Enzyme Catalysis

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

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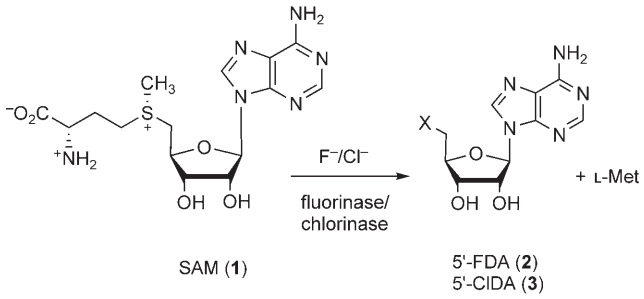
*Dedicated to the memory of Jonathan B. Spencer*

*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, $K_m$(SAM) = 39.2 μM, $k_{cat} = 0.14\ s^{-1}$). 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 $H_2{}^{18}O$ resulted in isotopically enriched [5′-$^{18}$O]-adenosine ([*M*+2]$^+$: 48%), as determined by GC–MS analysis after pertrimethylsilylation at the three hydroxy groups and the

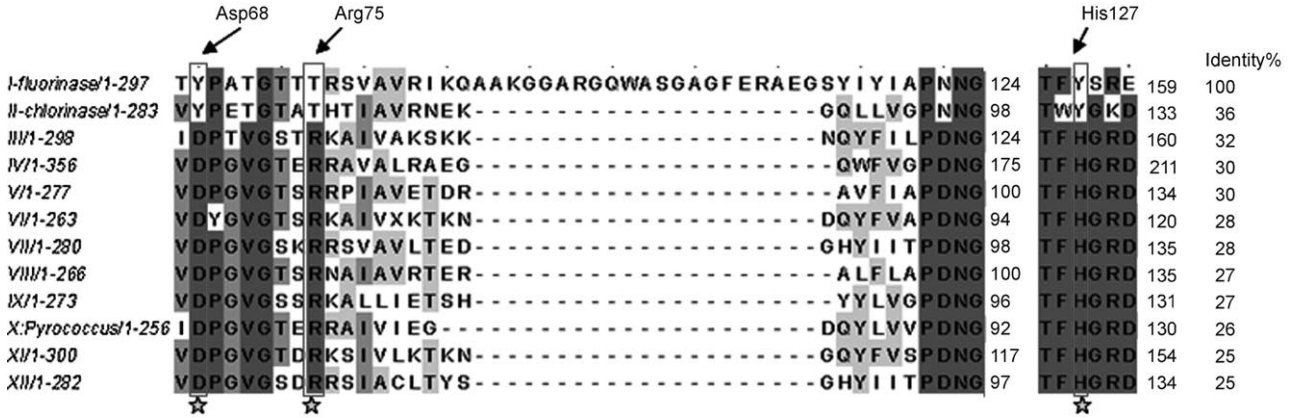



***Figure 1.*** Amino acid sequence alignment and identity (%) between the fluorinase from *S. cattleya* and DUF62 proteins from various organisms. I) Fluorinase from *Streptomyces cattleya*;[7] II) chlorinase from *Salinispora tropica*;[8] III) *Acidobacteria bacterium*; IV) *Salinibacter ruber*; V) *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).

