Isolation and characterisation of 5′-fluorodeoxyadenosine synthase, a fluorination enzyme from *Streptomyces cattleya*

Christoph Schaffrath, Hai Deng, David O'Hagan*

School of Chemistry and Centre for Biomolecular Sciences, University of St Andrews, North Haugh, St Andrews KY16 9ST, UK

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Abstract 5′-Fluorodeoxyadenosine synthase, a C–F bond-forming enzyme, has been purified from *Streptomyces cattleya*. The enzyme mediates a reaction between inorganic fluoride and *S*-adenosyl-L-methionine (SAM) to generate 5′-fluoro-5′-deoxyadenosine. The molecular weight of the monomeric protein is shown to be 32.2 kDa by electrospray mass spectrometry. The kinetic parameters for SAM (K_m 0.42 mM, V_{max} 1.28 U/mg) and fluoride ion (K_m 8.56 mM, V_{max} 1.59 U/mg) have been evaluated. Both *S*-adenosyl-L-homocysteine (SAH) and sinefungin were explored as inhibitors of the enzyme. SAH emerged as a potent competitive inhibitor (K_i 29 μM) whereas sinefungin was only weakly inhibitory.

Key words: 5′-Fluorodeoxyadenosine synthase; *S*-Adenosyl-L-methionine; Fluoride ion; *S*-Adenosyl-L-homocysteine; *Streptomyces cattleya*

1. Introduction

In 1986 Sanada and co-workers reported the identification of fluoroacetate and 4-fluorothreonine as secondary metabolites of *Streptomyces cattleya*, when the bacterium was grown in the presence of fluoride ion [1]. These metabolites are among the very few known fluorine-containing metabolites [2,3]. The toxin fluoroacetate is the most ubiquitous of the fluorine-containing metabolites and has been identified in over 40 plants with a global distribution [4]. Although some tentative efforts have been made over the years to explore fluoroacetate biosynthesis in plants, no substantial progress has been made [4]. The identification of fluoroacetate and 4-fluorothreonine from a bacterium, however, presented a more amenable biosynthetic system in which to study fluoroacetate biosynthesis [1,5]. As a result, a combination of isotopic labelling studies and cell-free experiments with *S. cattleya* has revealed some of the details of fluorometabolite biosynthesis including the intermediacy of fluoroacetaldehyde as a common precursor to fluoroacetate and 4-fluorothreonine [6]. The enzymes between fluoroacetaldehyde and both metabolites have been identified and purified [7,8]. Of particular significance, we have recently reported that the fluorination process, in cell-free studies, involves a reaction between *S*-adenosyl-L-methionine (SAM) and fluoride ion to generate 5′-fluoro-5′-deoxyadenosine (5′-FDA) [9]. This enzyme unusually generates a C–F bond and is the first formed product in fluorometabolite biosynthesis in *S. cattleya* as summarised in Fig. 1.

In this paper we report details of the purification and characterisation of 5′-FDA synthase, the fluorination enzyme from *S. cattleya* (Fig. 1).

2. Materials and methods

2.1. Materials

SAM and *S*-adenosyl-L-homocysteine (SAH) were purchased from Sigma, UK. 5′-FDA was prepared as previously described [6].

2.2. Preparation of cell-free extract

Cells of *S. cattleya* NRRL 8057 were grown in conical flasks (500 ml) containing the medium (90 ml) as described by Reid et al. [5]. After 6 days incubation (28°C, 180 rpm), the cells were harvested and washed with Tris–HCl buffer (50 mM, pH 7.8) and resuspended in the same buffer. The cell-free extract was prepared by sonication (5×1 min, 60% duty cycle) and centrifugation of the cell debris (20 000×*g* for 20 min at 4°C). Typically the protein concentration was 2.6 mg/ml.

