The Active-Site Arginine of *S*-Adenosylmethionine Synthetase Orients the Reaction Intermediate†

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Received May 12, 1998

ABSTRACT: *S*-Adenosylmethionine (AdoMet) synthetase catalyzes the formation of AdoMet and tripolyphosphate (PPP_i) from ATP and L-methionine and the subsequent hydrolysis of the PPP_i to PP_i and P_i before product release. Little is known about the roles of active-site residues involved in catalysis of the two sequential reactions that occur at opposite ends of the polyphosphate chain. Crystallographic studies of *Escherichia coli* AdoMet synthetase showed that arginine-244 is the only arginine near the polyphosphate-binding site. Arginine-244 is embedded as the seventh residue in the conserved sequence DxGxTxxKxI which is also found at the active site of inorganic pyrophosphatases, suggesting a potential pyrophosphate-binding motif. Chemical modification of AdoMet synthetase by the arginine-specific reagents phenylglyoxal or *p*-hydroxyphenylglyoxal inactivates the enzyme. ATP and PPP_i protect the enzyme from inactivation, consistent with the presence of an important arginine residue in the vicinity of the polyphosphate-binding site. Site-specific mutagenesis has been used to change the conserved arginine-244 to either leucine (R244L) or histidine (R244H). In the overall reaction, the R244L mutant has the k_{cat} reduced $\sim 10^3$-fold, with a 7 to 10-fold increase in substrate K_m values; the R244H mutant has an $\sim 10^5$-fold decrease in k_{cat}. In contrast, the k_{cat} values for hydrolysis of added PPP_i by the R244L and R244H mutants have been reduced by less than 2 orders of magnitude. In contrast to the wild-type enzyme in which 98% of the P_i formed originates as the γ-phosphoryl group of ATP, in the R244L mutant the orientation of the PPP_i intermediate equilibrates at the active site yielding equal amounts of P_i from the α- and γ-phosphoryl groups of ATP. Thus, the active-site arginine has a profound role in the cleavage of PPP_i from ATP during AdoMet formation and in maintaining the orientation of PPP_i in the active site, while playing a lesser role in the subsequent PPP_i hydrolytic reaction.

S-Adenosylmethionine synthetase (ATP:L-methionine *S*-adenosyltransferase E.C.2.5.1.6) catalyzes the only known route of biosynthesis of *S*-adenosylmethionine (AdoMet) which plays a myriad of biological roles and is considered to be the primary methyl group donor in all organisms (*1*−*4*). The overall synthetic reaction is composed of two sequential steps, AdoMet formation and the subsequent tripolyphosphate (PPP_i) hydrolysis which occurs prior to release of AdoMet from the enzyme, as shown below.

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

The structural and mechanistic properties of AdoMet synthetase have been extensively studied in the *Escherichia coli met*K enzyme, which is a tetramer of identical 383 residue subunits (*5*−*10*). Each active site binds two divalent metal ions (e.g., Mg^{2+}) and a monovalent cation (K^+), all of which are required for maximal activity. The recent report of the crystal structure of *E. coli* AdoMet synthetase by Takusagawa and co-workers has facilitated the design and interpretation of ongoing mechanistic investigations (*11*−*13*). While numerous studies have characterized the overall reaction and the AdoMet formation step, information on the PPP_i hydrolysis step has been elusive. Hindering elucidation of the kinetic and thermodynamic importance of the PPP_i hydrolysis step in the overall reaction has been the close physical and kinetic coupling of AdoMet formation and PPP_i hydrolysis. Thus, the importance of PPP_i hydrolysis in the mechanics and energetics of the overall reaction has remained unclear. This study has focused on determination of the roles of an active-site residue involved in processing of the PPP_i intermediate.

In many cases, protein-sequence comparisons have been successful in identifying catalytic or substrate binding sequences prior to the availability of three-dimensional structural data. In this study, a sequence homology comparison between AdoMet synthetase and inorganic pyrophosphatase has identified a potential pyrophosphate binding sequence common to both enzymes as DxGxTxxK(I, or V,

† This work was supported by National Institutes of Health Grants GM31186 and CA06927 and also supported by an appropriation from the Commonwealth of Pennsylvania. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute, or any other sponsoring organization.

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S0006-2960(98)01101-5 CCC: $15.00
Published on Web 09/05/1998

