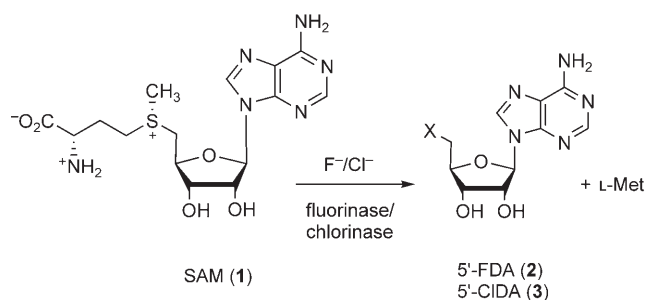


S-Adenosyl-L-methionine:Hydroxide Adenosyltransferase: A SAM Enzyme**

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Dedicated to the memory of Jonathan B. Spencer

S-Adenosyl-L-methionine (SAM, **1**) has a variety of roles in enzymology. It is most commonly involved in methyl-transfer reactions,^[1] in which it transfers its methyl group to O, N, S, and C atoms of various substrates. SAM also donates a methylene group in the cyclopropanation of unsaturated fatty acids.^[2] There is a SAM decarboxylase,^[3] which initiates polyamine biosynthesis, and, perhaps most exotically, SAM is the source of 5'-deoxyadenosyl radicals in at least three iron-sulfur enzymes.^[4] SAM acts as a precursor in the biosynthesis of several metabolites, including ethylene in plants,^[5] biotin, and epoxyqueuosine.^[6] Fluorination^[7] and chlorination^[8] enzymes have been reported that utilize SAM as a substrate; in each case, the halide ion mediates a nucleophilic attack at the 5'-position of SAM (**1**) to generate the corresponding 5'-halo-5'-deoxyadenosine (5'XDA) product **2** or **3** and L-methionine (L-Met; Scheme 1). These reactions, with



Scheme 1. Nucleophilic substitution of SAM with halide ions.^[7,8]

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SAM-dependent methyl transferases,^[1] are among the few enzymatic S_N2-type reactions known.^[9,10]

Examination of the fluorinase and chlorinase amino acid sequences with data derived from genome sequencing reveals an identity (<36%) to the DUF62 superfamily (Figure 1; DUF = domain of unknown function).^[11] No function has been assigned to the DUF62 proteins. The distribution of these genes is restricted; in general they are found only in extremophile and pathogen-related microorganisms. The protein products of four different *duf62* genes from four different extremophiles have been the subject of overexpression and X-ray crystal-structure evaluation in structural-proteomics screening programs.^[12]

The four DUF62 protein structures^[12] are nearly identical with each other and almost superimposable on the previously reported fluorinase^[10] and chlorinase^[8] structures. One of these enzymes is from *P. horikoshii* OT3, a microorganism with an optimum growth temperature of 98°C, which was isolated at a depth of 1395 m from an Okinawa trough vent in the Japanese Pacific Ocean.^[13] Examination of the structure reveals an adenosine molecule coordinated at an intersubunit position corresponding to the fluorinase active site (Figure 2). The fluorinase and chlorinase also cocrystallize with adenosine, and the adenosine molecule in the re-refined *P. horikoshii* structure is almost superimposable on that in the structures of the fluorinase and the chlorinase. For example, an aspartate carboxylate (Asp7) anchors the 2'- and 3'-OH groups of the adenosine ribose moiety. With this background, we recloned the DUF62 gene of *P. horikoshii* OT3 into *E. coli* and overexpressed and purified the protein.^[14] The protein was unable to mediate a fluorination or chlorination reaction with SAM after incubation at high concentrations (>10 mM) of halide ions.^[15] However, a novel activity was apparent in that the enzyme could catalyze the conversion of SAM into adenosine (**4**) by the attack of a hydroxide ion (from water) at C5' of SAM (**1**), in analogy with nucleophilic halide reactions (Scheme 2).

