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The fluorinase, the chlorinase and the duf-62 enzymes

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The fluorinase from *Streptomyces cattleya* and chlorinase from *Salinispora tropica* have a commonality in that they mediate nucleophilic reactions of their respective halide ions to the C-5′ carbon of *S*-adenosyl-L-methionine (SAM). These enzyme reactions fall into the relatively small group of S_N2 substitution reactions found in enzymology. These enzymes have some homology to a larger class of proteins expressed by the duf-62 gene, of which around 200 representatives have been sequenced and deposited in databases. The duf-62 genes express a protein which mediates a hydrolytic cleavage of SAM to generate adenosine and L-methionine. Superficially this enzyme operates very similarly to the halogenases in that water/hydroxide replaces the halide ion. However structural examination of the duf-62 gene product reveals a very different organisation of the active site suggesting a novel mechanism for water activation.

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Introduction

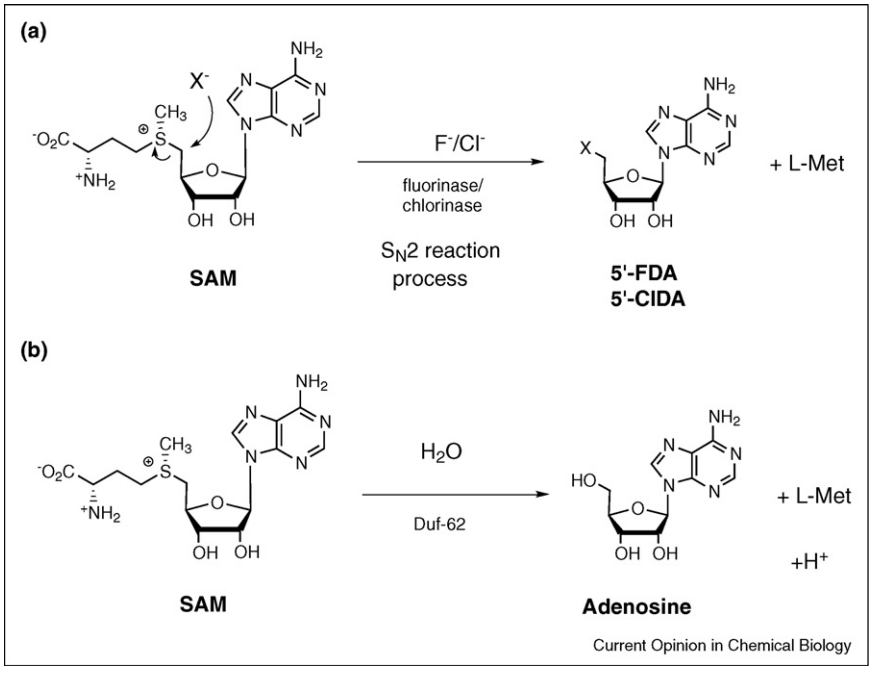
S_N2 substitution reactions at carbon are relatively rare in enzymology [1]. The most common class of such enzymes are *S*-adenosyl-L-methionine (SAM)-dependent methyltransferases, where C, N, O and S nucleophiles attack the methyl group of SAM to generate methylated products [2,3]. Those S_N2 reactions have the lowest possible reaction barriers due to the limited steric impact of the migrating methyl group. Beyond methyltransferases there are haloacid dehalogenases which use the carboxylate groups of Asp as a nucleophile to generate intermediate covalently bound enzyme–ester adducts which are subsequently hydrolysed by water [4–7]. There are also epoxide hydrolases, the majority of which also use Asp/Glu carboxylate groups as the nucleophile, though some less widely distributed epoxide hydrolases utilise water [8,9]. Our studies investigating the fluorinase enzyme involved in the biosynthesis of fluoroacetate and 4-fluorothreonine from the soil bacterium *Streptomyces cattleya* [10–15] have revealed another S_N2 reaction where fluoride ion attacks C-5′ of SAM [16•,17••,18]. More recently two other related enzymes in this category have emerged where chloride ion (chlorinase) [19••] and water/hydroxide (SAM adenosylhydroxide transferase) [20••] act as nucleophiles in similar substitution reactions to C5′ of SAM (Scheme 1). This *minireview* highlights the current mechanistic hypothesis of the fluorinase and the closely related chlorinase, and then explores the nature of the catalytic groups involved in the activation of water in SAM hydroxide adenosyltransferase, the most recently identified and the largest class of these enzymes. These three enzymes show very high similarities in their tertiary structures all of which are almost superimposable trimers (Figure 1).

The fluorinase from *Streptomyces cattleya*

The fluorinase enzyme from the bacterium *S. cattleya* mediates a substitution reaction whereby fluoride ion displaces L-methionine with formation of a C–F bond and cleavage of a C–S bond [16•,17••,18]. Fluoride ion is heavily hydrated in water and the intriguing feature of this enzyme is how it manages to strip fluoride of its water of hydration and release fluoride ion as a nucleophile. The first indication of an S_N2 pathway for the fluorinase came from an exploration of the stereochemical course of the reaction which was shown to occur with an inversion of configuration at the C-5′ carbon [21,22•,23••,24]. This was demonstrated after the preparation of a sample of SAM carrying deuterium at the 5′-*pro*-*S* site and showing that after incubation with the fluorinase, the resultant [$^2H_1$]-5′-FDA had the (*S*) configuration at the C-5′ 'chiral fluoromethyl group' [21]. This stereochemical outcome is consistent with an *inversion of configuration* (Scheme 2).

