AMPPNP, the tripolyphosphate derivative formed can reorient so that the P–O–P linkage occupies the β,γ-phosphoryl group site; thus, the tripolyphosphate analogue can be hydrolyzed to P_i and the corresponding pyrophosphate derivative, preventing determination of the equilibrium constant for the first step of the overall AdoMet synthetase reaction. An ATP analogue substituted in both the α,β- and β,γ-bridge positions could obviate this problem.

Recent synthetic advances have been developed that permit ready access to a broad variety of ATP analogues that are nonhydrolyzable at both the α,β- and the β,γ-positions (Davisson et al., 1987; Ma et al., 1988, 1989). In this paper we compare existing analogues as substrates and inhibitors of AdoMet synthetase, present for the first time a synthetic method for preparation of the bis-imido derivatives AMPNPNP and $AMP(NP)_2$, and provide a new route to the bis-methylene analogue adenosine 5′-(α,β:β,γ-dimethylenetriphosphate) (AMPCPCP). The structures of all of these ATP analogues are shown in Figure 1. Behaviors of the mono- and disubstituted imido and methylene analogues as substrates and inhibitors of AdoMet synthetase are compared.

Materials and Methods

^{31}P NMR spectra were obtained at 79.5 MHz on a Nicolet NTCFT-1180 NMR spectrometer. A sweep width of 4000 Hz, a probe temperature of 25 °C, and ^1H broad-band decoupling were used in ^{31}P NMR measurements. Chemical shifts were determined relative to 85% H_3PO_4 with positive shifts being downfield from the reference. High- and low-resolution liquid secondary ion mass spectra were taken on a Kratos MS-50 mass spectrometer, and a negative ion probe was used to measure the mass of the M − 1 peak.

PEI–cellulose plates were obtained from J. T. Baker Co. Other chemicals were from Aldrich. AMPCPP, AMPPCP, and AMPPNP were purchased from Sigma and showed single spots on PEI–cellulose thin-layer chromatography. [*methyl*-^{14}C]Methionine (51.8 mCi/mmol) was purchased from New England Nuclear.

Trichloro[(dichlorophosphoryl)imino]phosphorane was synthesized by the method of Emsley et al. (1971) and used as a precursor for the synthesis of both AMPNPNP and $AMP(NP)_2$.

[P,P-Dichloro-N-(dichlorophosphinyl)phosphinimyl]-phosphorimidic trichloride was prepared as described by Riesel and Somieski (1975). Trichloro[(dichlorophosphoryl)imino]phosphorane (17.8 g, 66 mmol) was dissolved in 65 mL of dry *s*-tetrachloroethane cooled to 0 °C in an ice bath. 1,1,1,3,3,3-Hexamethyldisilazane (10.9 g, 68 mmol) dissolved in 15 mL of *s*-tetrachloroethane was added to the stirring solution. After the mixture had been stirred at 60 °C for 12 h, phosphorus pentachloride (14.4 g, 69 mmol) was added, and the reaction was continued at 120 °C for another 12 h. Solvent was removed under vacuum, and product was purified by vacuum distillation at 165 °C (0.5 Torr). Colorless crystals formed after cooling to room temperature and weighed 17.5 g (69% yield). ^{31}P NMR ($CDCl_3$): δ 4.18 (1 P, d, J = 32 Hz), −10.49 (1 P, d, J = 30 Hz), −19.57 (1 P, t, J = 31 Hz).

