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Probing the Molecular Determinants of Fluorinase Specificity^a

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The molecular determinants of FIA1 fluorinase specificity was probed using 5'-chloro-5'-deoxyadenosine (5'-CIDA) analogs as substrates and FIA1 active site mutants. Modifications at F213 or A279 residues are beneficial towards these modified substrates, including 5'-chloro-5'-deoxy-2-ethynyladenosine, CIDEA (>10-fold activity improvement), and conferred novel activity towards substrates not readily accepted by wild-type FIA1.

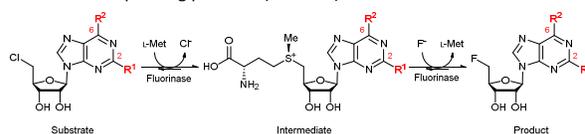
The identification and characterization of a growing number of naturally occurring fluorinase enzymes (FIA,^{1,2} FIA1,³ FIA3,³ Noba^{3,4} and FIA4^{5,6}) offer an exciting prospect of producing fluorinated compounds under mild conditions.⁷ These fluorinases catalyze the conversion of *S*-adenosyl-L-methionine (SAM) and fluoride ion to generate 5'-fluoro-5'-deoxyadenosine (5'-FDA) *via* a nucleophilic substitution (S_N2) mechanism.⁸⁻¹⁰ In order to expand the scope of application, substrate promiscuity was explored on non-native substrates. However successful examples were only limited to 5'-chloro-5'-deoxyadenosine (5'-CIDA),¹¹ 2'-deoxyadenosine,¹² L-methionine (L-Met) analogs,¹³ methylaza-analog of SAM,¹⁴ and C-2 modified substrates (5'-chloro-5'-deoxy-2-ethynyladenosine, CIDEA^{15,16} and di- and tetra-cyclic peptide constructs of CIDEA¹⁷). In addition, the catalytic efficiency of wild-type fluorinases on these non-native substrates is often reduced.¹³⁻¹⁶ There is only a handful of reports demonstrating limited success to enhance substrate specificity through enzyme modification.^{4,6,10,13,18} The challenge here is the lack of understanding in the fluorinase-substrate interactions to enable better engineered enzyme for fluorination.

Herein, we probed the molecular determinants of FIA1

fluorinase specificity towards non-native substrates which are modified at the adenine ring as varying this moiety can give rise to a variety of useful fluorinated nucleosides.^{15,16,19-24} We have recently demonstrated that the catalytic efficiency of FIA1 fluorinase on a non-native substrate 5'-CIDA can be improved *via* directed evolution.¹⁸ With the improved variants as a starting point, we probed their promiscuity against substrates that are modified at the C-2 (R¹ substitution) and the unexplored C-6 (R² substitution) positions of the adenine ring (Table 1).

O'Hagan's group reported that the reaction of CIDEA **2** to 5'-fluoro-5'-deoxy-2-ethynyladenosine (FDEA **2a**) using L-selenomethionine progressed at about 60% of the rate of conversion of 5'-CIDA **1** to 5'-FDA **1a** which demonstrated that the FIA fluorinase from *Streptomyces cattleya* has a specificity weakness at C-2 position.¹⁵ Similarly, FIA1 fluorinase from *Streptomyces* sp. MA37 also showed reduction in activity when 5'-CIDA **1** was substituted with CIDEA **2** in L-Met reaction (Table 2A). However, using our previously identified variants of fah2047 (A279L) and fah2081 (A279Y), we found that their activities for CIDEA **2** were either maintained or improved, respectively as compared to 5'-CIDA **1**. In particular, both variants also demonstrated >9-fold improvement in

Table 1. The substrates (**1 – 11**) tested for the two-step fluorinase reaction and their corresponding products (**1a – 11a**).