2.3. Enzyme assay

Enzymatic activity was assayed at 25°C, monitoring 5′-FDA production using a Waters HPLC system (Waters 2700 Sample Manager, 600 Controller, 2487 Dual Absorbance Detector and Fraction Collector II). Protein fractions (0.1 ml) were incubated at 37°C with SAM (0.2 mM) and KF (5.0 mM) in Tris–HCl buffer (50 mM, pH 7.8), in a final volume of 0.2 ml. Samples 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 on a Hypersil 5 μM C-18 column (250×4.6 mm, Macherey-Nagel) equilibrated with KH_2PO_4 (50 mM) and acetonitrile (95:5 v/v). Runs were monitored at 260 nm by gradient elution from a starting mobile phase of KH_2PO_4 (50 mM) and acetonitrile (95:5 v/v) to a final mobile phase consisting of KH_2PO_4 (50 mM) and acetonitrile (80:20 v/v). Samples were introduced through a high-pressure injector fitted with a 100 μl loop and the flow rate was maintained at 1.0 ml/min with a total time of elution of 20 min. The concentration of 5′-FDA was determined against a standard curve derived using synthetic 5′-FDA [6]. Protein concentrations were determined using the Coomassie blue binding method [10].

2.4. Purification of 5′-FDA synthase

The enzyme activity was partially purified by adding solid $(NH_4)_2SO_4$ to cell-free extracts until 45% saturation and removing the precipitate by centrifugation. The supernatant was adjusted to 60% saturation with solid $(NH_4)_2SO_4$ and the precipitate was collected after centrifugation. The pellet was then resuspended in Tris–HCl buffer (50 mM, pH 7.8) with 1 M $(NH_4)_2SO_4$, and the protein eluted from a phenyl Sepharose HP (Amersham Pharmacia Biotech)

*Corresponding author. Fax: (44)-1334-463808.
E-mail address: do1@st-andrews.ac.uk (D. O'Hagan).

Abbreviations: SAM, *S*-adenosyl-L-methionine; 5′-FDA, 5′-fluoro-5′-deoxyadenosine; SAH, *S*-adenosyl-L-homocysteine; ESI-MS, electrospray ionisation mass spectrometry

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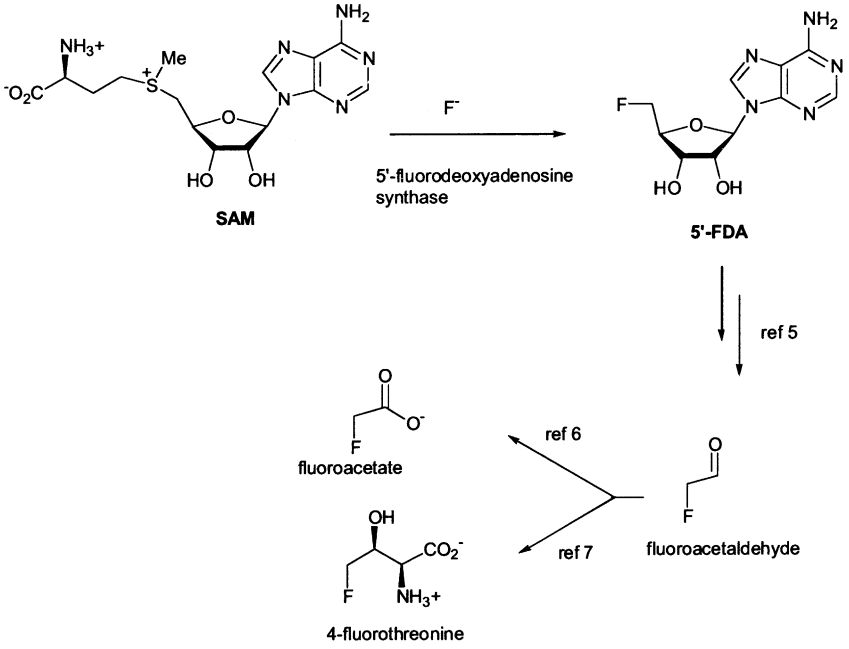

Fig. 1. Biotransformation of SAM and F^- to 5′-FDA in *S. cattleya*.