Crystallography studies on the fluorinase generated separate structures with SAM bound and 5′-FDA bound to the active site of the enzyme [17••]. Key binding residues were revealed for the adenosine ring, the ribose ring and the L-methionyl moiety of SAM. Ser-158 emerged as a key residue [17••]. In the 5′-FDA structure Ser-158 forms two hydrogen bonds through the main chain NH and the side chain OH, to the organic-bound fluorine atom, suggesting a putative site for fluoride ion binding, immediately before nucleophilic attack. This hypothesis was further supported by a QM/MM theory study [22•] where the conformation of the reactants (SAM + F^-) and products (5′-FDA + L-Met) in the before and after reactive complexes, and of the transition state have been

Scheme 1

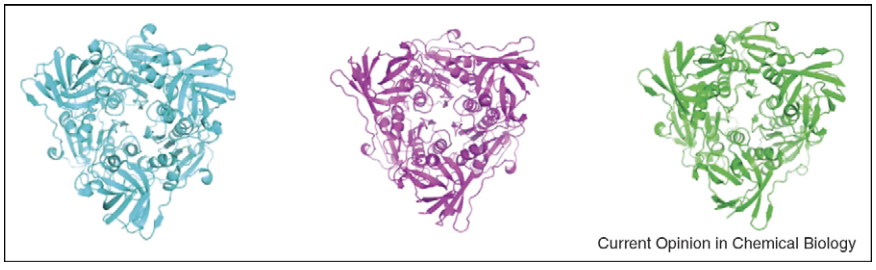


(**a**) Halide ion (fluorinase/chlorinase) reactions of SAM to generate 5′-XDA and L-methionine and (**b**) the SAM hydroxide adenosyltransferase reaction.

examined. Taken together the crystallographic and theory studies suggest that fluoride ion becomes completely dissociated from its hydrated water. There is no crystallographically located water hydrogen bonding to the fluorine. The theory study tracked the reaction through its transition state to 5′-FDA. Fluoride ion makes hydrogen-bonding contacts to both the NH and OH hydrogens of Ser-158 in the reactive complex and also to the OH side chain of Thr-80 as it progresses towards the transition state. This last hydrogen-bonding contact is not observed in the ground state X-ray derived structures but is a conclusion drawn for the QM/MM theory study (Figure 2). The enzyme appears to catalyse the reaction by predisposition of these hydrogen-bonding interactions. The calculated activation energy is 53 kJ mole^{-1}. This is a significant lowering of the activation barrier compared to the barrier calculated for the solution reaction (92 kJ mole^{-1}) and represents an enzymatic rate acceleration of one million fold (10^6) [22•].

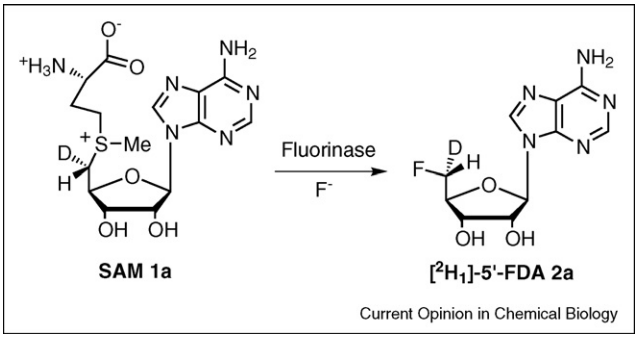
In order to probe the role of the putative hydrogen bond from Thr-80 to fluoride ion during the fluorinase reaction, a series of site-directed mutagenesis studies were carried out [23••]. The implication from the QM/MM study was that the Thr-80 OH, which in the ground state is hydrogen bonded to the peptide backbone carbonyl of Pro-154, breaks free of this interaction to assist the stabilisation of desolvated fluoride ion. Consistent with this working hypothesis, a T80A mutation, removed the OH group and reduced the catalytic efficiency of the enzyme to 3% relative to the wild-type enzyme. However a T80S mutation, in which an OH group is now retained in the new amino acid, resulted on a protein with 95% activity.

Figure 1



Trimer motifs of the fluorinase (blue), chlorinase (pink) and SAM hydroxide adenosyl transferase (green).

Scheme 2



The fluorinase reaction proceeds with an inversion of configuration.

The result suggests that this is an important hydrogen-bonding interaction for catalysis.

Kinetic studies on the fluorinase have revealed that in the forward direction fluoride ion binds before SAM for catalytic turnover. Fluoride ion actually has a very low affinity for the enzyme with a K_m in the high mM range. Intriguingly the K_m of fluoride ion increases (10–47 mM) with higher SAM concentrations (20–300 μM), indicative of competitive binding. This data suggests that fluoride ion passively diffuses into the active site with very low affinity, but when SAM binds, it traps fluoride ion in the active site, and the binding of SAM, which has a much lower K_m (6.5 μM) squeezes out any remaining water. This binding event acts to fully desolvate fluoride ion, driven by a high affinity of the enzyme for the adenosine, ribose and methionyl moieties of SAM. A representation of this process is illustrated in Scheme 3.