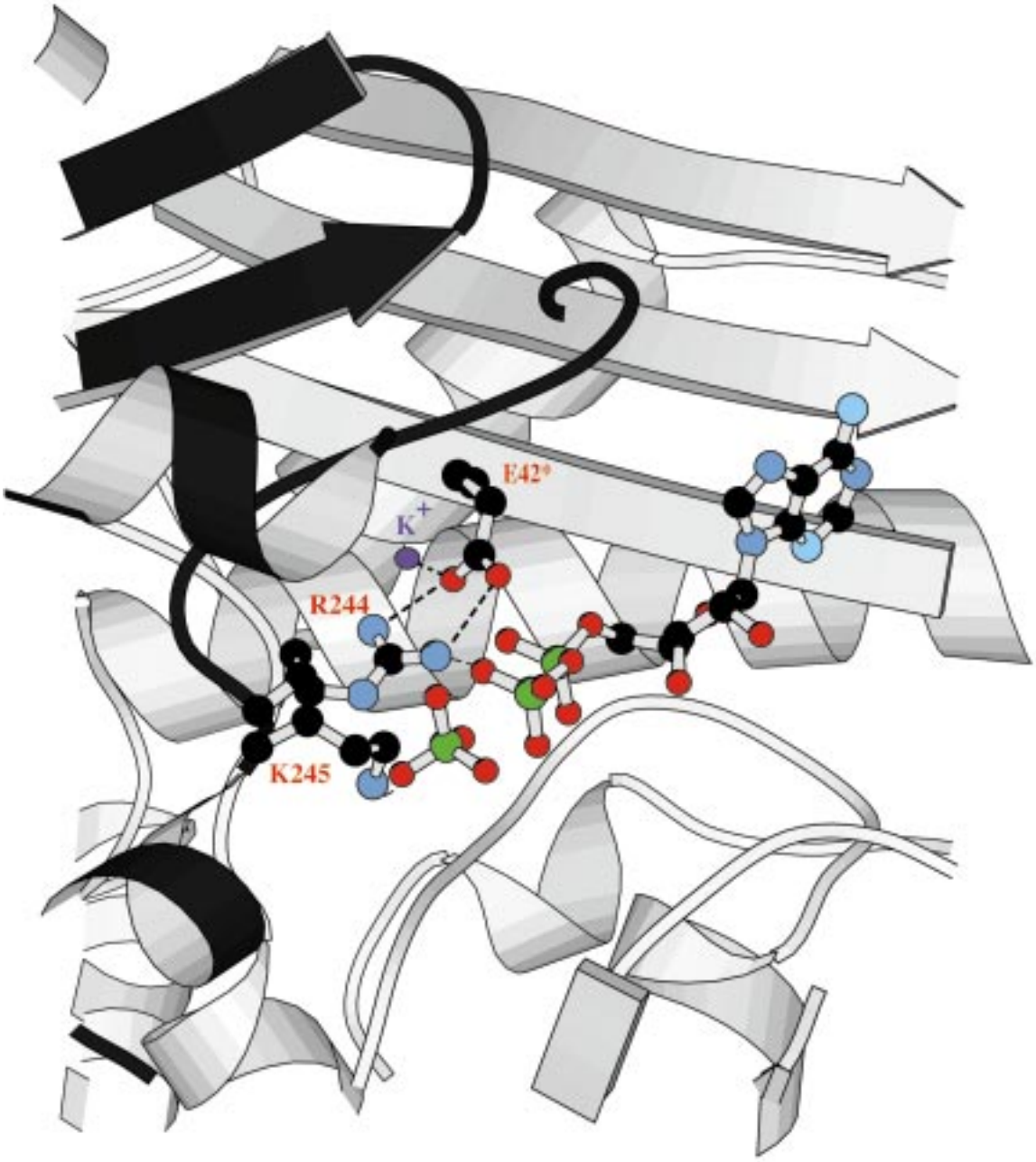

FIGURE 1: Illustration of the active site of AdoMet synthetase showing ADP, P_i, and selected side chains. The backbones of two subunits which contribute to the active site are shown in different shades of gray. The locations are shown for arginine-244, lysine-245, and glutamate-42; glutamate-42 is denoted with an asterisk (*) since it comes from a different subunit than residues 244 and 245. Coordinates were taken from the Protein Data Bank file 1mxb (*13*). This figure was prepared with MOLSCRIPT (*14*).

or L)I. Crystallographic results show that this sequence is present at the pyrophosphate binding site of *Saccharomyces cerevisiae* inorganic pyrophosphatase (*15*−*17*). X-ray crystallographic results for *E. coli* AdoMet synthetase in complexes with ADP and P_i, or PP_i and P_i, indicate that this sequence (encompassing residues 238−247 which contains two basic residues, arginine-244 and lysine-245) is present in the vicinity of the polyphosphate groups (*13*) (Figure 1). Arginine and lysine residues have been identified as functionally important components of the active sites of a number of enzymes that utilize nucleotides or phosphorylated substrates including adenylosuccinate synthetase, adenylate kinase, and inorganic pyrophosphatase (*17*−*20*). While this work was in progress, a report by Chamberlin et al. revealed that a naturally occurring alteration of the corresponding arginine to histidine in human liver AdoMet synthetase is associated with loss of enzyme activity and familial hypermethioninemia (*21*).

To provide a broad view of the importance of arginine residues in *E. coli* AdoMet synthetase function, both site-directed mutagenesis and chemical modification techniques have been used. The present studies show that AdoMet synthetase is inactivated when incubated in the presence of either phenylglyoxal (PGO) or *p*-hydroxyphenylglyoxal (HO-PGO), reagents which are specific for reaction with arginine residues (*22*). In contrast, chemical modification reagents specific for lysine residues are reported not to inactivate the enzyme (*5*). ATP and PPP_i protect the enzyme from inactivation, suggesting that functionally important arginine residue(s) are near the polyphosphate-binding region. To

complement the chemical modification studies of the wild-type enzyme, site-specific mutagenesis was used to change arginine-244 of AdoMet synthetase to a histidine (R244H) or a leucine (R244L) residue, and these mutants have been characterized in detail. The results reported herein indicate that arginine-244 is critical in interactions with the polyphosphate chain during AdoMet synthesis and to a lesser extent in PPP_i hydrolysis.

MATERIALS AND METHODS

L-Methionine, ATP, KCl, $MgCl_2$, $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, $NaAsO_2$, citric acid, ascorbic acid, acetic acid, malachite green, sodium dodecyl sulfate (catalog no. L4509), Tween 20, TRIS, Hepes, 2-mercaptoethanol, isopropyl-1-thio-β-D-galactopyranoside, and sodium tripolyphosphate were purchased from Sigma. AdoMet was purchased from Research Biochemicals International. PGO was purchased from Aldrich. HO-PGO was purchased from Pierce. Glycerol was purchased from Baxter Scientific. L-[Methyl-^{14}C]-methionine and [γ-^{33}P]ATP were purchased from DuPont NEN. [7-^{14}C]Phenylglyoxal was purchased from Amersham. Ecoscint scintillation fluid was purchased from National Diagnostics. Phosphocellulose P81 filters (2.5 cm) were purchased from Whatman. The Mutagene site-specific mutagenesis kit was purchased from Bio-Rad.

Cells and Plasmids. *E. coli* strains MV1190 (an *E. coli* K12 derivative) or RSR15(DE3) (an *E. coli* B derivative) were used. RSR15(DE3) is a *met*K derivative of BL21(DE3) and has been described previously (*10*, *23*); extracts of this strain have no detectable AdoMet synthetase activity. Strain MV1190 and the phagemid pTZ19U were components of the Mutagene kit.