column at 2 ml/min using an AKTA Prime FPLC system (Amersham Pharmacia Biotech) (first 40 ml Tris–HCl buffer with 1 M $(NH_4)_2SO_4$ and then a linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in Tris–HCl buffer (50 mM, pH 7.8) over a final volume of 80 ml). The active fractions were concentrated (1.5 ml) using a 10 kDa Macrosep (Pall Folton) centrifugal concentrator and the solution was applied to a HiLoad 16/60 Superdex 200 gel filtration column (Amersham Pharmacia Biotech) with Tris–HCl buffer (50 mM, pH 7.8). The fractions containing fluorination activity were pooled and subjected to a 10 ml Source 15 Q strong anion exchange column (Amersham Pharmacia Biotech) at 2 ml/min, with a linear gradient of 0–0.3 M KCl in Tris–HCl over 80 ml. All purification steps were conducted at 4°C.

3. Results

3.1. Purification analysis

The fluorination enzyme was purified to homogeneity following the procedure described in Section 2.4 and summarised in Table 1, Fig. 2. Using this procedure the enzyme was purified 26-fold with an overall yield of 17%. As there was no significant improvement in the level of purity between the gel filtration and anion exchange steps, characterisation was generally carried out on protein recovered after gel filtration chromatography.

Analysis of the protein fractions by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) after each purification stage is shown in Fig. 2.

3.2. Biochemical properties of 5′-FDA synthase

The molecular weight of the monomeric protein was estimated to be around 32 kDa by SDS–PAGE; however, a more accurate assessment (~32.2 kDa) of the molecular weight was obtained by electrospray ionisation mass spectrometry (ESI-MS). The resultant spectrum is shown in Fig. 3. Passing the purified enzyme through a HiLoad 16/60 Superdex 200 gel filtration column showed a native mass of approximately 180 kDa, indicating that this enzyme is probably a hexamer in solution. The effect of temperature on the enzyme was investigated by conducting an assay over a range of temperatures from 25 to 70°C. The temperature optimum was determined to be 55°C. Addition of EDTA (1 mM) increased enzyme activity by 25%, indicating that the enzyme may be sensitive to trace amounts of metal ions. However, the presence of Mg^{2+} ion (1 mM) did not affect the activity.

The N-terminal amino acid sequence (24 amino acids) of the purified synthase (determined using an Applied Biosystems Procise 491 sequencing instrument) was found to be Ala-Ala-Asn-Ser-Thr-Arg-Arg-Pro-Ile-Ile-Ala-Phe-Met-Ser-Asp-Leu-Gly-Thr-Thr-Asp-Asp-Asp-Val-Gln.

3.3. Substrate specificity of 5′-FDA synthase

5′-FDA synthase utilises SAM and fluoride ion to generate 5′-FDA (Fig. 1). The kinetic parameters of the enzyme were

Table 1
Purification of the fluorination enzyme from *S. cattleya*

Purification step	Total volume (ml)	Total protein (mg)	Specific activity (U/mg)	Total activity (U)[a]	Purification (fold)	Yield (%)
Cell-free extract	100	263.34	0.055	14.5	1	100
Desalted $(NH_4)_2SO_4$ fraction	10	72.77	0.14	10.2	2.5	70.3
Hydrophobic interactive	1.5	7.49	1.04	7.8	18.9	53.7
Gel filtration	6	3.99	1.36	5.4	24.7	37.4
Anion exchange	8	1.73	1.44	2.5	26.2	17.2

[a]One unit is defined as the amount of enzyme required to synthesise 1 nmol of 5′-FDA per minute.