The enzyme was assayed^[16] at 37°C and found to have optimal activity at pH 8.5, and to be inactive below pH 5 and above pH 10 (pH 8.5, $K_m(\text{SAM}) = 39.2 \mu\text{M}$, $k_{\text{cat}} = 0.14 \text{ s}^{-1}$). Consistent with a hyperthermophilic protein, the enzyme retains full activity after being heated at 80°C for 30 min and then cooled to 37°C. An assay of the *P. horikoshii* SAM hydroxide adenosyltransferase in a buffer enriched with H₂¹⁸O resulted in isotopically enriched [5'-¹⁸O]-adenosine ([M+2]⁺: 48%), as determined by GC-MS analysis after pertrimethylsilylation at the three hydroxy groups and the

	Asp68	Arg75		His127			
<i>I</i> -fluorinase/1-297	TYPATGTTT	RSVAVRI	KQAAKGGARGQWASGAGFERAEGSYIYIAPNNG	124	TEYSRE	159	100
<i>II</i> -chlorinase/1-283	VYPETGTATHT	IAYRNEK	-----GQLLVGPNNNG	98	TWYCKD	133	36
<i>III</i> /1-298	IDPTVGGSTRKAI	IVAKSKK	-----NQYFILLPDNG	124	TFHGRD	160	32
<i>IV</i> /1-356	VDPGVGTERRAVALRAEG		-----QWFGVPDNG	175	TFHGRD	211	30
<i>V</i> /1-277	VDPGVGTSRRPI	AVETDR	-----AVFIA PDNG	100	TFHGRD	134	30
<i>VI</i> /1-263	VDPGVGTSRKAI	IVXKTKN	-----DQYFVAPDNG	94	TFHGRD	120	28
<i>VII</i> /1-280	VDPGVGSKRRSVAVLTED		-----GHYIITPDNG	98	TFHGRD	135	28
<i>VIII</i> /1-266	VDPGVGTSRNALAVRTER		-----ALFLAPDNG	100	TFHGRD	135	27
<i>IX</i> /1-273	VDPGVGSSRKALLIETSH		-----YYLVGPDNG	96	TFHGRD	131	27
<i>X</i> : <i>Pyrococcus</i> /1-256	IDPGVGTERRAIVIEG		-----DQYLVVPDNG	92	TFHGRD	130	26
<i>XI</i> /1-300	VDPGVGTDKRSIVLKTKN		-----GQYFVSPDNG	117	TFHGRD	154	25
<i>XII</i> /1-282	VDPGVGSDRRSIACLTYS		-----GHYIITPDNG	97	TFHGRD	134	25
	★	★			★		

Figure 1. Amino acid sequence alignment and identity (%) between the fluorinase from *S. cattleya* and DUF62 proteins from various organisms. I) Fluorinase from *Streptomyces cattleya*;[7] II) chlorinase from *Salinispora tropica*;^[8] III) *Acidobacteria bacterium*; IV) *Salinibacter ruber*; V) *Prosthecochloris aestuarii*; VI) *Thermotoga maritima*; VII) *Listeria monocytogenes* EGD-e; VIII) *Chlorobium ferrooxidans*; IX) *Staphylothermus marinus* F1; X) *Pyrococcus horikoshii* OT3; XI) *Escherichia coli* O157:H7; XII) *Staphylococcus aureus* subsp. *aureus*. Star: conserved amino acids in DUF62 proteins (Asp68, Arg75, and His127).