Site-Directed Mutagenesis. Mutagenic oligonucleotides were prepared in the Core Facility at the Fox Chase Cancer Center. To construct the R244L mutant, mutagenesis was performed on the plasmid pTZK, which consists of the *E. coli* metK gene inserted between the *Pst*I and *Eco*RI sites in vector pTZ19U. The uracil enrichment method of Kunkel was used (*24*). The R244H variant was constructed using the Quickchange kit (Strategene) on plasmid pT7K (*10*), which has the *E. coli met*K gene inserted between the *Pst*I and *Eco*RI sites of plasmid pT7-6; AdoMet synthetase expression from this plasmid is ~10-fold higher than obtained from the pTZK plasmid. Following transformation and selection for ampicillin resistance, plasmid DNA was extracted using the QIAGEN Plasmid Prep (QIAGEN Inc., Chatworth, CA) for restriction digestions and nucleotide sequencing.

Screening and Nucleotide Sequencing of MetK Mutants. The mutagenic oligonucleotide for the R244L mutant was designed such that, along with encoding the nucleotide sequence to generate the desired mutation, codon degeneracy was exploited to introduce a unique *Afl*II restriction endonuclease recognition site to facilitate identification. Potential pTZK/R244L transformants were initially identified by restriction digestion of the purified plasmid DNA with *Afl*II. The complete nucleotide sequence was determined for the selected *met*K/R244L mutant using the procedure of Sanger et al. (*25*) with the Sequenase kit (U.S. Biochemical Corp.). The R244H mutant was identified directly by DNA sequencing on an ABI automated sequencer. In both cases, complete DNA sequences confirmed that only the desired mutations were introduced.

Expression, Purification, and Characterization of Mutant AdoMet Synthetases. Plasmids pTZK/R244L and pT7/K/R244H were transformed into strain RSR15(DE3). Cultures were grown in LB media and following isopropyl-1-thio-β-D-galactopyranoside induction the R244L mutant protein accounted for ~20% of total cellular protein. Comparable protein levels were obtained for the R244H mutant in the absence of induction.

A standard purification protocol was used to isolate both wild-type and mutant AdoMet synthetases (*10*). Both wild-type and mutant AdoMet synthetases were purified to electrophoretic homogeneity as judged by PAGE analysis on a Pharmacia Phast System. Purifications were monitored by PAGE on 10 to 15% gradient gels containing SDS. Following purification, purified wild-type and mutant AdoMet synthetases were analyzed by native gel electrophoresis on 8 to 25% gradient gels.

Circular dichroism spectra were recorded on an Aviv model 62A spectrapolarimeter. Samples (0.3 mg/mL protein in 25 mM Tris/HCl and 25 mM KCl, pH 8.0) were placed in 1 mm path length cells and spectra were recorded from 200 to 260 nm.

AdoMet Synthetase Assays. AdoMet synthetase activity was determined by the [^{14}C]AdoMet filter binding method (*10*). The tripolyphosphatase activity was determined in the presence or absence of AdoMet by quantitating orthophosphate production (*26*, *27*). AdoMet activation was evaluated as described previously (*10*).

Substrate saturation data were evaluated using the kinetic programs of Cleland (*28*) or the Enzfitter program (Elsevier Biosoft). The distribution of products formed in the overall reaction by the R244L mutant was determined by monitoring product formation from [γ-^{33}P]ATP. Reactions contained 5 mM [γ-^{33}P]ATP (40 mCi/mmol), 5 mM methionine, 20 mM $MgCl_2$, 50 mM KCl, and 1.0 mg/mL R244L AdoMet synthetase in 0.1 M Hepes/$(CH_3)_4N^+$, pH 8.0. Aliquots (15 μL) of the reaction were removed at various times from 1 to 60 min, and the reactions were quenched by mixing with 40 μL of 0.1 M EDTA, pH 7.0. Spots(1 μL) were placed on polyethyleneimine (PEI) cellulose thin layers and air-dried, and then the plates were washed in methanol and air-dried before development in 0.9 M LiCl and 50 mM EDTA, pH 7. In this system the R_f values for ATP, PPP_i, PP_i, and P_i are 0.25, 0.08, 0.20, and 0.8, respectively. To remove ATP and thus facilitate observation of the PP_i, parallel quenched samples were mixed with 5 mg of acid-washed Norit in 30 μL of water for 30 min and centrifuged before spotting; Norit treatment quantitatively removed ATP from the samples. To verify the ratio of PP_i to P_i produced, reactions were also conducted in the presence of 8 units/mL yeast inorganic pyrophosphatase. The amounts of products formed were quantified using a Fuji MacBas2000 imaging plate system. AdoMet formation under these conditions was determined by the usual method.

Enzyme Modification by PGO and HO-PGO. Purified wild-type (24 μM subunit) or mutant R244L (120 μM subunit) AdoMet synthetase was incubated with varying concentrations of PGO or HO-PGO at 25 °C in the dark[1] in 50 mM Hepes·KOH at pH 8.0 with 50 mM KCl and 20 mM $MgCl_2$. Stock solutions of PGO and HO-PGO were prepared

in 100% ethanol. Reactions were initiated by the addition of the glyoxal. At various intervals, aliquots were removed and added to assay mix with 10 mM L-arginine to quench the modification and allow quantification of the residual activity. In protection experiments, conditions were identical to those described above except that 10 mM HO-PGO was used and substrates or products were present in the reaction sample at saturating concentrations (i.e., $\geq$10 times K_m or K_i) values.

