Substrate specificity in enzymatic fluorination. The fluorinase from *Streptomyces cattleya* accepts 2′-deoxyadenosine substrates†

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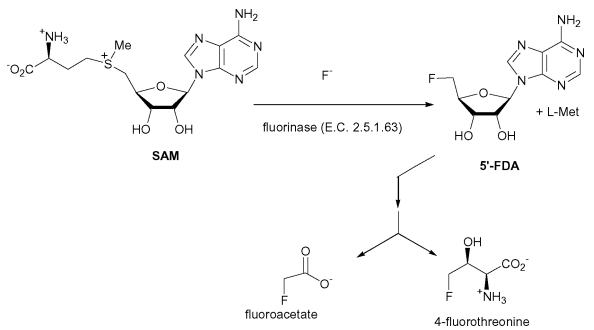
The fluorinase enzyme from *Streptomyces cattleya* displays an unusual ability in biocatalysis in that it forms a C–F bond. We now report that the enzyme will accept 2′-deoxyadenosine in place of adenosine substrates, and structural evidence reveals a reorganisation in hydrogen bonding to accommodate this substrate series. It emerges from this study that the enzyme does not require a planar ribose conformation of the substrate to catalyse C–F bond formation.

Introduction

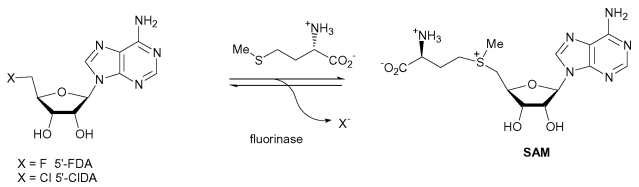
The fluorinase enzyme from *Streptomyces cattleya*[1,2] is the first committed enzymatic step on the biosynthetic pathway to the fluorometabolites fluoroacetate and 4-fluorothreonine.[3] The enzyme catalyses the reaction of *S*-adenosyl-L-methionine (SAM) and fluoride ion to generate 5′-fluoro-5′-deoxyadenosine (5′-FDA) and L-methionine (L-Met) (Scheme 1).



Scheme 1 The fluorinase enzyme of *S. cattleya* is the first committed step on the biosynthetic pathway to fluoroacetate and 4-fluorothreonine.

We have recently reported[4] that the fluorinase reaction is reversible and that the fluorinase can catalyse the conversion of L-Met and either 5′-FDA or the chlorinated analogue 5′-ClDA to SAM, with concomitant release of the corresponding halide ion. In fact, in the reverse direction 5′-ClDA is the more efficient substrate, as chloride is the better leaving group (Scheme 2). Also the selenium analogue of SAM, SeAM, is a more efficient substrate than SAM.

The ability to run the reaction in reverse has opened up the possibility of exploring details of the substrate specificity of the fluorinase, as analogues of 5′-FDA are more readily prepared than analogues of SAM. In terms of trying to assess where structural variations can or cannot be made, non-covalent contacts between the substrate/product and the surface of the enzyme can provide a useful backdrop in guiding this. The X-ray structure of the enzyme has revealed what appear to be important hydrogen bonding interactions between the carboxylate group of Asp-16 and the 2′- and 3′-hydroxyl groups of the ribose ring of 5′-FDA.[5] The ribose ring is held in a strained conformation as a consequence of this hydrogen bonding interaction. The C2′–OH and C3′–OH bonds (torsion angle 1°) are eclipsed in the substrate (SAM) structure and relax a little (torsion angle 12°) in the product (5′-FDA) structure (Fig. 1). The consequence of this ring conformation to the reactivity of the substitution reaction is not clear even after a detailed theoretical analysis of the reaction.[6] In this *Communication* we report that the fluorinase will accept the 2′-deoxy analogues 2′d-FDA and 2′d-ClDA as substrates to



Scheme 2 The fluorinase operates in reverse with both 5′-FDA and 5′-ClDA.

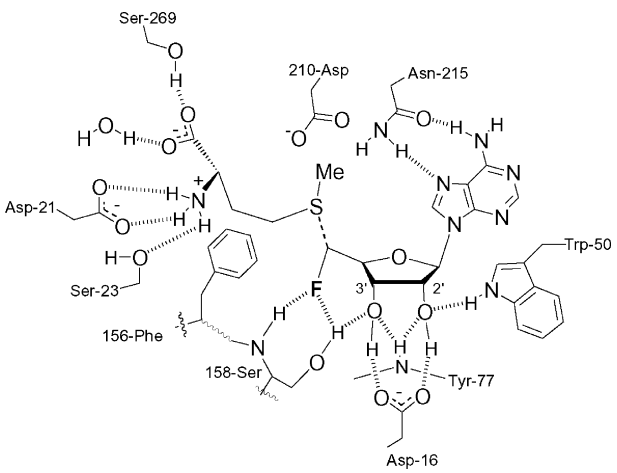


Fig. 1 Representation of 5′-FDA and L-Met bound to the fluorinase from X-ray structure analysis. The graphic highlights two hydrogen-bonds to the fluoromethyl group from Ser-158. There also are hydrogen bonding interactions between Asp-16-CO_2^- and the 2′- and 3′-OH groups of the ribose ring.