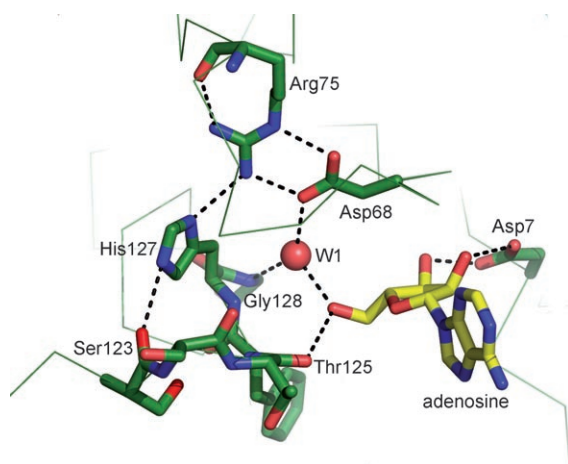
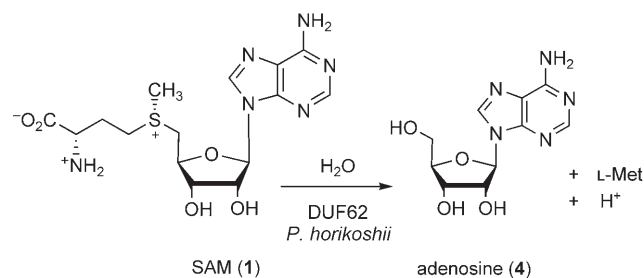
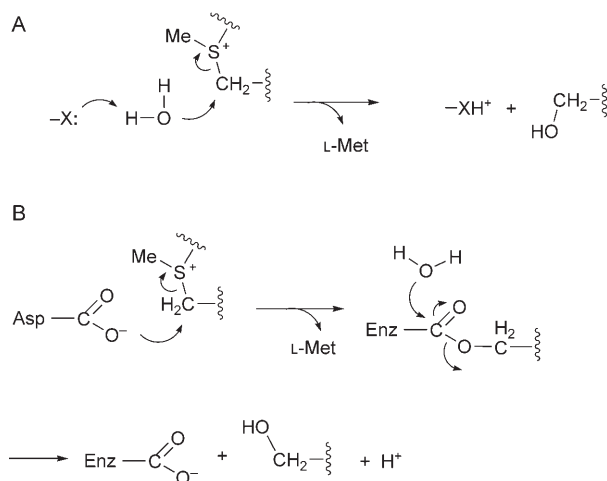


Figure 2. Adenosine bound to SAM hydroxide adenosyltransferase from *P. horikoshii*.



Scheme 2. The SAM hydroxide adenosyltransferase reaction.

amino group.^[17] This result suggests that the general mechanism A in Scheme 3 operates. However, an examination of the active-site residues of the protein from its X-ray crystal structure (Figure 2) reveals an aspartate carboxylate residue, Asp68, in relatively close contact to the ribose ring of adenosine (bound product). Asp and Glu carboxylate groups are known to be involved as nucleophiles in haloacid dehalogenase reactions to generate a substrate-bound ester



Scheme 3. Two putative mechanisms for the *P. horikoshii* SAM hydroxide adenosyltransferase reaction with SAM.

in the catalytic cycle. This ester is then hydrolyzed to release an alcohol and recover the carboxylate group.^[18] Putative mechanism B in Scheme 3 presents an analogous possibility for SAM hydroxide adenosyltransferase. To distinguish mechanisms A and B and probe the role of Asp68 in this reaction, we examined isotopic labeling of the protein.