The pentasodium salt of diimidotriphosphate was synthesized by mixing [*P,P*-dichloro-*N*-(dichlorophosphinyl)-phosphinimyl]phosphorimidic trichloride (1.86 g, 4.8 mmol) with 20 mL of 0.5 N HCl and stirring at room temperature overnight. Solvent was removed below 30 °C in vacuum. The residue was separated on a QAE-Sephadex column (3.5 × 50 cm) eluted with a 0–0.6 M gradient of triethylammonium bicarbonate, and solvent was removed as before. Four aliquots of 50 mL of methanol were added and then evaporated to remove triethylammonium bicarbonate and remaining water. The residue was passed through a SP-Sephadex column (Na^+ form, 2 × 50 cm). The fractions containing PNPNP were pooled, and a white powder weighing 1.25 g (71% yield) was obtained. The compound gave a single spot (R_f = 0.3) on PEI–cellulose thin-layer chromatography (Rowley & Kenyon, 1974) using 1.0 M TEA–HCO_3^- eluent. ^{31}P NMR (D_2O, pD 7.5): δ −0.12 (2 P, d, J = 5 Hz), −1.82 (1 P, t, J = 5 Hz) LSIMS spectrum showed the expected parent ion of the free acid from [255 (M − 1)].

AMPNPNP and AMP(NP)$_2$ were prepared by an adaptation of the method of Davisson et al. (1987). The pentasodium salt of PNPNP (366 mg, 1 mmol) was passed through a SP-Sephadex C-25 column (H^+ form, 2 × 50 cm) and the appropriate fractions were pooled and neutralized with tetrabutylammonium hydroxide to pH 8. Solvent was removed, and the residue was dried by repeated evaporation of three aliquots of 50 mL of methanol and two aliquots of 30 mL of dry acetonitrile. The resulting tetrabutylammonium salt of PNPNP was dissolved in dry acetonitrile, and 5′-tosyladenosine (211 mg, 0.5 mmol) was added. After the mixture had been stirred at room temperature for 26 h with protection from moisture, the solvent was removed, and the residue was dissolved in 20 mL of H_2O. The solution was loaded on a SP-Sephadex column (H^+ form, 2.5 × 50 cm), eluted with H_2O, and 2-mL fractions were collected. AMPNPNP was eluted first (fractions 41–45) followed by $AMP(NP)_2$ (fractions 46–51). Both AMPNPNP and $AMP(NP)_2$ were further purified by DEAE-Sephadex A-25 column chromatography and converted to their respective sodium salts by the method described previously (Ma et al., 1988).

The sodium salt of AMPNPNP was obtained as a white powder and weighed 21 mg (7% yield). It gave a single spot

(R_f = 0.2) on PEI–cellulose thin-layer chromatography with 0.5 M TEA–HCO_3^- eluent. ^{31}P NMR (D_2O, pD 7.8, ^1H broad-band decoupling on): δ 0.59 (1 P, d, J = 4.1 Hz, α-P), −0.96 (1 P, d, J = 5.1 Hz, γ-P), −4.4 (1 P, t, J = 4.5 Hz, β-P). ^{31}P NMR (D_2O, pD 7.8, ^1H broad-band decoupling off): δ 0.59 (1 P, m, α-P), −0.96 (1 P, d, J = 5.1 Hz, γ-P), −4.4 (1 P, t, J = 4.5 Hz, β-P). The ^{31}P NMR pH titration curves (Ma et al., 1989) were fully consistent with the structure. Accurate mass measured parent ion of its free acid form (M − 1): calculated for $C_{10}H_{16}O_{11}N_7P_3$, 504.0197; found 504.0195.

AMP(NP)$_2$, as its sodium salt, was also isolated as a white powder and weighed 17 mg (5.7% yield). It showed a single spot (R_f = 0.2) on PEI–cellulose thin-layer chromatography with 0.5 M TEA–HCO_3^- eluent. ^{31}P NMR (D_2O, pD 4.8 ^1H broad-band decoupling on): δ 5.21 (1 P, t, J = 7.5 Hz, α-P), −3.99 (2 P, d, J = 7.5 Hz, β,β′-P). ^{31}P NMR (D_2O, pD 4.8, ^1H broad-band decoupling off): δ 5.2 (1 P, m, α-P), −3.98 (2 P, d, J = 7.5 Hz, β,β′-P). ^{31} P NMR pH titration curves were fully consistent with the proposed structure. The liquid secondary ion mass spectrum gave a molecular weight of 505, as expected.

AMPNPP was synthesized enzymatically from AMPNP and phosphocreatine in the same way as described previously (Ma et al., 1988), but AMPNP was prepared by the alternative method of Tomasz et al. (1988). ATP contamination was ruled out by examining AMPNPP in the RNA polymerase reaction (Promega Biotec, 1988).