The stoichiometry of incorporation of [7-^{14}C]PGO into wild-type and R244L mutant AdoMet synthetases was determined. Reactions were initiated by the addition of [7-^{14}C]PGO, and at various times, aliquots were removed and diluted into buffer containing a 10-fold excess of L-arginine with respect to PGO. Time point samples were assayed for residual PPP_iase activity, and for incorporation of [7-^{14}C]PGO into the enzyme as trichloroacetic acid precipitable radioactivity. For quantitation of [7-^{14}C]PGO incorporation, 50 μL of sample was diluted into an equal volume of 10% TCA/90% ethanol. Samples were then centrifuged at 10^4g for 15 min at 0 °C, the supernatant was removed by aspiration; finally the protein pellets were washed five times with 0.2 mL of 10% TCA in 90% ethanol before scintillation counting to assess the degree of protein modification.[2]

RESULTS

Identification of a Putative Pyrophosphate-Binding Sequence. It has long been known that AdoMet synthetase is inactivated by the arginine specific reagent phenylgloxal but not by the lysine reagent trinitrobenzenesulfonic acid, although no protection or other studies were reported (*5*). Sequence comparison between *E. coli* AdoMet synthetase and *S. cerevisiae* inorganic pyrophosphatase identified the sequence DxGxTxKxI, which the crystal structures of both enzymes showed to be located at their active sites (*13*, *16*). A search of the SWISSPROT protein sequence database using this consensus sequence revealed 46 matches including several inorganic pyrophosphatases, all AdoMet synthetases, and several other proteins, in which PP_i is either a substrate or a product, or which have been hypothesized to utilize ATP. Restricting the consensus to DxGxTxxK(I, or V, or L)I, where position 9 is one of the branched chain amino acids (isoleucine, valine, leucine), refined the search to 39 matches which include several inorganic pyrophosphatases, all AdoMet synthetases, and a hypoxanthine-guanine phosphoribosyl pyrophosphate transferase in which pyrophosphate is a product. The sequences identified in this search are depicted in Table 1. Among the AdoMet synthetase sequences, stricter homology is observed as DxGxTGRKII which includes arginine-244. The results of this sequence search, in conjunction with the early results indicating that AdoMet synthetase was inactivated by PGO, prompted further characterization of the effects of arginine modification in both the wild-type and an arginine-244 mutant enzyme. Consistent with the variability of the second position of the consensus sequence, a C239A mutant of AdoMet synthetase has less than a 10-fold change in any kinetic parameter (*10*).

Table 1: Pyrophosphate-Binding Sequence[a]

enzyme	sequence[b]
concensus	DxGxTxxK(I,L,V)I
AdoMet synthetase[c]	DcGlTg**R**KI I
inorganic pyrophosphatase[d]	DeGeTdwKV I
hypoxanthine-guanine[e] phosphoribosyl transferase	DtGkTitKL I

[a] The residues in the consensus sequence are shown in capital letters. [b] The identified sequence (DxGxTxxKxI) is not a universal pyrophosphate-binding sequence since it did not identify sequences for the pyrophosphate utilizing or forming enzymes such as PP_i-dependent phosphofructokinase, NAD^+ pyrophosphorylase, UDP-glucose pyrophosphorylase, ATP sulfurylase, or the aminoacyl-tRNA synthetases. [c] The 34 AdoMet synthetases included those from *H. influenza*, *E. coli* (Met K), *S. cerevisiae* (Met K and MetL), *D. caryophyllus*, *P. crispum*, *A. thaliana* (Met K and Met L), *O. sativa*, *L. esculentum* (isozymes 1−3), *D. melanogaster*, *R. norvegicus* (α, β, γ forms), and *H. sapiens* (α, β, γ forms). The underlined **R** is conserved in AdoMet synthetases and is the residue studied herein. [d] The inorganic phosphatases were from *B. taurus*, *H. sapiens*, *K. lactis*, *S. pombe*, and *S. cerevisiae*. [e] The hypoxanthine-guanine phosphoribosyl transferase was from *S. mansoni*.

PGO and HO-PGO Modification of Wild-Type and R244L Mutant AdoMet Synthetase. Figure 2 depicts the results of treatment of wild-type or R244L mutant AdoMet synthetase with PGO or HO-PGO. The wild-type enzyme is inactivated by both reagents. The R244L mutant remains susceptible to inactivation by both glyoxals; however, it must be remembered that 100% of R244L mutant specific activity is <0.1% of wild-type specific activity (see below). Thus, modification of arginine residues of minor importance in the wild-type enzyme could account for loss of the residual activity. The crystal structure of the wild-type enzyme complexed with ADP and P_i (*13*) shows that in the R244L mutant the closest of the remaining 17 arginines would be arginine-272, ~11 Å away from the phosphate groups.

The rate of inactivation of wild-type AdoMet synthetase by PGO shows a nonlinear time course (Figure 2A), indicating modification of multiple arginine residues at distinctly different rates. In contrast, inactivation of both enzymes by HO-PGO produced linear inactivation kinetics (Figure 2B). HO-PGO modification of wild-type and mutant AdoMet synthetase was examined at various HO-PGO concentrations and the results were analyzed as described by Schloss (*29*). Second-order rate constants of 0.014 and 0.008 M^{-1} min^{-1} were obtained for wild-type and R244L mutant, respectively, with reaction orders of 1.8−1.9 with respect to HO-PGO for both enzymes. Since glyoxals form arginine adducts with a stoichiometry of two modifying groups per residue (*22*, *35*), the reaction order indicates that inactivation correlates with the modification of a single arginine residue per subunit.

The linear inactivation time courses with HO-PGO facilitated investigation of protection by substrates and products as summarized in Table 2. ATP and PPP_i protect wild-type AdoMet synthetase from HO-PGO inactivation; however, only PPP_i protects the R244L mutant. In contrast, while

[1] Washing [7-^{14}C]PGO-labeled wild-type AdoMet synthetase or the R244L mutant with aqueous 10% TCA was inadequate for removal of noncovalently associated [7-^{14}C]PGO or the [7-^{14}C]PGO-arginine adduct from labeled protein and produced spurious and irreproducible levels of ^{14}C incorporation. However washing the precipitated, labeled protein with either 10% TCA in ethanol or acetone produced reproducible results for ^{14}C incorporation.

[2] M. S. McQueney, K. S. Anderson, and G. D. Markham, unpublished results.