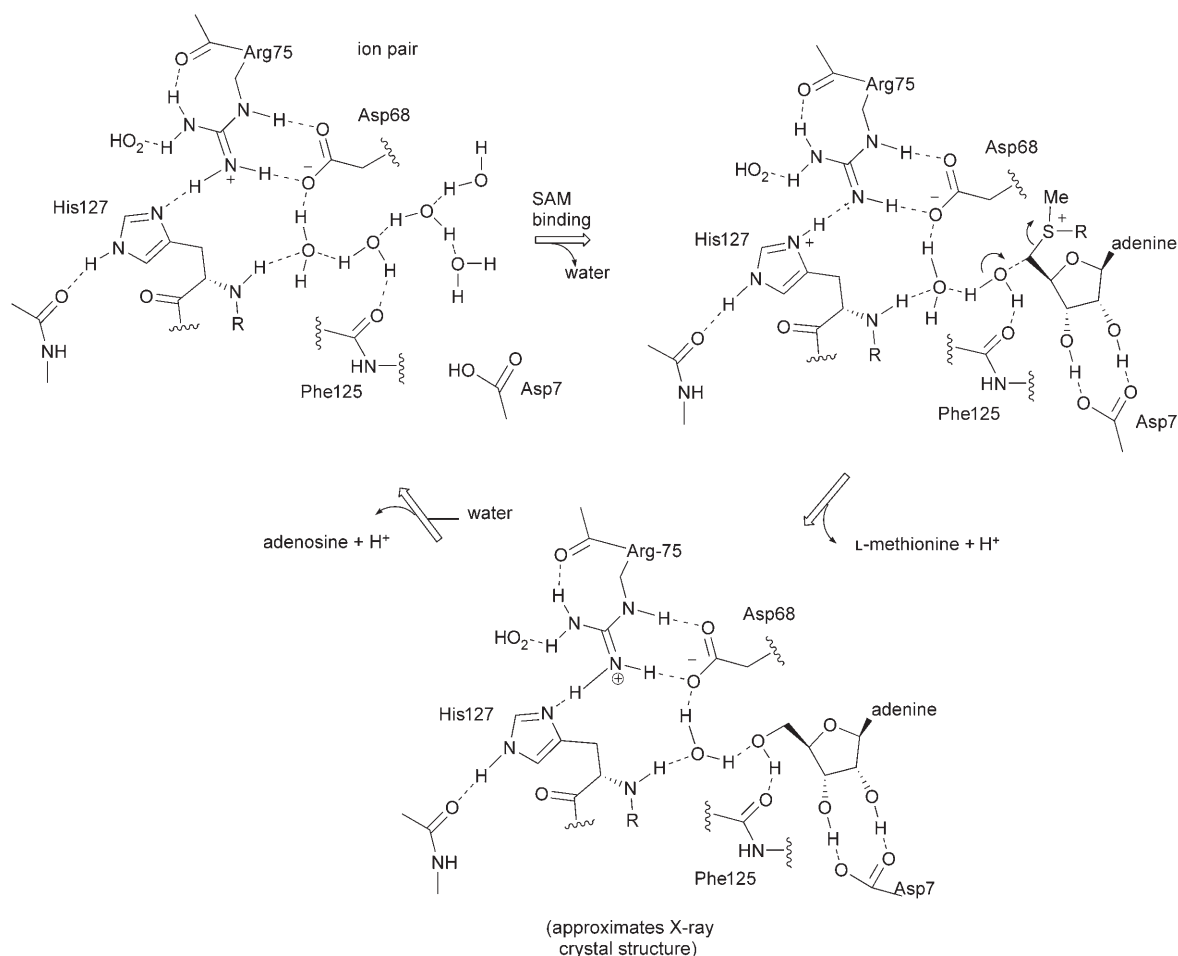
Assays were carried out with $H_2^{18}O$ (64 atom %). Mechanism A would label the product adenosine with ^{18}O at C5', but not the enzyme. Mechanism B would load the Asp carboxylate group with ^{18}O , and multiple turnover would result in both enzyme and product labeling (at C5'). To probe isotope incorporation into the protein, subsequent trypsin digestion of the DUF62 protein in heavy-isotope-free buffer was followed by analysis of a fragment containing the Asp68 residue by mass spectrometry.^[17] MS/MS analysis did not provide any evidence for heavy-isotope incorporation into the peptide fragment containing Asp68. In a control experiment, the enzyme was digested with trypsin in $H_2^{18}O$ (95 atom %) buffer (in-gel digestion),^[19] and the fragment containing the

Asp68 residue was analyzed by MS/MS mass spectrometry. In this case, heavy-isotope incorporation was very clear: $[M+2]^+$ and $[M+4]^+$ ions were observed, which is consistent with the trypsin-mediated incorporation of ^{18}O into the newly released terminal carboxylate residue. The MS method is clearly capable of detecting ^{18}O incorporation into the protein; however, in the SAM hydroxide adenosyltransferase assays, no incorporation of ^{18}O was observed. These experiments suggest that the enzyme operates by general mechanism A (Scheme 3).

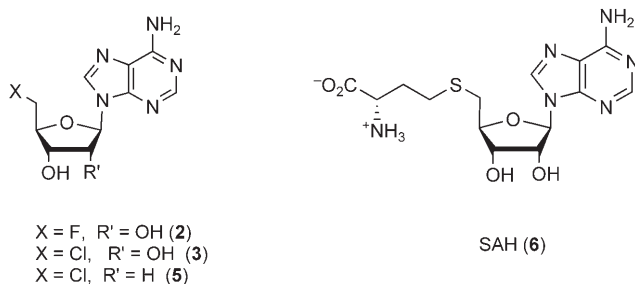
Examination of the active-site residues of the enzyme in the X-ray crystal structure reveals an intriguing juxtaposition of substituents. Asp68 forms an ion-pair dimer with the guanidinium residue of Arg75. The guanidinium residue is H bonded to the imidazole unit of His127. These three amino acid residues are absolutely conserved in all of the putative hydroxide adenosyltransferases (≈ 200) identified in BLAST^[20] searches of the DUF62 enzymes (see the Supporting Information), ten of which are shown in Figure 1. Interestingly, there are no equivalent residues in the fluorinase or chlorinase enzymes, and the arrangement of the active site for nucleophilic attack in this enzyme bears no obvious resemblance to those of the halide transferases. It is anticipated that substantial active-site desolvation occurs as a consequence of efficient SAM binding, in a similar manner to