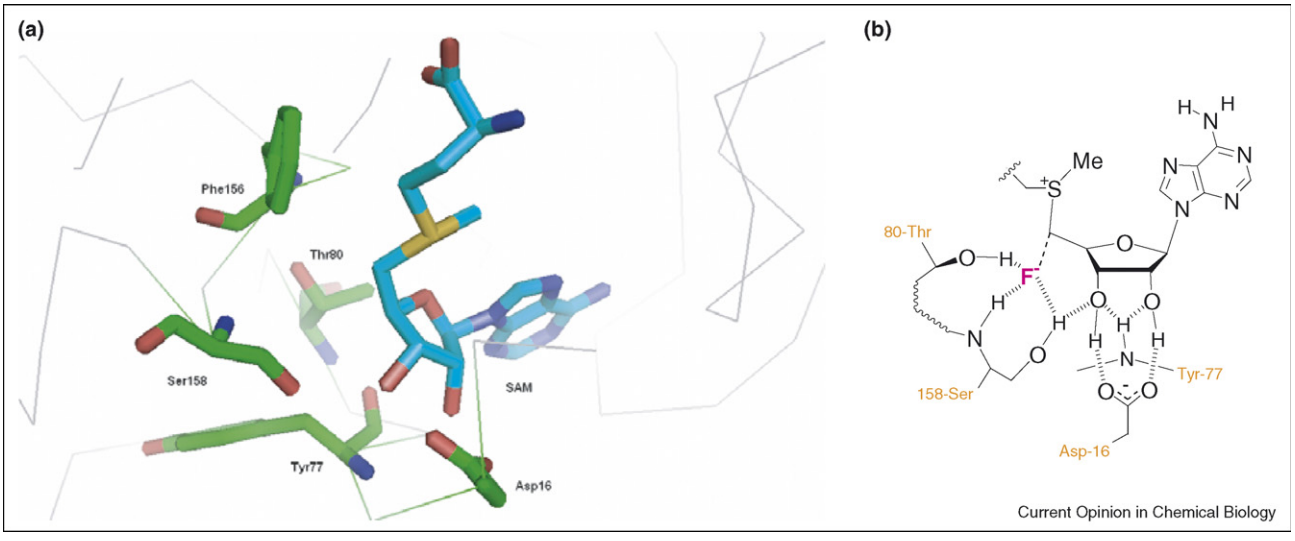
Another theory study which modelled this reaction in a water matrix, has argued that the electrostatic interaction between F^- ion and the positively charged sulfur (R_3S^+) of SAM also confers some stability to this bound complex [24]. Thus fluoride ion gains additional stability by the approach of the sulfonium on binding. With fluoride ion now desolvated and trapped, it then hits back by attacking the C-5′carbon of SAM by an S_N2 type process to generate the new C–F bond and release L-methionine. Investigations on the order of debinding reveal that L-methionine is released from the enzyme surface followed by 5′-FDA [23••].

The chlorinase from *Salinispora tropica*

In 2007 a genome sequence-guided study led to the identification of a gene cluster from a marine actinomycete *Salinispora tropica* [25]. This organism is responsible for the production of the chlorinated natural product salinisporamide A (Scheme 4) [26,27], a compound in Phase II clinical trials. A SAM-dependent chlorinase, a product of the *SalL* gene, emerged as an analogue of the fluorinase and has been shown to generate 5′-ClDA as the first step in salinisporamide A biosynthesis (Scheme 4). The chlorinase has 35% amino acid identity to the fluorinase (Figure 5) and is also a homotrimer by crystallography and in solution (Figure 1). Although the chlorinase binds adenosine/5′-ClDA in a similar manner to the fluorinase, the key residues for co-ordinating their respective halide ions are different. Gly-131 replaces Ser-158 in the fluorinase and Tyr-70 replaces the Thr-80 residue in the fluorinase [19••] (Figure 3).

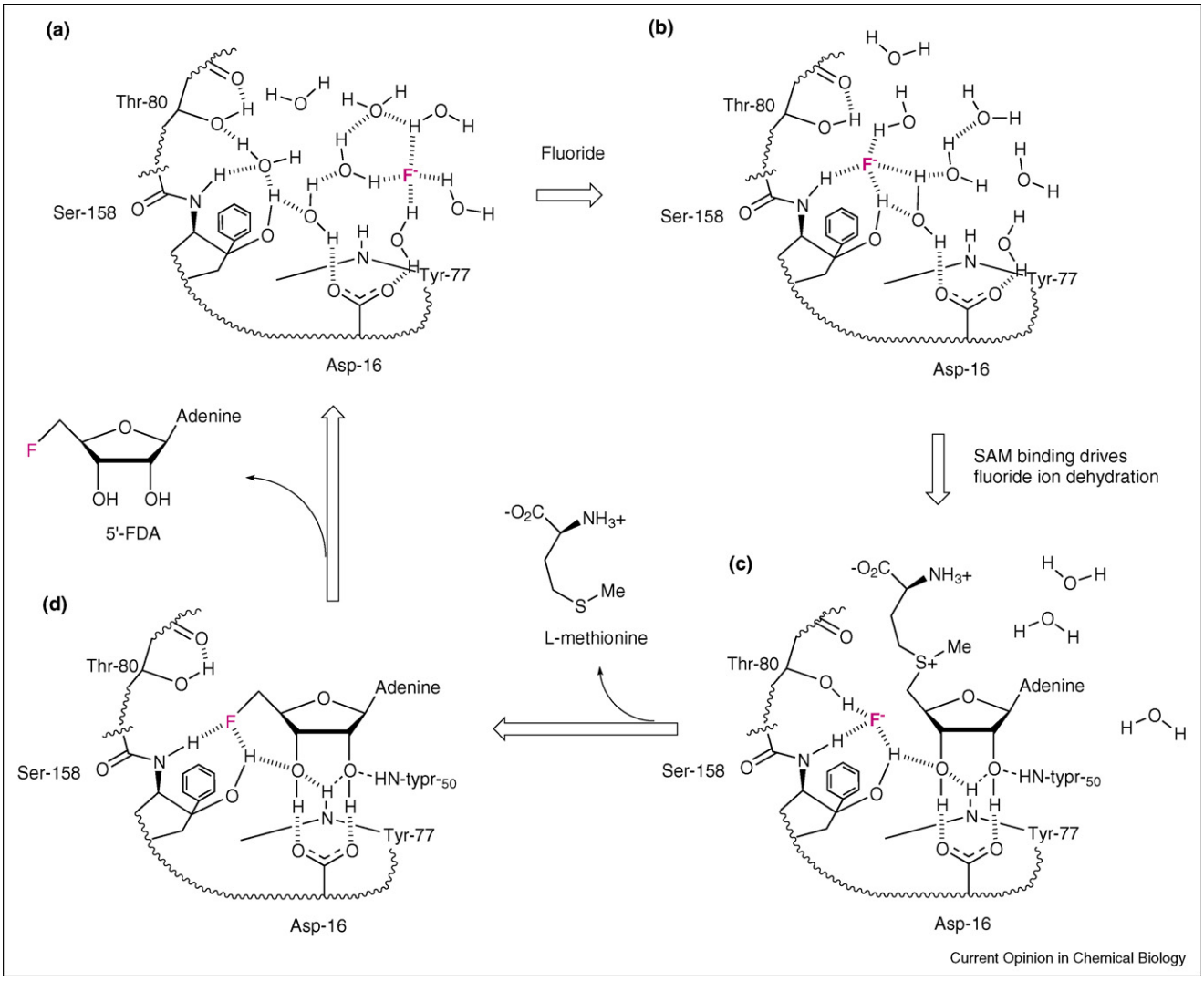
Although this enzyme will also utilise Br^- and even I^- it does not fluorinate. Replacement of Gly-131 by serine to engineer a similarity to the fluorinase resulted in instability