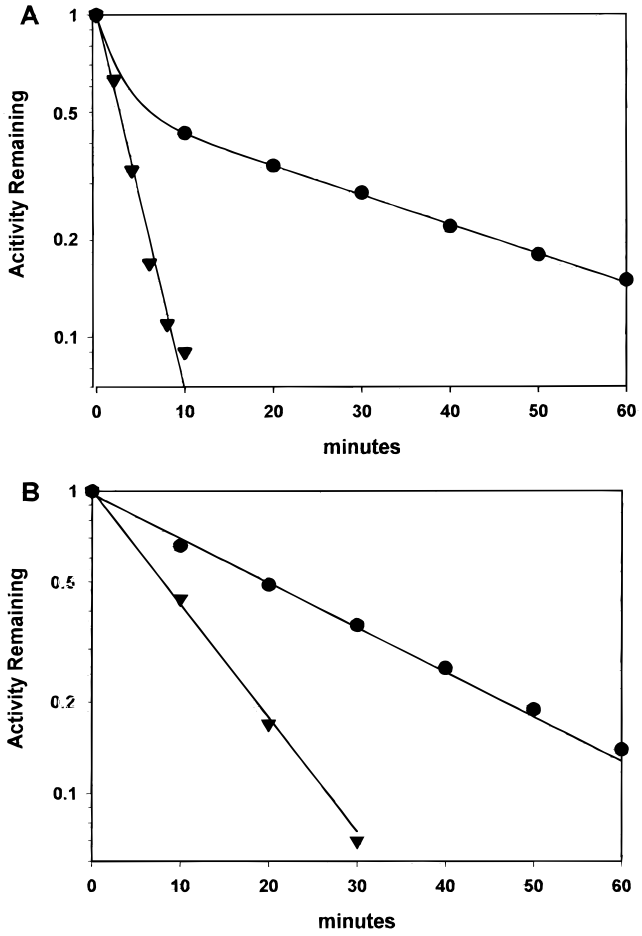

FIGURE 2: Time courses of inactivation of AdoMet synthetases by glyoxals. Enzymes [wild-type AdoMet synthetase at 1.0 mg/mL (●), and R244L mutant at 5.0 mg/mL (▼)] were incubated with (A) PGO (at 10 mM for the wild-type enzyme and 12 mM for the R244L mutant) or (B) HO-PGO (at 15 mM for the wild-type enzyme and 20 mM for the R244L mutant) in 50 mM Hepes·KOH at pH 8.0 with 50 mM KCl and 10 mM $MgCl_2$ at 25 °C in the dark. At various times, aliquots were removed for assay of residual AdoMet synthetase activity. Corresponding control samples for both enzymes retained 100% activity for the duration of the experiment.

L-methionine and AdoMet slightly protect the R244L mutant from inactivation, neither compound protects wild-type enzyme, consistent with inactivation resulting from modification of a different residue.

Although HO-PGO could be used to characterize the kinetics of arginine modification and enzyme inactivation, the availability of [7-^{14}C]PGO allowed determination of the stoichiometry of arginine modification as shown in Figure 3. Two arginine residues are modified in wild-type AdoMet synthetase coincident with 95% inactivation. With the wild-type enzyme, 50% inactivation reflects incorporation of 2 equiv of PGO per subunit, presumably the modification of one arginine since the rate-determining step in formation of the $(PGO)_2$·arginine adduct is formation of the initial PGO·arginine adduct (*35*). In contrast, only one arginine residue is modified in the R244L mutant enzyme coincident with 99% inactivation. Unfortunately, instability of the modifications thwarted attempts to identify the modified residues.

Characterization of R244L and R244H AdoMet Synthetase Mutants. Both mutant AdoMet synthetases are similar to the wild-type enzyme in secondary structure, as judged by indistinguishable circular dichroism spectra, and are clearly tetrameric according to native polyacrylamide gel electrophoresis. However, the mutations have profound effects on the catalytic behavior of the enzyme. The AdoMet synthetase activity of the R244H mutant is reduced by ~10^5-fold from the wild-type enzyme, precluding detailed investigations. In contrast, the 10^3-fold reduction in activity of the R244L mutant allowed functional characterization.

The activator constants for the R244L mutant for K^+ and Mg^{2+} are increased ~5-fold relative to wild-type enzyme to ~10 mM in each case. K^+ activates the R244L mutant enzyme only ~2-fold, in contrast to the ~100-fold activation

Table 2: Substrate and Product Protection from Inactivation by HO-PGO[a]

protector	$t_{1/2}$ (min)	protection (fold)
(A) Wild-type AdoMet Synthetase[b]		
none	35	0
L-methionine, 6 mM	34	0
AdoMet, 1 mM	35	0
PPP_i, 5 mM	>270	>8
ATP, 10 mM	>240	>7
(B) R244L Mutant AdoMet Synthetase[c]		
none	8.5	0
L-methionine, 50 mM	12.4	0.5
AdoMet, 2 mM	12.4	0.5
PPP_i, 4 mM	30.1	2.5
ATP, 10 mM	6.1	−0.3

[a] Solutions contained 50 mM Hepes·KOH, 50 mM KCl, and 20 mM $MgCl_2$, pH 8.0, and 10 mM HO-PGO. Concentrations of protectors were chosen to approximate saturating conditions based on K_i or K_m values of the individual protectors. [b] Protection evaluated using the [^{14}C]AdoMet filter binding assay (*5*). [c] Protection evaluated using the colorimetric P_i assay (*26*, *27*). Fold protection is defined as $(t_{1/2+protector})/(t_{1/2-protector}) - 1$.