Substrates	Products	R ¹	R ²
1	1a	H	NH ₂
2	2a	C≡CH	NH ₂
3	3a	Cl	NH ₂
4	4a	I	NH ₂
5	5a	NH ₂	NH ₂
6	6a	NH ₂	Cl
7	7a	H	Cl
8	8a	NH ₂	H
9	9a	H	H
10	10a	NH ₂	OMe
11	11a	NH ₂	O

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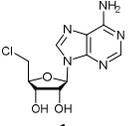
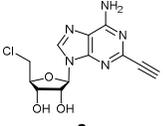
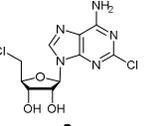
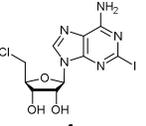
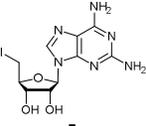
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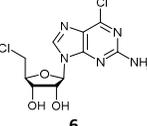
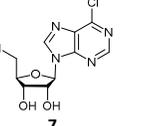
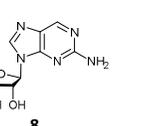
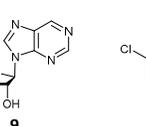
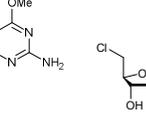
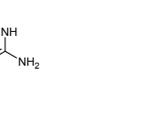
activity over FIA1 for CIDEA **2**. These results suggest that position 279 could play a role in facilitating binding to the ethynyl group of the substrate at C-2 position. When R¹ substituents are bulkier groups (**3** and **4**), the % yield dropped further for all the enzyme variants, suggesting that the C-2 position is sensitive to steric bulk. Nonetheless, most of our enzyme variants showed improved activity over FIA1 for substrates **1** – **4**, except for frh2066 (Y77W) which only showed improved activity for substrate **1**. Thus, the mutations F213Y and/or A279LY have led to improved activity against a wider range of R¹ substituted substrates. When R¹ substituent is an amino group **5**, the % yield for all the fluorinases increased as compared to that of 5'-CIDA **1**, with all our enzyme variants still outperforming the fluorination efficiency of FIA1. This is an unprecedented improvement in the fluorination efficiency on 5'-CIDA analogs. Our results showed that having a hydrogen donor such as an amino group at the C-2 position of the adenine group enables the modified substrate to interact better with the fluorinase enzyme and hence achieving higher % yield of the corresponding fluorinated product.

With the improvement in activity observed due to the amino

group at the C-2 position (substrate **5**), we went on to explore changes at the C-6 position of the adenine group (R² substitution). When R² is either a chloro or hydrogen substituent (**6** and **8** respectively), the % yield of all the fluorinases dropped significantly compared to substrate **5** at 4 h reaction (Table S1). To enable better comparison between the C-6 modified substrates, the reaction time was lengthened to 24 h (Table 2B). Three of our enzyme variants (fah2019, fah2047 and fah2114) showed improved activity (up to 27-fold) over FIA1 for substrates **6** and **8**. However, no activity was seen for any of the fluorinases for substrates **10** or **11** even with an amino group at the C-2 position. These results suggested that F213Y and A279L mutations are beneficial for selected R² substituents such as chlorine or hydrogen. When R¹ was changed from an amino group to a hydrogen atom for substrates **6** and **8** (to give **7** and **9** respectively), there was a decrease in % yield similar to what was observed between **5** and **1**. For substrate **7**, there was no detectable activity for wild-type FIA1 whereas activity could be observed with fah2114 (F213Y, A279L). For substrate **9**, marginal activity was observed for FIA1 and fah2047 (A279L) whereas improved activity (4 – 6-fold) was observed for fah2019 (F213Y) and

Table 2. % product yield and fold improvement (Fold imp) of fluorinases over FIA1 at 37°C. Reaction conditions: Synthesized substrate (0.2 mM), L-Met (0.1 mM), NaF (80 mM), and fluorinase (50 μM) at a single time point of 4 h or 24 h. % yield (mean of triplicates) = (concentration of product generated / concentration of substrate) x 100%. Fold imp = % yield of evolved variants / % yield of FIA1. A. 4 h reactions using substrates **1** – **5**. B. 24 h reactions using substrates **6** – **11**.