Figure 2



Location of the fluoride ion binding site as deduced by (**a**) X-ray structure analysis of the fluorinase with SAM and (**b**) a schematic diagram derived after QM/MM evaluation of the reaction transition state [17,22•].

Scheme 3



Stepwise illustration of the fluorinase reaction. (**a**) An open hydrated binding site. (**b**) Fluoride ion has a low affinity diffusing into the active site where hydrogen bonds to two water molecules are replaced by contacts to Ser-158. (**c**) SAM then binds with high affinity and displaces water from the active site and acts to desolvate fluoride ion. Fluoride ion is then partially stabilised by a further interaction with Thr-80. (**d**) The juxtaposition of fluoride and SAM leads to nucleophilic attack by fluoride ion to generate 5′-FDA. L-Methionine is released followed by 5′-FDA [23••].

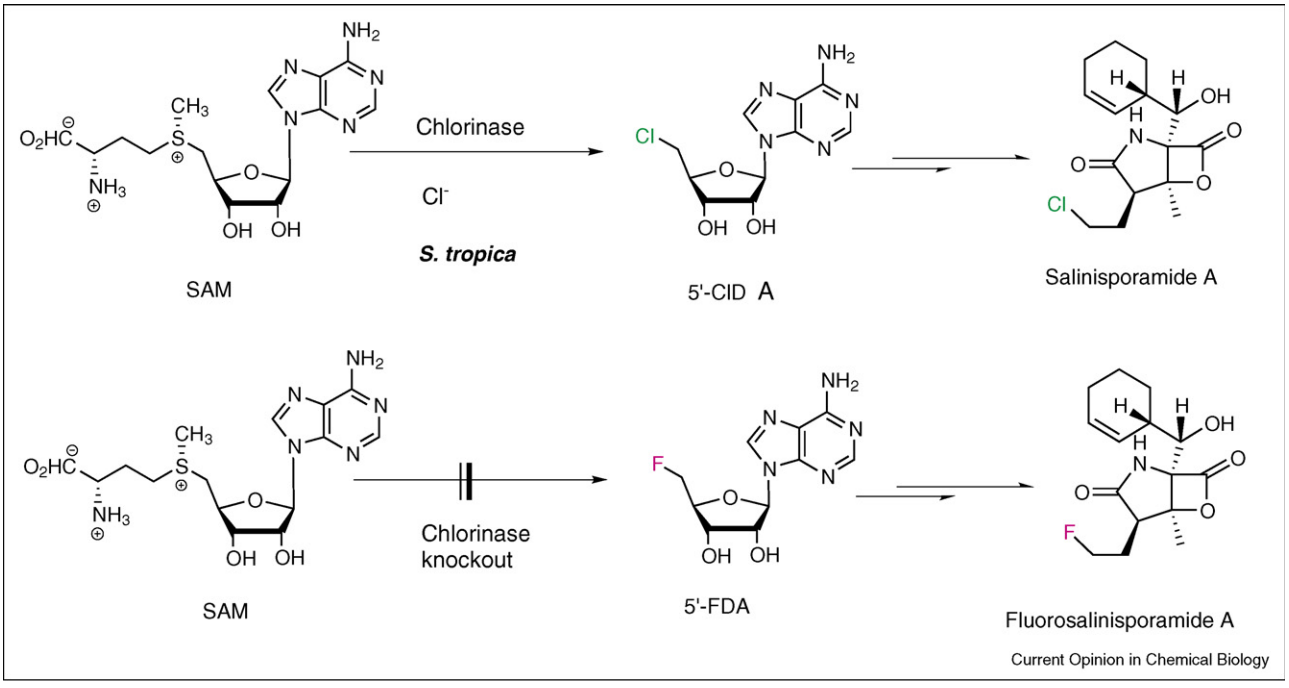
and arrested chlorination. Replacement of Tyr-70 by threonine led to a 10^3 fold loss of chlorination activity. However, crystallography of the Y70T mutant allowed chloride ion to be crystallographically located and revealed hydrogen bonds to two water molecules in the active site. The chloride ion adopts a position approximately 180° from the electrophilic C-5′ carbon of SAM, consistent also with an S_N2 mechanism as described for the fluorinase [19••]. Presumably there is a similarity in the overall catalysis between the fluorinase where SAM binding promotes desolvation and positions the nucleophilic halide ion proximate to C-5′ of SAM.