AMPCPCP was synthesized in a similar fashion to AMPNPNP. The pentasodium salt of PCPCP was prepared by the method described by Trowbridge et al. (1972). The sodium salt (364 mg, 1 mmol) was then converted into its tetrabutylammonium salt and treated with 5′-tosyladenosine (211 mg, 0.5 mmol). The AMPCPCP was purified in the same way as described in the AMPNPNP synthesis. The product, as a sodium salt, was a white powder and weighed 54 mg (18% yield). The liquid secondary ion mass spectrum of the free acid form of AMPCPCP showed an expected parent ion (M − 1, 502). ^{31}P NMR (D_2O, pD 7.7, ^1H broad-band decoupling on): δ 27.8 (1 P, t, J = 9.5 Hz, β-P), 18.5 (1 P, d, J = 10 Hz, α-P), 15.3 (1 P, d, J = 8.8 Hz, γ-P). The ^{31}P NMR pH titration curves were consistent with the structure.

AdoMet synthetase was purified to homogeneity from *Escherichia coli* strain DM22pK8 as described previously (Markham et al., 1980; Markham, 1981). Enzyme concentrations are expressed as active site concentrations using a subunit molecular mass of 41.9 kDa.

AdoMet synthetase assays measured the incorporation of [*methyl*-^{14}C]methionine into AdoMet (Markham et al., 1980). At the high (up to 0.5 mM) enzyme levels used in this study, 5% of a single turnover can readily be measured by this assay. Experiments were performed at 22 °C.

Results and Discussion

Synthesis and Characterization of ATP Analogues. Although PNPNP has been well characterized (Quimby et al., 1960; Irani & Callis, 1961; Nielson & Pustinger, 1964; Pollard et al., 1964; Töpelmann et al., 1979), AMPNPNP has not been previously reported. It was synthesized by treating 5′-tosyladenosine with the tetrabutylammonium salt of PNPNP as described for similar syntheses by Davisson et al. (1987). In the course of purification two products were obtained, the expected AMPNPNP and a second nucleoside imidotriphosphate derivative. Further characterization of the second product showed it to be AMP(NP)$_2$, a compound in which the adenosine moiety is attached to the central phosphorus of the triphosphate chain. In contrast, reaction of 5′-tosyladenosine with tripolyphosphate according to the method of Davisson et al. (1987) yielded only ATP with no detectable isomeric AMP(OP)$_2$ (<1%). A search of the literature revealed that the ATP isomer AMP(OP)$_2$ has only been described once and it was shown to be a substrate for neither hexokinase nor *E. coli* aminoacyl-tRNA synthetase, the only enzymes with which it was tested (Kozarich et al., 1973).

Table I: Interaction of Methylene Analogues of ATP with *S*-Adenosylmethionine Synthetase[a]

compound	steady-state rate (min^{-1})	K_i (mM)
ATP	96	
AMPCPP	0.02	2.0
AMPPCP	<0.003	8.5
AMPCPCP	$<5 \times 10^{-5}$	>20

[a] AMPCPP and AMPPCP were tested as substrates at 10 mM. A single turnover with AMPPCP has a >4-h half time. There is <5% of a single turnover from 2 mM AMPCPCP is 2 h. Inhibition by AMPCPCP was not detectable (<5%) at 3 mM nucleotide. Inhibition experiments were performed in 50 mM Hepes/KOH, 50 mM KCl, 25 mM $MgCl_2$, 0.1 mM ATP, and 0.5 mM methionine (K_m values for ATP and methionine are 0.12 and 0.08 mM, respectively) (Markham et al., 1980). Substrate activity tests were performed in 50 mM Hepes/KOH, pH 8.0, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM [*methyl*-^{14}C]methionine, and nucleotide. Enzyme active site concentrations between 0.03 and 0.5 mM were used.