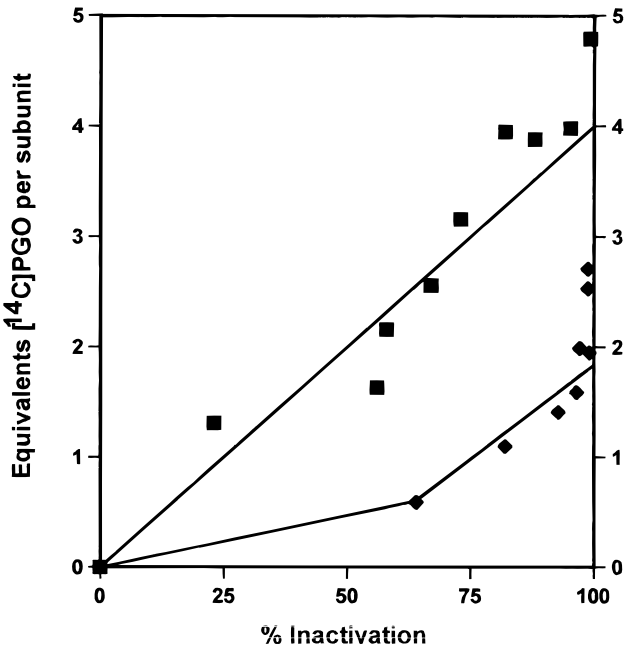

FIGURE 3: Correlation of [7-^{14}C]PGO incorporation into AdoMet synthetases and loss of enzymatic activity. 1.0 mg/mL wild-type (■) and 5.0 mg/mL R244L mutant (◆) AdoMet synthetase were incubated with 40 mM and 12 mM [7-^{14}C]PGO (specific activity = 0.35 mCi/mmol), respectively, in 50 mM Hepes·KOH at pH 8.0 with 50 mM KCl and 10 mM $MgCl_2$ at 25 °C in the dark. At various times, aliquots were removed for assay of residual activity and quantitation of [7-^{14}C]PGO incorporation.

Table 3: Kinetic Parameters for AdoMet Synthetase Activity of Wild-Type, R244L Mutant AdoMet Synthetases[a]

enzyme	$K_{m,ATP}$ (mM)	$K_{m,L-Met}$ (mM)	k_{cat} (s^{-1})	$k_{cat}/K_{m,ATP}$ (M^{-1} s^{-1})	$k_{cat}/K_{m,L-Met}$ (M^{-1} s^{-1})
wild-type MetK[b]	0.11	0.08	1.5	1.4×10^4	1.9×10^4
MetK/R244L[c]	0.74	0.88	0.0012	1.6	1.4
MetK/R244H	ND[d]	ND[d]	$\sim 3 \times 10^{-5}$[e]		

[a] AdoMet synthetase activity was measured as described in the Materials and Methods. [b] Values from Markham et al. (*5*). [c] MetK/R244L assays were performed in the presence of 1.0 to 5.0 mM L-[^{14}C-methyl]methionine (1.9 mCi/mmol), 2 to 15 mM ATP ($Tris^+$ form) in 100 mM Hepes·$(CH_3)_4N^+$ at pH 8.0 with 50 mM KCl and 50 mM $MgCl_2$. [d] ND, not evaluated since the low activity precluded accurate determinations. [e] Apparent value measured at substrate concentrations of 10 mM ATP and 0.2 mM methionine (51 mCi/mmol).

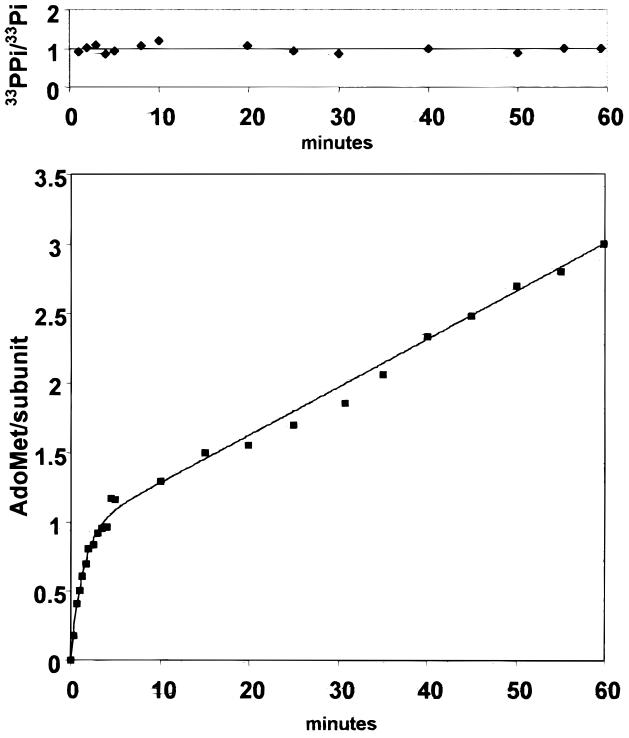

FIGURE 4: Time course and stoichiometry of product formation by the R244L mutant AdoMet synthetase. Samples contained 1.0 mg/mL R244L mutant AdoMet synthetase, 5 mM ATP (including [γ-^{33}P]ATP for samples quantifying $^{33}PP_i$ and $^{33}P_i$ production) and 5 mM L-methionine (including L-[methyl-^{14}C]methionine for samples in which AdoMet was quantified) in 100 mM Hepes·$(CH_3)_4N^+$, 50 mM KCl and 20 mM $MgCl_2$, pH 8.0, at 25 °C. At various times, aliquots were removed and assayed for [^{14}C]AdoMet formation (bottom panel), or $^{33}PP_i$ and $^{33}P_i$ formation (top panel) as described in Materials and Methods.

of the wild-type enzyme. Although arginine-244 is spatially close to the three metal ions bound at the active site, crystallographic results do not indicate a direct role for arginine-244 in metal binding (*13*). Arginine-244 is however hydrogen bonded to glutamate-42 whose side chain provides a ligand to the K^+ activator (Figure 1); thus, the loss of this hydrogen bond may affect the position of glutamate-42 and may account for the diminished monovalent cation activation (*9*). The kinetic parameters for AdoMet synthesis by the R244L mutant are summarized in Table 3. The R244L mutant has ~0.08% of the maximal catalytic activity (k_{cat}) of the wild-type enzyme and the K_m values for ATP and L-methionine are increased by factors of ~7 to 10, respectively. The slow steady-state rate of AdoMet production with the R244L mutant is preceded by a more rapid initial single turnover of AdoMet formation (Figure 4). The rate of this first turnover is 0.015 s^{-1}, ~12 times faster than the steady-state rate, but still only ~1% of the rate of AdoMet synthesis by the wild-type enzyme. During this initial time period, equimolar amounts of AdoMet, P_i, and PP_i are produced, indicating that a late step in the reaction limits the steady-state rate.