Interestingly a chlorinase knockout mutated strain of *S. tropica* was able to biosynthesis the fluorinated analogue of salinosporamide A, when the medium was supplemented with 5′-FDA [28]. Thus all of the subsequent enzymes on the biosynthetic pathway in *S. tropica* between 5′-ClDA and salinisporamide were able to process the analogous fluorinated intermediate, 5′-FDA.

S-adenosyl-L-methionyl hydroxide adenosyltransferase

A BLAST database search of the fluorinase amino acid sequence reveals the homology table shown in Figure 4. The fluorinase is most closely related (36%) to the chlorinase from *S. tropica*, and then has a homology (32–25%) to a series of domains of unknown function-62 (duf-62) proteins. [20••]. For brevity, Figure 4 shows only 10 of over 200 or so duf-62s identifiable in the database and

Scheme 4

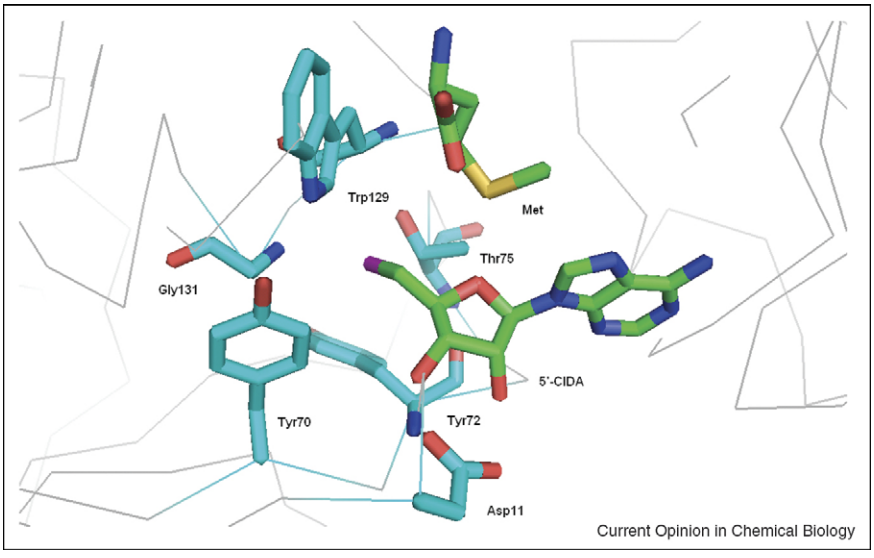


The biosynthesis of salinisporamide A and fluorosalinisporamide A [19].

presents only a section of this homology which has been selected to highlight key differences and similarities. Poignantly the fluorinase has a unique 21 amino acid 'insert' (AAKGGARGQWASGAGFERAEG) which forms a clearly defined loop in the tertiary structure of the protein. This is absent in all of the related proteins. Interestingly four gene sequencing/protein over-expression programmes have generated X-ray structures of duf-62 proteins [29]. All four of these structures have almost identical tertiary/quaternary structures to each other and to the fluorinase/chlorinase structures (Figure 1).

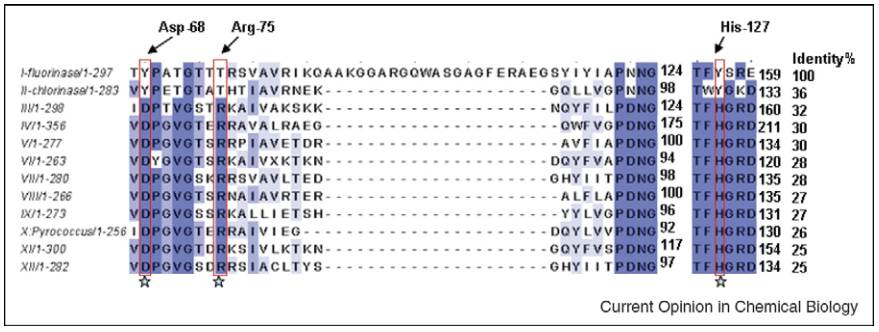
The duf-62 protein from *Pyrococcus horikoshii* was recently over-expressed in *E. coli* to assess its function [20••]. The

Figure 3



Active site of the chlorinase from *Salinispora tropica* with 5′Cl-DA and L-methionine bound [19••].