In view of the recent success of the method of Davisson et al. (1987) in preparation of a variety of ATP analogues, we decided to synthesize AMPCPCP by that method since it is more convenient than the earlier method (Trowbridge et al., 1972). Properties of the compound prepared by the two methods were identical. In the synthesis of AMPCPCP from 5′-tosyladenosine and PCPCP, none of the isomeric AMP(CP)$_2$ (<1%) could be detected by ^{31}P NMR spectroscopy.

Studies with AdoMet Synthetase. (A) Methylene Analogues of ATP. ATP analogues containing single methylene substitutions in both the α,β- and β,γ-bridge positions of the polyphosphate chain have been known for some time (Myers et al., 1963, 1965), and these monosubstituted derivatives are available commercially. These analogues have been used with several enzymes with a mixed record of binding productively either as substrates or as potent inhibitors (Ashman & Keech, 1975; Burger & Lowenstein, 1970; Cooke & Murdoch, 1973; Cuee et al., 1968; Horak & Zalik, 1976; Krug et al., 1973; Larsen et al., 1969; Mannherz & Goody, 1976; Milner-White & Rycroft, 1983; Morley & Stadtman, 1970; Rose et al., 1979; Taketa et al., 1971). The disubstituted compound AMPCPCP, which was previously reported (Trowbridge et al., 1972), has not previously been tested with enzymes.

Table I reports results of the use of the methylene bridging analogues with AdoMet synthetase. Both AMPCPP and AMPPCP are poor substrates for the enzyme (maximally 0.02% of the activity of ATP), and AMPCPCP has no detectable substrate activity (<10^{-5}% of the activity of ATP). For AMPCPP the rate of product formation is linear with time from within 10% of a single enzyme turnover, indicating that product release is not rate limiting. Although AMPPCP is clearly a substrate, the rate of even a single turnover with AMPPCP (half-time ~4 h) is so slow as to prevent determination of the steady-state rate.

The monosubstituted compounds are at best poor inhibitors with K_i values at least 20 times greater than the K_m for ATP (0.12 mM under these conditions as illustrated in Figure 2A). At best, only extremely weak inhibition was observed with the disubstituted AMPCPCP. Between the monosubstituted compounds, AMPCPP was both the better substrate and the better inhibitor, indicating that the enzyme has a greater tolerance of geometrical distortions at the α,β-bridge position,

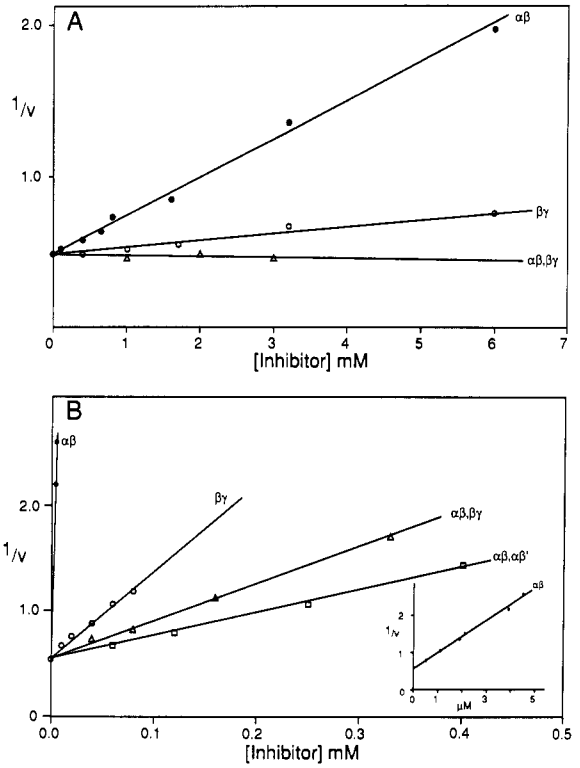

FIGURE 2: Inhibition of AdoMet synthetase by ATP analogues. The position of substitution for oxygen is shown adjacent to the corresponding data. (A) Inhibition of AdoMet synthetase by methylene analogues of ATP. (B) Inhibition of AdoMet synthetase by imido analogues of ATP. The insert expands the region showing inhibition by AMPNPP. Solutions contained 50 mM Hepes/KOH, 50 mM KCl, 25 mM $MgCl_2$, 0.1 mM ATP, and 0.5 mM methionine. V is expressed as nanomoles of AdoMet formed per minute.