the fluoride-ion-desolvation process of the fluorinase, to force two water molecules into the active-site cavity.^[15] The inner water molecule is hydrogen-bonded to the Asp68 carboxylate group. We propose that the outer water molecule is made nucleophilic by coordination to both a backbone amide carbonyl group and the inner water molecule (present in the product crystal structure). On SAM binding, the outer water molecule is also forced close to the electrophilic carbon atom of SAM. Also, the basicity of the Asp68 carboxylate group will increase upon the weakening of the Asp68/Arg75 ion pair. Such a weakening will be promoted by hydrogen bonding of the guanidinium residue to the imidazole residue of His127. The subtlety of this hydrogen-bonding arrangement in activating a water molecule for nucleophilic attack in the enzyme remains to be evaluated; however, the weakening of very similar ion-pair interactions in this manner has been the focus of model reactions in supramolecular chemistry.^[21,22] Scheme 4 illustrates the putative interplay of the active-site residues for catalytic turnover.

The enzyme was assayed with the adenosine analogues **2**, **3**, and **5** in place of SAM to explore its ability to catalyze the displacement of halides as leaving groups. Intriguingly, 5'-CIDA (**3**)^[23] served as a substrate; however, adenosine was generated with much lower efficiency than from SAM (**1**; $K_m(\mathbf{3}) = 410 \mu\text{M}$, $k_{\text{cat}} = 0.31 \text{ min}^{-1}$). Thus, chlorine could



Scheme 4. Topographical images of the active-site residues of SAM hydroxide adenosyltransferase: A putative reaction cycle.



replace the methionyl moiety as a leaving group. No new products were detected by HPLC or NMR spectroscopy after the incubation of the enzyme with SAM and F^- , Cl^- , or NH_4^+ ions at high mM concentrations, although in all cases adenosine (**4**) was generated in a manner consistent with normal enzymatic turnover, with water providing the nucleophile. *S*-Adenosyl-L-homocysteine (SAH, **6**), the neutral, demethylated form of SAM, is often a potent inhibitor of SAM-dependent enzyme reactions^[24] and also emerged as an efficient inhibitor of this enzyme ($K_i(\mathbf{6}) = 3.6 \mu\text{M}$).

In conclusion, we have identified a SAM enzyme that generates adenosine and L-methionine in a reaction with water. SAM is more commonly converted into adenosine, after demethylation to SAH (**6**), by the action of SAH lyase.^[25] This latter enzyme does not catalyze a direct nucleophilic reaction but utilizes NAD^+ in an oxidation–elimination–reduction process. Thus, there is no mechanistic analogy between SAM hydroxide adenosyltransferase and SAH lyase. The metabolic role of SAM hydroxide adenosyltransferase is not clear. The enzyme takes a high-energy compound (SAM) and generates lower-energy metabolites (adenosine and L-methionine), which are available from other metabolic sources. A similar observation was made with NAD glycohydrolase (NADase), the role of which is also not clear, although recent studies are revealing the sophisticated regulation of its activity in bacteria.^[26] Intriguingly, SAM hydroxide adenosyltransferase generates a proton with each turnover, and the enzyme becomes deactivated at pH 5. The enzyme is most active at pH 8.5, which perhaps suggests a role in regulating intracellular pH values in these extremophiles. To date, the *duf62* gene has been found in up to 200 microorganisms, most of which are extremophiles. Despite its very close (superimposable) structural similarity to the fluorinase and chlorinase enzymes, the DUF62 enzyme activates the nucleophile in a very different manner.^[9] SAM hydroxide adenosyltransferase appears to have a novel mode of action in this respect.

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