Table 4: Kinetic Parameters for the Tripolyphosphatase Activity of Wild-Type, R244L, and R244H Mutant AdoMet Synthetases in the Presence and Absence of AdoMet[a]

$$PPP_i + H_2O \xrightarrow[\pm AdoMet]{AdoMet\ synthetase} PP_i + P_i$$

	-AdoMet			+AdoMet		
enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
wild-type MetK	3	0.066	2.2×10^4	13	1.23	9.4×10^4
MetK/R244L	130	0.010	7.7×10^1	16	0.13	8.1×10^3
MetK/R244H	19	0.0012	6.3×10^1	25	0.085	3.3×10^3

[a] Tripolyphosphatase activity was measured in the presence and absence of 40 μM AdoMet as described in the Materials and Methods. All assays were performed at 25 °C and contained 3 to 500 μM PPP_i (Na^+ form) in 50 mM Hepes·$(CH_3)_4N^+$ at pH 7.8 with 10 mM KCl and 10 mM $MgCl_2$.

Following AdoMet formation, the tightly bound tripolyphosphate derived from ATP is hydrolyzed to pyrophosphate and orthophosphate. AdoMet synthetase also catalyzes the hydrolysis of added tripolyphosphate and the reaction rate is stimulated by AdoMet (*5*, *31*−*34*). For both the R244L and R244H mutants, maximal AdoMet activation of the mutants is observed at an AdoMet concentration of 40 μM, which is comparable to that required for maximal activation of the wild-type enzyme (*10*). The kinetic parameters for PPP_i hydrolysis in the presence and absence of AdoMet are summarized in Table 4. For both the R244L and R244H mutants, the reductions in hydrolytic rates are modest compared to the effects on the overall reaction. In the absence of AdoMet, there is a 43-fold increase in the K_m for PPP_i and a 6-fold decrease in k_{cat} for the R244L mutant, whereas there is a 6-fold increase in K_m and a 50-fold decrease in V_{max} for the R244H mutant. In the presence of AdoMet there is at most a 15-fold decrease in k_{cat} relative to wild-type enzyme with less than a 2-fold change in K_m for either mutant. Thus, the kinetic characteristics of the tripolyphosphatase activity of both mutants demonstrate that the catalytic handicaps incurred by the arginine-244 mutations are expressed primarily in the AdoMet synthesis step.

Reorientation of the PPP_i Intermediate in the R244L Mutant. Since arginine-244 is positioned to directly interact with the polyphosphate chain, the fate of PPP_i formed during the overall reaction was evaluated by the formation of $^{33}P_i$ and $^{33}PP_i$ from [γ-^{33}P]ATP. As illustrated in Figure 4, during the time course of the reaction, the R244L mutant produces

equivalent amounts of $^{33}PP_i$ and $^{33}P_i$, indicative of randomization of the orientation of the endogenously formed $^{33}PPP_i$ prior to hydrolysis. This result contrasts with that observed for wild-type AdoMet synthetase where 98% ±2 of the P_i formed in the overall reaction is derived from the Pγ of ATP (*5*). $^{33}PPP_i$ does not accumulate measurably through the course of the reaction (<0.1 equiv/subunit), as would be expected since the k_{cat} for hydrolysis is ~9-fold greater than even the rate of the initial burst of AdoMet formation in the mutant.

DISCUSSION

The basic amino acid residues arginine and lysine have been identified at the active sites of a variety of enzymes that utilize nucleotides or metabolize phosphorylated substrates or products (*18*−*20*, *30*). Presumably, in their protonated forms they provide favorable binding interactions for the anionic moieties of substrates or products. Furthermore, in reactions that involve development of a negatively charged species, such as PPP_i in the AdoMet synthetase reaction, these residues can play a catalytic role by charge stabilization. X-ray crystallographic results of AdoMet synthetase show that arginine-244 is the only arginine residue in the immediate vicinity of the polyphosphate group (Figure 1) (*13*). Arginine-244 is located in the interface between the two subunits that contribute to each active site. A naturally occurring variant at the corresponding position in the human AdoMet synthetase, R264H in the MAT1α isozyme, inactivates the enzyme by >1000-fold and hinders oligomer formation (*21*), although detailed studies of this enzyme have not been reported. In contrast, our studies show that the *E. coli* AdoMet synthetase R244L and R244H mutants remain tetrameric with no obvious change in secondary structure, which may reflect the intrinsically higher stability of the bacterial tetramer over the human form (*8*, *21*).