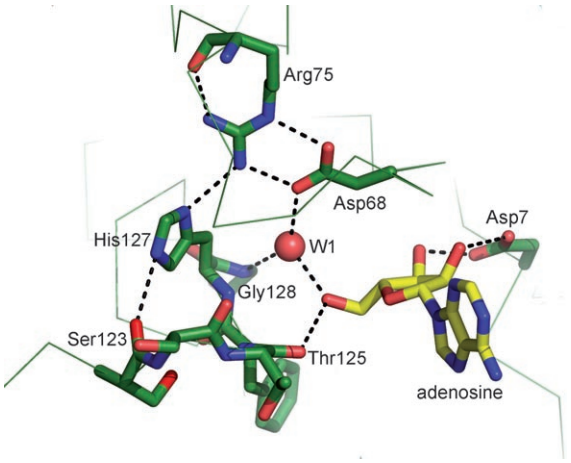
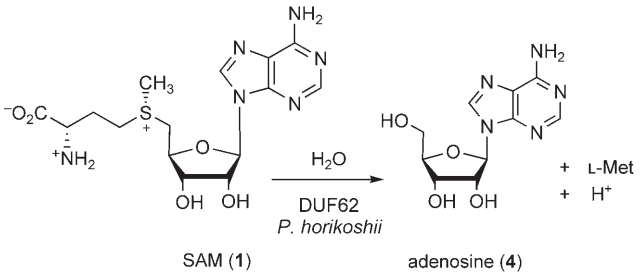
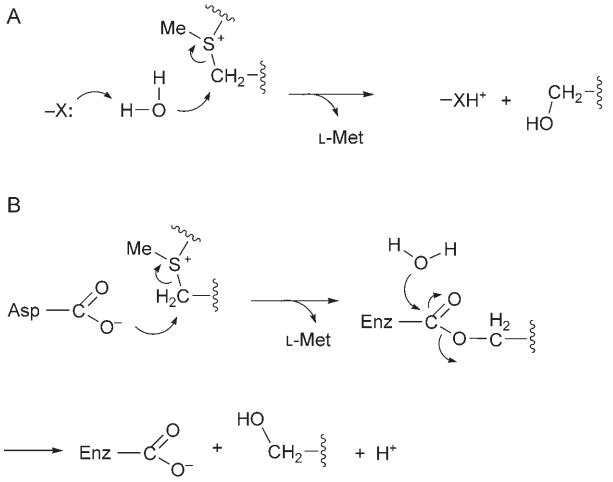



***Figure 2.*** Adenosine bound to SAM hydroxide adenosyltransferase from *P. horikoshii*.




***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.

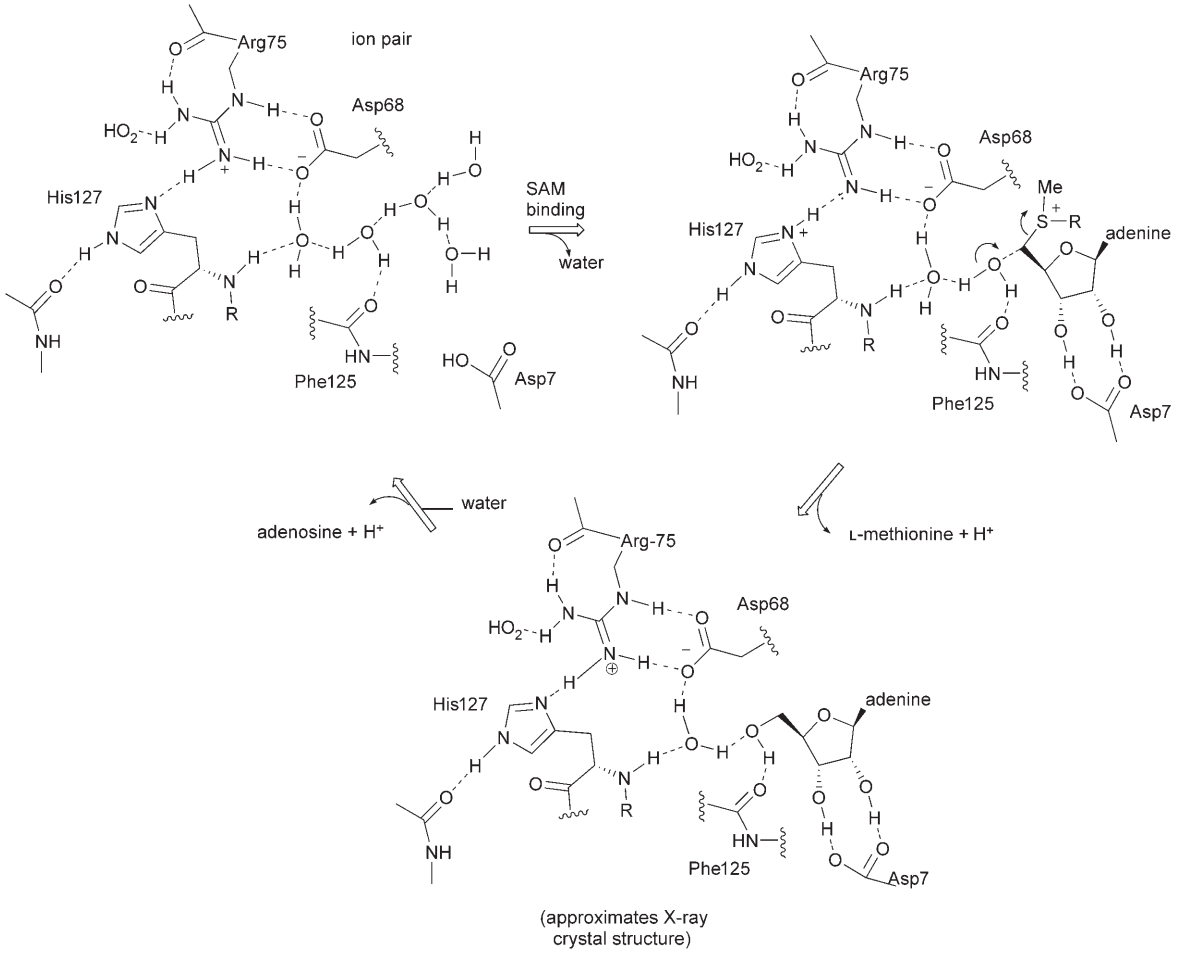
Assays were carried out with $H_2{}^{18}O$ (64 atom %). Mechanism A would label the product adenosine with $^{18}O$ at C5′, but not the enzyme. Mechanism B would load the Asp carboxylate group with $^{18}O$, and multiple turnover would result in both enzyme and product labeling (at C5′). To probe isotope 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).