A											
Fluorinase (Mutation changes)											
	% yield	Fold imp	% yield	Fold imp	% yield	Fold imp	% yield	Fold imp	% yield	Fold imp	
FIA1	6.6 ± 0.1	-	2.3 ± 0*	-	3.5 ± 0.1	-	0	-	28.8 ± 0.3	-	
fah2019 (F213Y)	12.5 ± 0.2	1.9	10.3 ± 0.1	4.5	7.8 ± 0*	2.2	0.2 ± 0*	-	35.4 ± 0.5	1.2	
fah2047 (A279L)	19.9 ± 0.3	3.0	20.5 ± 0.5	9.0	8.2 ± 0.1	2.3	0.2 ± 0*	-	38.0 ± 0.2	1.3	
frh2066 (Y77W)	13.9 ± 0.2	2.1	0.3 ± 0*	0.1	0.1 ± 0*	0	0	-	31.6 ± 0.4	1.1	
fah2081 (A279Y)	15.3 ± 0.1	2.3	23.9 ± 0.6	10.5	8.5 ± 0.2	2.4	2.2 ± 0*	-	37.7 ± 0.2	1.3	
fah2114 (F213Y, A279L)	18.1 ± 0.3	2.7	13.5 ± 0.3	6.0	9.4 ± 0.1	2.7	0.1 ± 0*	-	37.6 ± 0.5	1.3	

B												
Fluorinase (Mutation changes)												
	% yield	Fold imp	% yield	Fold imp	% yield	Fold imp	% yield	Fold imp	% yield	Fold imp	% yield	Fold imp
FIA1	0.2 ± 0*	-	0	-	15.2 ± 0.3	-	0.1 ± 0*	-	0	-	0	-
fah2019 (F213Y)	1.4 ± 0*	6.6	0	-	33.2 ± 0.3	2.2	0.5 ± 0*	6.5	0	-	0	-
fah2047 (A279L)	2.0 ± 0.1	9.8	0	-	37.4 ± 0.7	2.5	0.1 ± 0*	0.9	0	-	0	-
frh2066 (Y77W)	0	0	0	-	1.2 ± 0.1	0.1	0	0	0	-	0	-
fah2081 (A279Y)	0.4 ± 0*	2.0	0	-	7.5 ± 0.1	0.5	0	0	0	-	0	-
fah2114 (F213Y, A279L)	5.8 ± 0.1	27.7	0.1 ± 0*	-	30.2 ± 0.4	2.0	0.4 ± 0*	4.7	0	-	0	-

[*] Standard deviation less than 0.1

fah2114 (F213Y, A279L). These observations suggest the benefits of a single F213Y mutation for the improved activity on substrate **9**.

To elucidate the rates of the best performing substrates, time-course reactions were ran for FIA1 and fah2018 with substrates **1**, **2** and **5** (Figure 1). The initial rates for the substrates tested were significantly disparate; with FIA1 showing rates in the order of $5 \gg 1 \gg 2$ whereas fah2081 of $5 \gg 1 \approx 2$. The interesting anomaly was substrate **2** which gave the highest % yield with fah2081 at 24 h whereas the rates of the rest showed trends of considerable slowdown after ~ 7 h. This suggest that tyrosine at 279 position has imparted additional benefit towards substrate **2** even though it has a slower initial rate. As for FIA1, substrate **5** still gave the highest % yield after 24 h despite the rate was plateaued after ~ 7 h. The much lower activity seen for R² substitution as compared to R¹ substitution suggests that C-6 position, which is largely unexplored, plays a more important role than C-2 position in the binding of the substrate to the enzyme. Control experiments had ruled out the possibility that the inactivity observed was due to substrate or product degradation under the reaction conditions. It is also interesting to note that frh2066 (Y77W) only showed improved activity over FIA1 for two of the substrates (5'-CIDA **1** and **5**). Dong *et al* reported that the Y77 residue is involved in facilitating halide binding for FIA.⁹ This implies that Y77 residue is critical for the fluorination reaction and should not be modified.⁶ Y77 residue is conserved in FIA1, which showed 88% identity to FIA.³ For our previous work of directed evolution of FIA1 with 5'-CIDA **1** and L-Met substrates, saturation mutagenesis of Y77 residue showed that Y77F (unpublished) and Y77W mutations displayed improvement over FIA1. This suggests that Y77 residue may play an additional role in FIA1 enzymatic reaction, although the model complexes for Y77W do not show any obvious reason for the improved activity towards substrates **1** and **5**.