The R244L mutant AdoMet synthetase has 0.08% of the k_{cat} of the wild-type enzyme. The K_m values for both substrates are increased ~10-fold yielding a reduction in catalytic efficiency (k_{cat}/K_m) of 4 orders of magnitude. In contrast, the R244H mutant has only ~0.003% of the AdoMet synthetase activity, precluding steady-state kinetic studies. In either mutant, the catalytic efficiency of the tripolyphosphatase half-reaction was reduced by less than 2 orders of magnitude in the presence or absence of AdoMet, which could not account for the hindrance of the overall reaction in either case. The finding that the R244H mutation causes a larger diminution in AdoMet synthesis activity than the R244L conversion, while both mutations have comparable impediments of PPP_i hydrolysis rates, was unanticipated. While the physical bases for the differences in these behaviors are not totally clear, the observations indicate that the presence of the basic, potentially uncharged, imidazole side chain of histidine is more detrimental to AdoMet synthesis than is the hydrophobic group of leucine. The side chain of histidine-244 is likely to be unprotonated due to proximity to the adjacent positively charged lysine-245 (Figure 1), and an interaction between these groups could hinder the lysine's contribution to catalysis, as well as remove the favorable catalytic interactions of the guanidinium group of the natural arginine-244. The findings that both arginine-244 mutations preferentially impair the AdoMet-forming reaction suggest that the location of the phosphate groups observed in the crystal structure of the enzyme·ADP·P_i complex is not the precise location present during catalysis, as was suggested in the original analysis of the crystallographic data (*13*). It may be that the nucleotide moves further into the active-site cleft in the catalytically active conformation, which would place arginine-244 nearer the α-phosphoryl group, which is involved in AdoMet formation (*13*). During the overall reaction with the R244L mutant, the PPP_i intermediate randomizes orientation before hydrolysis, in contrast to the wild-type enzyme where ~98% of the P_i originates as the γ-phosphoryl group of ATP (*5*). From the ~2% reorientation of PPP_i in the wild-type enzyme, a PPP_i reorientation rate of ~0.05 s^{-1} can be estimated (*5*); while this rate is comparable to the hydrolytic k_{cat} of the R244L mutant (0.13 s^{-1}), simulations using KINSIM (*36*) suggest that at least a 20-fold increase in PPP_i reorientation rate in the mutant would be required to account for the randomization of the PPP_i formed in the overall R244L-catalyzed reaction. Since there is no detectable accumulation of PPP_i during the reaction, it appears that reorientation occurs within the active-site cavity which is ~30 Å deep (*13*). An analogous reorientation of thiopyrophosphate bound at the active site of valyl t-RNA synthetase was reported by Rossomando et al. (*37*).

AdoMet synthetase is inactivated by PGO and HO-PGO which specifically react with arginine residues (*35*). The wild-type AdoMet synthetase is protected from HO-PGO inactivation by ATP and PPP_i, suggesting that a crucial arginine residue is involved in polyphosphate binding. The R244L mutant enzyme remains susceptible to PGO inactivation; however, this is not surprising since two arginine residues are modified in wild-type enzyme upon inactivation and 17 arginines remain in the mutant. In contrast, one arginine residue is modified in the R244L mutant upon enzyme inactivation. While the guanidinium group of arginine-272, the closest remaining arginine, is ~11 Å from the phosphate groups in the enzyme·ADP·P_i complex (*13*) modification by the bulky PGO moiety might still interfere with enzyme function. The patterns of substrate/product protection from PGO inactivation for the mutant are consistent with the modification of an arginine residue perhaps involved in methionine binding causing inactivation of the mutant enzyme. The methionine-binding site of the enzyme has not yet been located crystallographically.

The requirement for tripolyphosphate hydrolysis in the AdoMet synthetase reaction has been an enigma since it was initially observed (*31*). One role for the tripolyphosphatase activity is that through hydrolysis reversal of the reaction is prevented. However, the equilibrium constant for the formation of enzyme-bound AdoMet and PPP_i suggests that the equilibrium of the reaction highly favors AdoMet formation ($K_{eq} > 10^4$) even without PPP_i hydrolysis, and as such, the significance of this possible role for the tripolyphosphatase is diminished (*5*). Furthermore, the equilibrium constant for hydrolysis of enzyme-bound PPP_i to bound PP_i plus P_i is <10 suggesting little importance for this role.[2] Another role for the tripolyphosphatase activity is to convert PPP_i to the less tightly bound forms PP_i and P_i. Since AdoMet binds more tightly to the enzyme in the presence of PPP_i, if PPP_i were not hydrolyzed, both products of the reaction would significantly inhibit the enzyme through

formation of the enzyme·AdoMet·PPP_i complex as has been observed with the reaction of methionine with the nonhydrolyzable ATP analogue AMPPNP (*5*). Alternatively, during catalysis the enzyme may undergo a conformational change and assume a conformation which may require energy in order to escape and complete the catalytic cycle. This is analogous to the oncogenic Rasp21 protein which undergoes a conformational change between the GTP- and GDP-bound states (*38*). Evidence of a conformational change during formation of the enzyme·AdoMet·PPP_i complex has been obtained by stopped-flow fluorimetry;[2] however, the structure of this altered conformation has not yet been elucidated.

Many studies of active-site architecture focus on the direct role of residues involved in stable enzyme−substrate interactions or in the covalent chemistry of catalysis. In contrast, the roles of residues that make important contacts with reactant molecules primarily during their transformation to products, or residues present in a region of an enzyme which undergoes a conformational change during catalysis, are often overlooked. In part, this is due to the fact that one can more readily assign a functionality to an amino acid residue if its side chain is capable of direct interaction with substrate. It is more difficult to decipher a functionality for an amino acid residue whose side chain renders it nonreactive but which can provide a favorable noncovalent interactions during catalysis or which facilitates the movement of a portion of the enzyme during the reaction. It appears that the catalytic contributions of arginine-244 may largely reside in its role in polyphosphate binding during AdoMet formation, whereas it plays a lesser role in the subsequent hydrolysis of the PPP_i intermediate. A curious property of the R244L mutant is the occurrence of one (relatively) rapid turnover of the overall AdoMet synthetase reaction before ∼12-fold slower steady-state rate is observed. Since equimolar AdoMet, PP_i, and P_i are produced in the first turnover, neither chemical reaction is rate limiting. Furthermore, the >100-fold more rapid steady-state rate of the PPP_i hydrolytic reaction indicates that product release is not rate limiting. Thus, a step between product release and subsequent turnovers of AdoMet synthesis has become rate limiting. This process that may reflect "recycling" of the enzyme between product and substrate-binding forms, a process akin to that reported for furmarase where the enzyme forms apparently reflect different protonation states of active-site residues (*39*). The physical basis for this slow step in the R244L AdoMet synthetase is yet to be elucidated.

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BI9811011