Table II: Interaction of Imido Analogues of ATP with *S*-Adenosylmethionine Synthetase[a]

compound	burst rate (min^{-1})	steady-state rate (min^{-1})	K_i (mM)
ATP	ND	96	
AMPNPP	2.8	0.10	0.0022
AMPPNP	3.5	0.04	0.09
AMPNPNP	0.6	<0.001	0.16
iso-AMPNPNP		<0.001	0.22

[a] ND, not determined. All compounds were tested as substrates at 1.1 mM. Other conditions are as described in Table I.

perhaps not surprisingly since no enzymatic reactions occur at this position. The effects of methylene substitution appear to be cumulative since AMPCPCP was a poorer inhibitor (at least 2-fold) than either of the monosubstituted compounds.

(*B*) *Imido Analogues of ATP.* The interaction of AdoMet synthetase with the imido analogues proved to be considerably more intriguing than with the methylene analogues. At saturating concentrations (see below) all of the linear NH bridge containing ATP analogues (AMPNPP, AMPPNP, and AMPNPNP) were reasonably good substrates on a single turnover basis (Table II), although AMPNPNP gave only ~1% of the activity of ATP. As is illustrated in Figure 3 for AMPNPNP, the reactions with the above-three imido derivatives showed a burst of product formation followed by a slower steady-state rate; the burst corresponds to formation of enzyme-bound products, and the slow steady-state rate evidently results from the slow rate of product release from the [enzyme–AdoMet–tripolyphosphate analogue] complex as was previously shown for AMPPNP (Markham et al., 1980). The burst rates for formation of enzyme-bound products were approximately the same for AMPNPP and

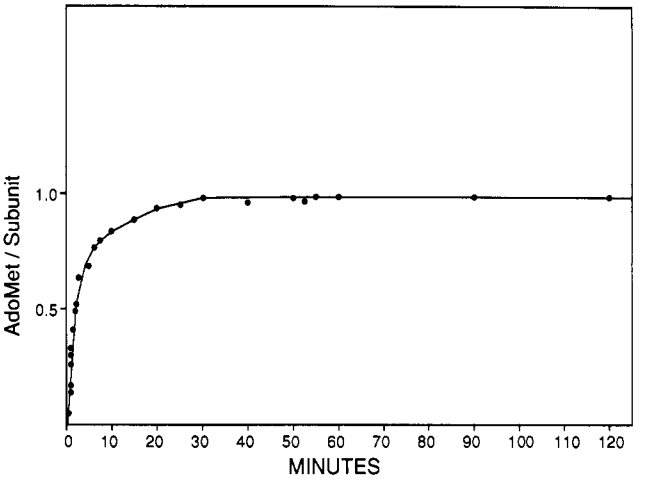

FIGURE 3: Time course of product formation from AMPNPNP and methionine. Solutions contained 1.1 mM AMPNPNP and 0.5 mM [*methyl*-^{14}C]methionine in 50 mM Hepes/KOH, pH 8.0, 50 mM KCl, and 10 mM $MgCl_2$. Enzyme active site concentrations between 0.03 and 0.5 mM were used. Data are expressed as moles of AdoMet formed per mole of enzyme active sites.

AMPPNP; however, the burst rate was ca. 5-fold slower with the disubstituted AMPNPNP. Furthermore, while multiple turnovers were observed with AMPNPP and AMPPNP, subsequent turnovers with AMPNPNP occurred at an unmeasurably slow rate (<0.05 turnover/h). In contrast to the linear analogues, the bifurcated $AMP(NP)_2$ had no detectable substrate activity (<10% of a single turnover in 2 h).