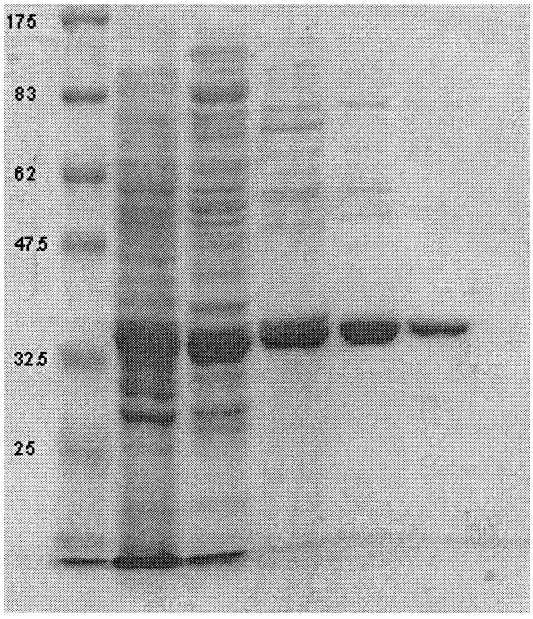

Fig. 2. SDS–PAGE of pooled fractions containing the fluorination enzyme during each purification step. Lanes: 1, marker MBP-β-galactosidase (175 kDa), MBP-paramyosin (83 kDa), glutamic dehydrogenase (62 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa) and β-lactoglobulin A (25 kDa); 2, crude cell-free extract; 3, $(NH_4)_2SO_4$ precipitate fractionation; 4, hydrophobic interactive chromatography; 5, gel filtration; 6, anion exchange chromatography.

determined from Lineweaver–Burk plots. The assays were conducted using a range of SAM concentrations (0.1–0.6 mM) while the fluoride ion concentration was kept constant at 5 mM, and then a range of fluoride ion concentrations (2.5–15 mM) while the SAM was held at 0.2 mM. The K_m value for SAM was calculated to be 0.42 mM (Table 2). The

Table 2
Kinetic parameters of substrates for enzymatic fluorination

Substrate	K_m (mM)[a]	V_{max} (U/mg)[a]
SAM	0.42[b] ± 0.05	1.28[b] ± 0.09
KF	8.56[c] ± 0.52	1.59[c] ± 0.13

[a]Values are the means of triple experiments; the variation around the mean was no more than 8%.
[b][SAM] varied from 0.1 to 0.6 mM with [F^-] at 5 mM.
[c][F^-] varied from 2.5 to 15 mM with [SAM] at 0.2 mM.

K_m value for fluoride ion was 20-fold higher than that for SAM (8.56 mM), perhaps indicating a poor affinity for the enzyme. One possible explanation for this higher value may be the well-known ability of fluoride ion to be readily solvated. Clearly, this high energy of solvation requires to be overcome for enzyme binding. Chloride ion was also explored in this study up to 10 mM but was not a substrate or inhibitor of the enzyme.

SAH (Fig. 4) has been reported to inhibit reactions of SAM-dependent enzymes [11]. Therefore SAH was explored as an inhibitor of the enzyme. In the event 5′-FDA production was inhibited (95% loss of enzyme activity) when the enzyme was incubated with SAM (0.4 mM), KF (10 mM) and SAH (0.4 mM). The values derived from Lineweaver–Burk plots indicated that SAH was a competitive inhibitor of the enzyme with a K_i value of 29 μM. The antibiotic sinefungin (Fig. 4) is also known as an inhibitor of SAM-dependent enzymes [12]. The presence of sinefungin (0.4 mM) caused only 15% loss of enzyme activity after 1 h incubation with SAM (0.4 mM) and KF (10 mM), indicating that it is a weak inhibitor of this enzyme.