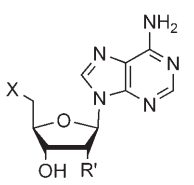
Examination of the active-site residues of the enzyme in the X-ray crystal structure reveals an intriguing juxtaposition of substituents. Asp68 forms an ion-pair dimer with the guanidinium residue of Arg75. The guanidinium residue is H bonded to the imidazole unit of His127. These three amino acid residues are absolutely conserved in all of the putative hydroxide adenosyltransferases ($\approx 200$) 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′-ClDA (**3**)[23] served as a substrate; however, adenosine was generated with much lower efficiency than from SAM (**1**; $K_m(\mathbf{3}) = 410\ \mu M$, $k_{cat} = 0.31\ min^{-1}$). Thus, chlorine could

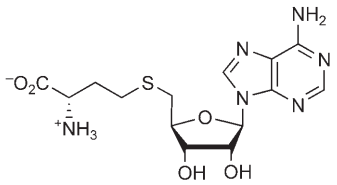



***Scheme 4.*** Topographical images of the active-site residues of SAM hydroxide adenosyltransferase: A putative reaction cycle.



X = F, R' = OH (**2**)
X = Cl, R' = OH (**3**)
X = Cl, R' = H (**5**)



SAH (**6**)

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 ($K_i$(**6**) = 3.6 μM).

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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**Keywords:** archaea · enzyme catalysis · fluorinase · nucleophilic substitution · SAM enzymes

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[12] There is no literature on these structures; however, the DUF62 X-ray crystal-structure coordinates have been deposited routinely in the Protein Data Bank. The four structures have the following PDB codes: *Methanococcus Jannaschii* DSM 2661, PDB 2F4N; *Pyrococcus horikoshii* OT3, PDB 1WU8; *Thermus Thermophilus* Hb8, PDB 2CW5; *Thermotoga maritima*, PDB 2ZBU.
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[14] The clone containing open reading frame (ORF) PH0463 from the marine archaeon *P. horikoshii* OT3 was purchased from NBRC (NITE-DOB, Japan; NBRC No.: NBRC G01-000-971, clone ID: S2_2219, EMBL accession No.: BAA29549). The ORF PH0463 was subcloned into pET28a(+) vector (Novagen) by using the primers PH0463-F (GCAGGAGGAATTCATATGATAACCTTAACCACCGACTTCGG) and PH0463-R (CGCCGCTCGAGTCATAGCAACCTCACCCTTAGCTC) with restriction sites NdeI and XhoI, respectively. The resultant plasmid pET-PH0463 was transferred into competent *E. coli* BL21 C43(DE3) cells and grown in Luria broth containing kanamycin (100 μg mL$^{-1}$) at 37 °C until an absorbance of 0.6 at $\lambda$ = 600 nm was reached. Overexpression was induced by adding isopropylthiogalactoside (IPTG) to 1 mM and continuing the incubation at 16 °C for 24 h. Cells were collected and lysed by sonication, and the apoenzyme was purified to homogeneity as described previously.[15]
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[17] See the Supporting Information for GC–MS, LC–ESI–MSMS, and MALDI–MSMS experimental details.
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