Figure 2B compares the inhibition of AdoMet synthetase by the four imido-ATP analogues. All were good inhibitors, although $AMP(NP)_2$ was the poorest. Further experiments with $AMP(NP)_2$ confirmed that it was a linear competitive inhibitor with respect to ATP with a K_i value of 0.10 mM compared to the ATP K_m of 0.12 mM (not shown). Remarkably, AMPNPP was a very good inhibitor of the enzyme with a K_i value that is ~60-fold lower than the K_m for ATP and 1000-fold less than the K_i value for AMPCPP. AMPNPP is thus the most potent known monosubstrate inhibitor of AdoMet synthetase, although certain bisubstrate analogues are better inhibitors (Kappler et al., 1988). Determination of the steady-state kinetic behavior with AMPNPP as substrate was complicated by the low K_m and low V_{max}, which necessitated use of enzyme concentrations comparable to the substrate concentration; an upper limit on the K_m of 5 μM was obtained, consistent with the measured K_i value. In contrast to the methylene analogues, the imido substitution does not cause cumulative effects on the affinity for the enzyme since AMPNPNP is a poorer inhibitor than either AMPNPP or AMPPNP.

Conclusions

The reaction catalyzed by AdoMet synthetase with AMPNPNP reveals several new features regarding enzyme function. The rapid formation of a single equivalent per subunit of enzyme-bound products provides evidence that the equilibrium constant for the reaction

[enzyme·AMPNPNP·methionine] ⇄ [enzyme·AdoMet·PNPNP]

lies far toward products; with AMPPNP, the equilibrium constant for the corresponding reaction was estimated to be $>10^4$ (Markham et al., 1980), and a similar estimate has been obtained for the reaction with ATP (unpublished results). Thus, at least at the active site, formation of the sulfonium

compound AdoMet is a thermodynamically favorable process. The extremely slow steady-state rate of product formation from AMPNPNP and the lack of substrate activity of AMPCPCP has, however, thwarted our attempts to determine the equilibrium constant for interconversion of free substrates and products. Determination of the thermodynamics of the first step of the overall AdoMet synthetase reaction will seemingly require an as yet unknown variant of AdoMet synthetase which either has lower affinity for the ternary product complex or lacks hydrolytic activity entirely; attempts to create such a variant by in vitro mutagenesis are underway.

The extraordinarily slow rate of turnover with AMPNPNP provides a novel use of the compound as an active site titrant for AdoMet synthetase, in the same fashion that *p*-nitrophenyl esters have been used to quantify active site concentrations of serine proteases (Bender et al., 1962). Combined with the resistance of P–N bonds to most phosphatases and nucleotidases, this stoichiometric reaction with AMPNPNP should find practical use in quantifying AdoMet synthetase levels in cellular extracts.

The entire imido class of ATP analogues has substantially higher affinity for, and substrate activity with, AdoMet synthetase than the methylene class. This is presumably due to the facts that the electronegativity, lone pair availability, and geometry of the imido compounds are more similar to the natural oxygen-bridged species (Larsen et al., 1969). Since the true substrate for AdoMet synthetase is a Mg(II)–nucleotide complex, the enhanced affinity for AMPNPP over ATP may reflect an altered distribution among the numerous metal–chelate complexes present in solution. More insight into the behavior of these analogues may emerge from crystal structures of the metal–nucleotide complexes.

AdoMet synthetase is quite tolerant in binding analogues with substitutions along the polyphosphate chain, which is emphasized by the finding that $AMP(NP)_2$ is a competitive inhibitor with a K_i value equal to the K_m for ATP. In contrast, under the same conditions we find that 15 mM ADP causes no inhibition, indicating that ADP has a K_i value at least 200-fold higher than the K_m for ATP; since $AMP(NP)_2$ can be viewed as an α-substituted ADP analogue, this high affinity was completely unexpected. The enzyme is more conservative, however, in accepting modified compounds as substrates, and the lack of substrate activity of $AMP(NP)_2$ is not surprising since even the conservative sulfur substitution present in the two diastereoisomers of ATPαS causes complete loss of substrate activity (Markham et al., 1980). As the crystallographic studies of the enzyme progress, the reason for this remarkable affinity for $AMP(NP)_2$ may become more evident.

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