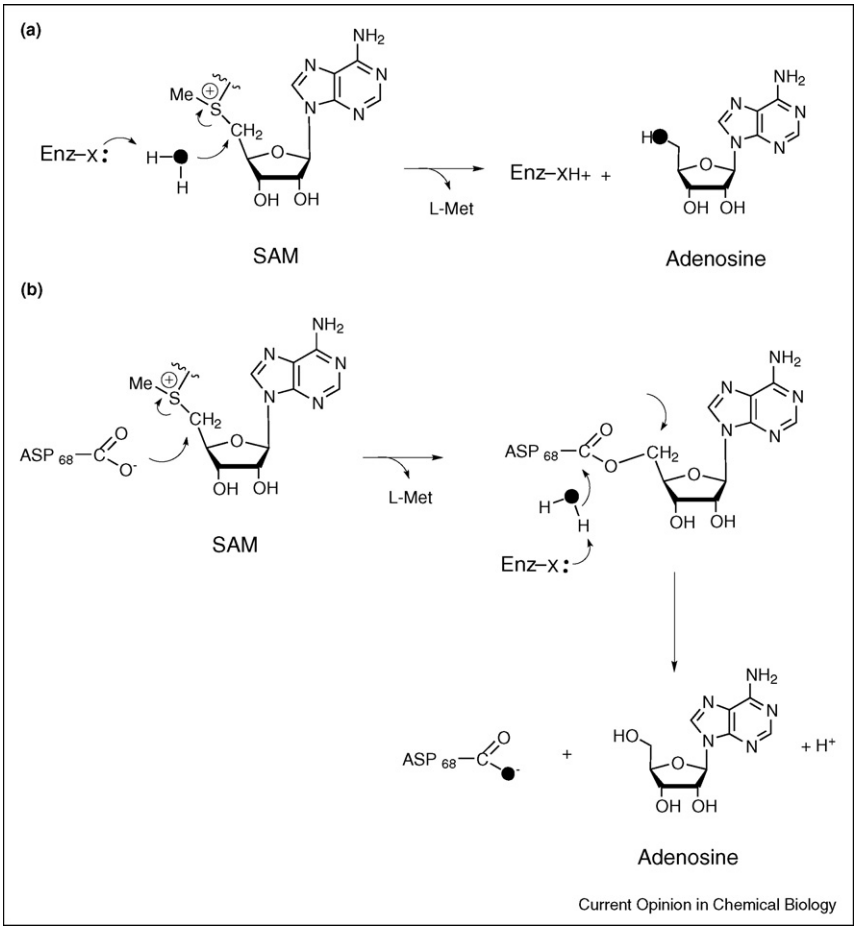
Figure 4



Amino acid sequence alignment and conserved amino acids in ten duf-62 proteins.

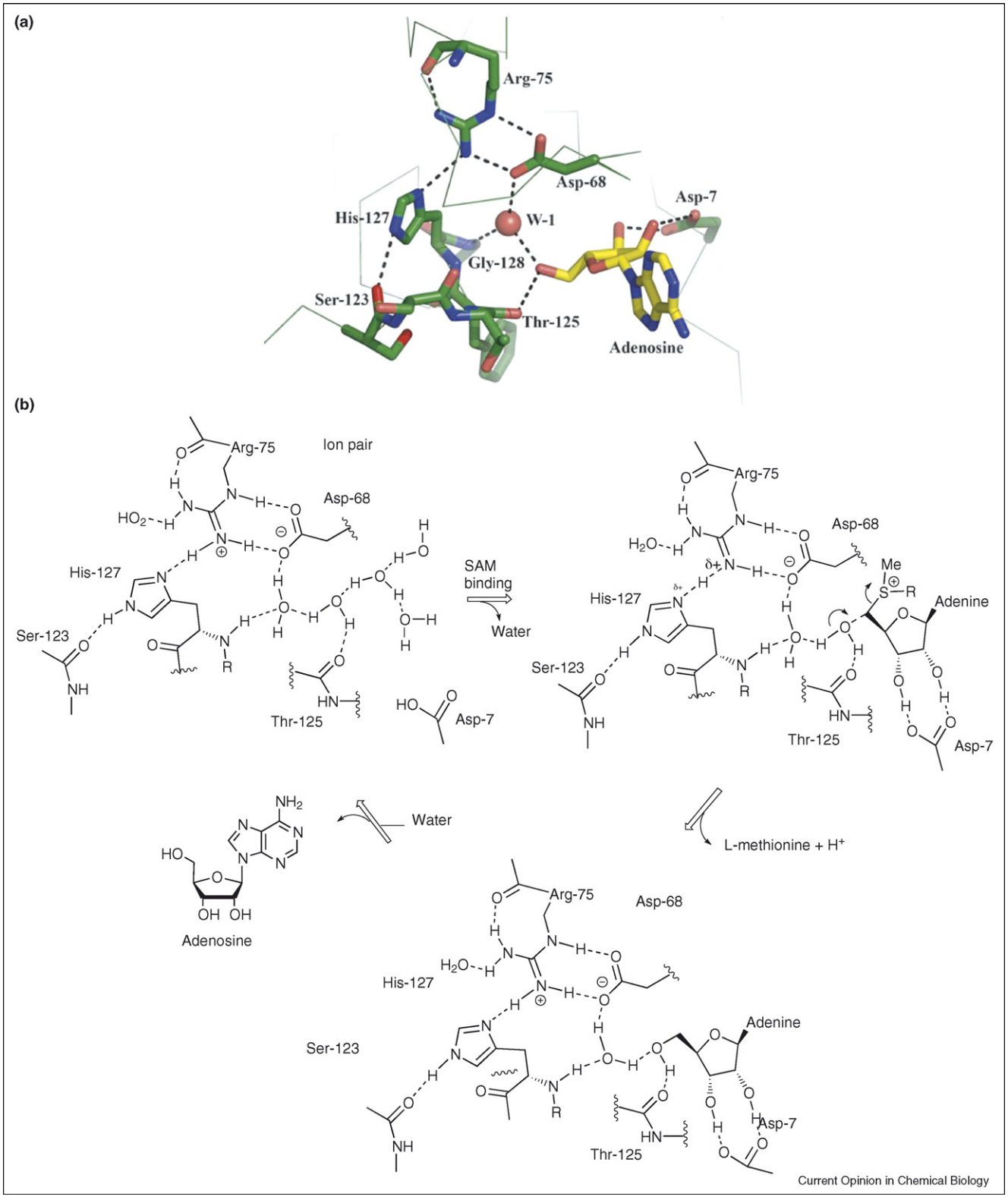
protein was unable to mediate a fluorination or a chlorination reaction with SAM **1** even after incubation at high (>10 mM) concentrations of halide ions. However it was apparent that the enzyme could catalyse the conversion of SAM to adenosine and thus water appeared to act as a nucleophile, attacking the same C-5′ carbon of SAM, analogous to the nucleophilic halide reactions (Scheme 5). The *P. horikoshii* enzyme was assayed at 37 °C and was optimal at pH 8.5 (SAM K_m = 39.2 μM, k_{cat} = 0.14 s^{-1}). Consistent with a protein from a hyperthermophilic

Scheme 5



Two candidate mechanisms A and B emerged for nucleophilic attack of SAM by the duf-62 enzymes. The image shows the expected isotopic labelling pattern from a single turnover using $^{18}OH_2$ water, Mechanism A is established as the more correct after isotopic labelling studies with $^{18}OH_2$ [20••].