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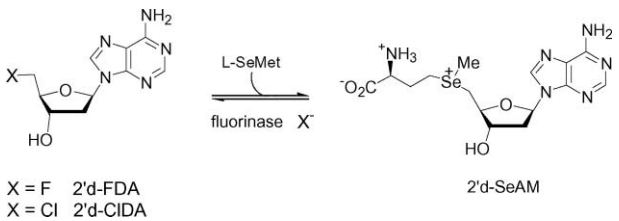
generate 2′d-SAM. We also report structural data which reveals how the 2-deoxy substrates bind at the active site.

Results and discussion

In order to explore the role of the 2′-OH hydroxyl group of the substrate during fluorinase catalysis, 2′d-FDA and 2′d-ClDA were synthesised in a straightforward manner following previously described methods.[7,8]

The 2′-deoxy analogue 2′d-FDA was then assessed as a substrate in the reverse direction with both L-Met and L-[^{13}C-*methyl*]-Met. In each case 2′d-SAM was generated and ESI-MS clearly indicated the incorporation of the ^{13}C-isotope in the latter experiment (ESI *m/z* 384.2 *versus* 383.3 $[M + H]^+$). 2′d-ClDA was also a good substrate for the fluorinase. Although both 2′d-FDA and 2′d-ClDA were substrates, they were processed less efficiently than 5′-FDA. Again the chloro-analogue 2′d-ClDA was the more efficient, with a relative rate[9] of approximately 50% of the natural substrate, 5′-FDA. 2′d-FDA was processed at approximately 10% of the rate of 5′-FDA. It is clear from this study that although the 2′-hydroxyl group of the ribose ring has been removed these substrates retain a significant level of activity.



Scheme 3 The fluorinase can catalyse the conversion of 2′d-FDA and 2′d-ClDA substrates with L-SeMet to generate 2′d-SeAM.

Although chloride is the better leaving group, fluoride ion is a better substrate/nucleophile in the forward direction. This is demonstrated in a transhalogenation reaction. When 2′d-ClDA was incubated with the fluorinase and L-selenomethionine (L-SeMet) in the presence of 2 mM fluoride, then as the reaction progresses (Scheme 3), the resultant 2′d-SeAM is arrested by fluoride ion and converted to 2′d-FDA in a simultaneous fluorinase-catalysed reaction. This is illustrated by the HPLC time course of the transhalogenation reaction in Fig. 2. These transhalogenation reactions are most efficient with the more nucleophilic L-SeMet.

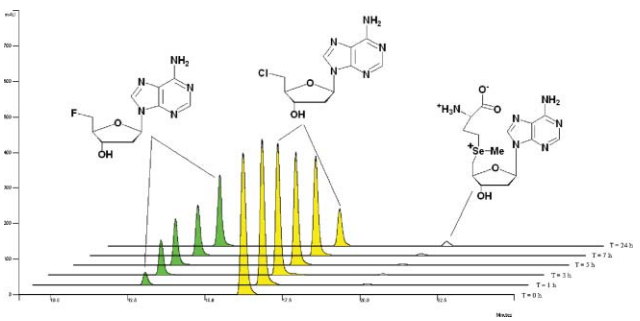


Fig. 2 HPLC profile of the transhalogenation from 2′d-ClDA to 2′d-FDA *via* 2′d-SeAM, catalysed by the fluorinase.

To gain some insight into how these substrates bind at the active site, a co-crystallisation of 2′d-FDA with the fluorinase was carried out for X-ray analysis. This resulted in crystals suitable for diffraction analysis, and the resultant structure is shown in Fig. 3. For comparison, 2′d-FDA is overlaid with the previously reported structure[5] of 5′-FDA bound to the active site. The 3′-OH of the 2′-deoxy substrate retains hydrogen bonding contacts to the OH of Ser-158 and the NH of Tyr-77, however the 3′-OH moves to accommodate a more central bifurcated hydrogen bond with oxygens O1 and O2 of Asp-16, a situation that does not arise with 5′-FDA. In both structures the fluorine atom is almost identically positioned, but the C–F bonds are not co-aligned. The ribose ring is puckered in 2′d-FDA (C–C–C–C torsion 30.4°) relative to the planar situation found with 5′-FDA (C–C–C–C torsion 1°). This suggests that the strained planar conformation of bound 5′-FDA is not a determining factor in driving the substitution reaction, an observation consistent with these experimental observations and a recent theoretical analysis of this reaction.[6]