4. Conclusion

We report the purification of the first native fluorination

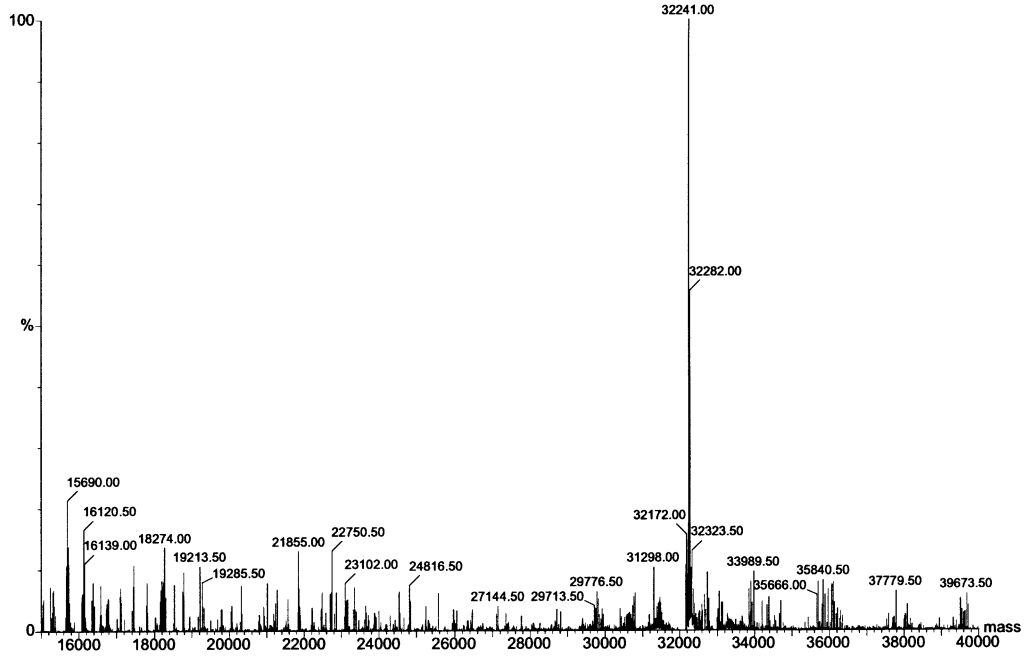

Fig. 3. ESI-MS spectra (Micromass LCT) of the synthase obtained from infusion of a protein solution. The analytic sample was applied to a HPLC (Waters 2795 LC unit) equipped with a guard column (Waters Xterra MS C8, 2.1×10 mm). A linear gradient of 2% solvent A (MeCN and 2% formic acid) to 2% solvent B (water and 2% formic acid) at 0.2 ml/min was used.

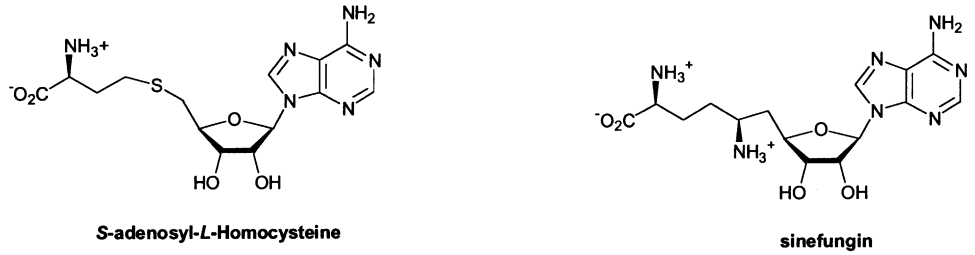

Fig. 4. The structure of SAH and sinefungin.

enzyme from an organism which has the capacity to biosynthesise organo-fluorine compounds as secondary metabolites. The enzyme mediates the first committed step in the biosynthesis of fluoroacetate and 4-fluorothreonine in *S. cattleya*.

There are some other reports of enzymes which can process fluoride ion and generate covalently bound fluorine. In 1957 Ochoa and co-workers demonstrated a 'fluorokinase' activity which co-eluted with pyruvate kinase, and had the ability to generate fluorophosphate from ATP and fluoride ion [13]. More recently the enzymatic synthesis of C–F bonds using mutant glycosidase enzymes has been reported [14,15]. These mutants were devoid of the key glutamate residue which stabilises the oxonium intermediate and had that residue replaced by other amino acid residues after site-directed mutagenesis e.g. by serine. These mutants generated glycosyl fluorides when incubated with glycosyl-ODNP substrates and fluoride ion at 2 M and clearly fluoride ion has intercepted the activated intermediate to generate a C–F bond. The present report outlines the isolation of an enzyme which has *evolved* a capacity to generate C–F bonds as its primary function. The over-expression of this enzyme and a fuller evaluation of its mechanism are under study.

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