Figure 5



(**a**) Active site residues from X-ray crystallography of SAM hydroxide adenosyltransferase from *P. horikoshii* showing the Asp-68–Arg-75–His-127 triad and adenosine bound to the enzyme active site. (**b**) The scheme illustrates a putative mechanism of water activation for the enzyme reaction involving the amino acid triad [20••].

organism, *P. horikoshii* [30,31], the enzyme retained full activity after heating to 80 °C for 30 min and then cooling to 37 °C.

In order to explore the role of water further the *P. horikoshii* enzyme reaction was carried out using $^{18}OH_2$ water (Scheme 5). Assay of the SAM hydroxide adenosyltransferase in buffer, enriched with $H_2{}^{18}O$ (50%), resulted in isotopically enriched [5′-^{18}O]-adenosine as determined by GC–MS analysis [20••].

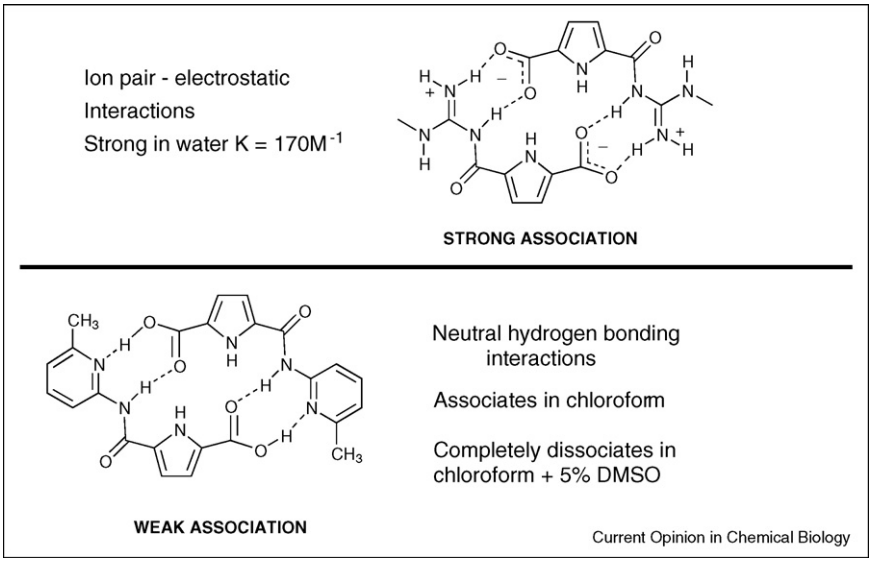
A mechanistic hypothesis for SAM hydroxide adenosyltransferase

In the X-ray structure (Figure 5a) of the enzyme (*P. horikoshi*), adenosine is bound to the enzyme clearly locating the active site. Examination of the active site amino acid residues from the structure reveals Asp-68 located close to the C-5′ carbon of adenosine. This residue emerged as a possible nucleophile in the reaction. Amino acid side chain carboxylate groups are known to be involved as nucleophiles in haloacid dehalogenase reactions [4–7] and in the majority of epoxide hydrolases [8–9,32•], generating a substrate-bound ester in the catalytic cycle which gets hydrolysed to release an alcohol with concomitant recovery the carboxylate group. The putative mechanism B in Figure 5b presents an analogous possibility for SAM hydroxide adenosyltransferase. To distinguish mechanisms A and B and probe a role for Asp-68 in the reaction, isotopic labelling of the protein was then examined by MS-MS mass spectrometry after assays were carried out with $H_2{}^{18}O$ [20••]. This analysis *did not* provide any evidence for isotope incorporation into the side chain carboxylate group of the peptide fragment containing Asp-68 supporting general mechanism A and *not* B (Scheme 5).

An investigation of the active site amino acid residues of the *P. horikoshii* and three other duf-62 structures [29] suggests an intriguing putative mechanism for nucleophilic activation of water (Figure 5b).

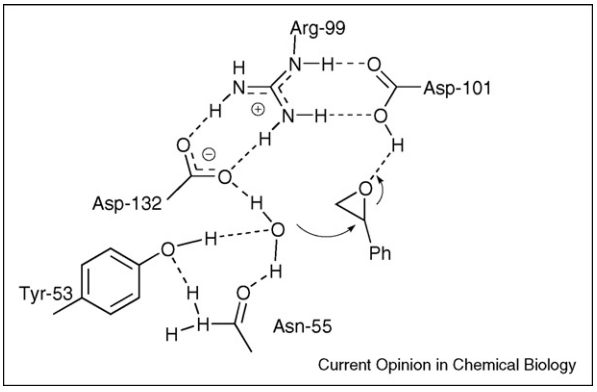
Substitution by hydroxide ion is almost certainly an S_N2 substitution process, similar to the fluorinase [17••]. Three key amino acids (Asp-68–Arg-75–His-127) are hydrogen bonded together. These amino acids are absolutely conserved in all of the duf-62 proteins but they *are not* present in the fluorinase or the chlorinase enzymes (see Figure 4) so nucleophile activation is different. It can be seen in Figure 5 that Asp-68 forms an ion pair dimer with the guanidinium residue of Arg-75. The guanidinium residue is H-bonded to the imidazole ring of His-127. An inner water molecule is hydrogen bonded to the Asp-68 carboxylate. The developing hypothesis suggests that the outer water is made nucleophilic by co-ordination to a backbone amide carbonyl of Thr-125 and it is also co-ordinated to the inner water molecule (present in the product crystal structure). This water molecule is also forced close to the electrophilic carbon on SAM binding. Clearly the basicity of the Asp-68 carboxylate will increase if the Asp-68/Arg-75 ion pair is weakened, and such a weakening would be promoted by increased hydrogen bonding of the guanidinium to the imidazole residue of His-127. Weakening of very similar ion-pair interactions in this manner has been explored quite extensively by Schmuck and Wienland [33,34] in their studies in supramolecular chemistry. They have shown that guanidinium–carboxylate ion-pairs generate very strong associations through H-bonding and electrostatics, even in water (Figure 6). However neutral molecules designed to be spatially similar with the same number of hydrogen bonds associate much more weakly in chloroform