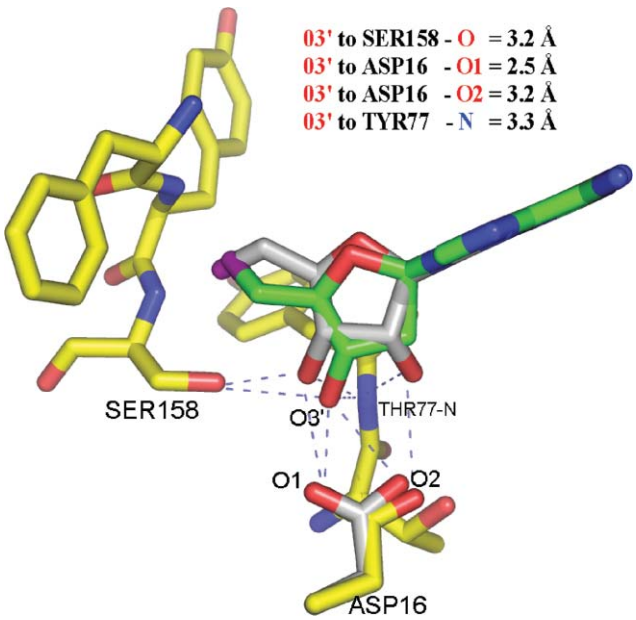


Fig. 3 Structure of the 2′d-FDA-fluorinase co-complex (ribose carbons are green), overlaid with the structure of 5′-FDA (ribose carbons are white) bound to the enzyme. The conformation of the ribose ring is more puckered in 2′d-FDA, and the 3′-OH group and the carboxylate group of Asp-16 move towards each other to accommodate a bifurcated hydrogen bond between the 3′-OH and O1 and O2 of Asp-16.

We have recently reported[10] the utility of the fluorinase for preparing [^{18}F]-FDA and [^{18}F]-5-fluoro-5-deoxyribose for positron emission tomography (PET) from ^{18}F fluoride ion, and clearly the current observation suggests possibilities for labelling the corresponding 2′-deoxy (DNA) structural series using the fluorinase. Also, the protocols described here offer a convenient method for the synthesis of 2′d-SAM, a compound which could find utility in substrate specificity studies of SAM enzymes. 2′d-SAM has previously been prepared[11] by methylation of the corresponding 2-deoxy-*S*-adenosylhomocysteine; however, the synthetic procedures to generate this compound are complex.[12,13]

Experimental

HPLC and NMR time course

The reaction mixtures for HPLC were incubated at 37 °C with 0.3 mM 2′d-ClDA, 0.08 mM L-SeMet and 75 mM KF and fluorinase (7×10^{-3} units μm^{-1} min^{-1}), in a final volume of 650 μL. Samples (60 μL) for HPLC analysis were boiled at 95 °C (3 min) and the precipitated protein was removed by centrifugation. An aliquot (20 μL) of the clear supernatant was used for HPLC analysis (Varian 9012 UV-Vis detector at 260 and 9050 solvent delivery module).

Preparation of ESI-MS samples

A 250 μL sample for ESI-MS analysis was incubated at 37 °C for 12 h with 2′d-FDA (0.4 mM), L-Met, L-[^{13}C-*methyl*]-Met or L-Se-Met (15 mM) and the fluorinase (1.4×10^{-3} units). The mixture was boiled at 95 °C (3 min) and the precipitated protein removed by centrifugation. The supernatant was analysed directly by ESI-MS (MicroMass, Manchester, UK) using MeCN as the solvent. Products were detected in +ve ion mode. L-Met gave 2′d-SAM EI-MS *m/z* 383.3 $[M + H]^+$ and L-[^{13}C-*methyl*]-Met gave 2′d-SAM EI-MS *m/z* 384.2 $[M + H]^+$, consistent with the incorporation of the isotope. L-Se-Met gave 2′d-SeAM, which underwent ready fragmentation to 5′-methylseleno-2′d-Ado. ESI-MS analysis revealed a characteristic Se isotope profile with the major ^{80}Se isotopomer measuring *m/z* 330.5 $[M + H]^+$ for 5′-methylseleno-2′d-Ado.

Crystallisation of 2′-deoxy-5′FDA co-complex

The fluorinase was purified by a slightly modified protocol to that previously described.[5] The enzyme was treated with tobacco etch virus protease to remove the His tag, and it was then treated with adenine deaminase to remove any endogenous adenosine bound to the enzyme by its conversion to inosine, which does not bind. This generates an apo-fluorinase which is then more amenable to binding substrate analogues than the immediately purified enzyme, which carries adenosine. Fluorinase at a concentration of 4 mg mL^{-1} was incubated with 2′d-FDA (20 mM, 4 h, 298 K). The solution was then crystallised by vapour diffusion against a reservoir containing 30% PEG 1000, 0.1 M phosphate–citrate pH 4.5, 0.2 M Li_2SO_4. A single crystal was selected and flash-cooled to 100 K in a nitrogen stream.[14] Data were recorded to 2.4 Å in-house using a Rigaku Micromax-007 rotating anode with Osmic mirrors ($\lambda = 1.5418$ Å) on a Rigaku RaxisIV^{++} image plate detector. Data were indexed and integrated using MOSLFM[15] and scaled using SCALA.[16] The structure was solved by molecular replacement, using MOLREP,[17] using the original FDAS structure (1rqr) and refined using Refmac.[18–20] The final *R*-factor was 22.0% with a *R*-free of 28.6%. The PDB entry code is 2c5b.

Acknowledgements

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