Figure 6



An ion paired dimer has a much greater affinity due to electrostatic stabilisation, than a geometrically similar H-bonded dimeric system [33,34].

Figure 7



A representation of the key active site residues of limonene-1,2-epoxide hydrolase from *R. erythropolis*, with styrene oxide illustrated as a substrate. Crystallography suggests a push-pull type mechanism where different aspartate carboxylates sandwich the guanidinium residue of Arg-99. One carboxylate acts as a general acid, and the other as general base to catalyse hydrolytic epoxide ring opening [32•].

and completely dissociate in polar solvents and water. It follows that dissipation of the charge on the guanidinium residue will act to weaken the interaction and promote release of the Asp carboxylate as a base in these enzymes.

The exact nature and the extent of any positive charge dissociation through His-127, promoted perhaps by SAM binding, remains to be verified. The activation of water as a nucleophile in these enzymes has some homology to the rare group of water/hydroxide utilising epoxide hydrolyses, most intensively studied in limonene-1,2-epoxide hydrolase [8,32•]. In the limonene-1,2-epoxide hydrolase from *Rhodococcus erythropolis*, water also acts as a nucleophile in an S_N2 reaction and intriguingly the nucleophilic water is similarly hydrogen bonded to an aspartate carboxylate involved in an 132-AspCO_2^-...$^+N_3H_5$-Arg-99 dimerisation, and to an amide carbonyl, in this case Asn-55 [8,9,32•] (Figure 7).

In this process the developing hypothesis suggests that the carboxylate of Asp-132 co-ordinates with water at the moment that Asp-101 contributes a proton to hydrogen bond the epoxide oxygen [32•]. Both carboxylates are competing for the electrostatic stabilisation of the guanidinium residue, and clearly as Asp-101 become deprotonated, this will weaken the ion pair between Asp-99 and the guanidinium, and render the carboxylate an improved base to assist in water deprotonation. The electronics of this push-pull system are however more evenly balanced than that found in SAM hydroxide adenosyltransferase.

A role for the SAM hydroxide adenosyltransferase?

The co-factor SAM is generally metabolised, after demethylation to *S*-adenosyl-L-homocysteine (SAH), by the action of SAH-lyase [35]. This enzyme activity also generates adenosine, but the conversion occurs after SAM has used its metabolic energy to transfer a methyl group. Also SAH-lyase does not catalyse a nucleophilic substitution reaction but it utilises the co-factor NAD^+ in an oxidation, to then mediate C–S bond cleavage by an elimination reaction. There is no mechanistic similarity between SAM hydroxide adenosyltransferase and SAH-lyase. It is not clear what metabolic advantage SAM hydroxide adenosyltransferase confers on the host organism. On the face of it, it converts a high-energy intermediate metabolite (SAM) to adenosine and L-methionine, low-energy cellular constituents available from primary metabolic pathways. Such a contra-intuitive role has some precedent in other enzymes. For example, NAD glycohydrolase (NADase) mediates a hydrolytic reaction on NAD^+, in an apparently wasteful reaction in energy terms, though molecular biology studies are illuminating a sophisticated regulation of this enzyme's activity in bacteria, suggesting a more deep-seated function [36,37]. SAM hydroxide adenosyltransferase also generates a proton (H^+) with each catalytic turnover, and the enzyme becomes deactivated at pH 5. The enzyme is most active at pH 8.5, so perhaps it has a regulatory role in influencing the cellular pH in the host organism. Many, but not all of the duf-62 containing organisms are extremophiles.

Summary

The fluorinase and chlorinase enzymes catalyse S_N2 reactions with halide ion as a nucleophile. The duf-62 gene product/enzyme catalyses an intriguing new hydrolytic activity, and reveal a new SAM metabolising enzyme, *S*-adenosyl-L-methionine hydroxide adenosyltransferase. This group of enzymes mediate superficially similar nucleophilic reactions, in that fluoride (F^-), chloride (Cl^-) or hydroxide (HO^-) ions attack C-5′ of SAM with concomitant displacement of L-methionine. However, the mode of nucleophile activation is different in each case. The halide enzymes have a different halide active site organisation and both activate the nucleophile by a combination of hydrogen bonds to the protein and by desolvation, assisted by the SAM binding event. The duf-62 proteins utilise a rigorously conserved amino acid side chain triad (Asp-Arg-His) which may have a role in activating water to hydroxide ion. The halide enzymes are involved in the first step of two halogen containing antibiotic biosynthetic pathways. *S*-adenosyl-L-methionine hydroxide adenosyltransferase is much more widely distributed as a gene/enzyme however its role is less clear. The production of a proton (H^+) with each catalytic turnover perhaps points to a role in cellular pH regulation, though this remains speculative at present.

Note added in proof

The Duf-62 enzyme from the marine organism *Salinispora arenicola* has recently been over-expressed and

assayed. See; A.S. Eustáquio, J. Härle, J.P. Noel, B.S. Moore, *ChemBioChem*, 2008, in press.

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