To understand how the mutants impact substrates binding, structural models of the FIA1 mutants were generated using ROSETTA²⁵, with the recently solved crystal structure of FIA1 (PDB code 5B6I)¹⁸ as the template. Substrate coordinates were derived from the crystal structure of the *Streptomyces cattleya* fluorinase trimer complexed with substrate **2** (PDB code 4CQJ)¹⁵ and docked into the FIA1 mutant active site using PyMOL²⁶. All models were

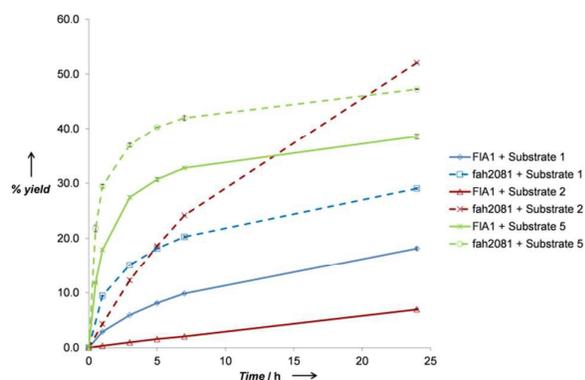


Figure 1. Comparison of reactions rates for FIA1 and fah2081 with substrates **1**, **2** and **5** at 37°C over 24 h. Reaction conditions: Synthesized substrate (0.2 mM), L-Met (0.1 mM), NaF (80 mM), and fluorinase (50 μ M) at different time points. % yield (mean of triplicates) = (concentration of product generated / concentration of substrate) \times 100%, error bars = standard deviation of triplicates.

then subjected to energy minimisation using AMBER.²⁷ The models identify key interactions that may have led to improved binding affinity translating to increased activity. The F213Y mutation is located in the binding pocket at a dimer interface, and is probably hydrogen bonded to D16 in the opposing monomer. This is likely to increase the stability of the enzyme, and may improve activity by increasing affinity towards the substrate. This is particularly noticeable with substrate **9** (Figure 2A). The A279Y also bridges the two monomers through a hydrogen bond between the tyrosine side-chain and the peptide carbonyl of W50 on an adjacent chain. These interactions translate into an overall improvement of activity for these mutations in their variants for most of the substrates.

The improved activity observed for our enzyme variants for R¹ substitutions on the adenine ring (**2** – **5**) is favourable for several reasons. Firstly, this C-2 position is pointing out into bulk solvent, and therefore should have minimal effect on the binding to FIA1 enzyme, similar to what was observed in FIA enzyme.¹⁵ Secondly, the A279L mutation, where the leucine side-chain directly interacts with the substituent, and is likely to form favourable van der Waals contacts with the substituent atoms (Figure 2B). As for the A279Y mutation, the tyrosine side-chain brings the enzyme in closer contact to the ethynyl substituent of substrate **2**, hence forming favourable van der Waals interactions. Thirdly, the amino group substituent is able to form favourable hydrogen bonds with the enzyme through the peptide carbonyl of P78 and the amide side-chain of N278 (Figure 2C). The iodinated substrate **4** has much lowered activity for all mutants, and this might be because P78 carbonyl and N278 side-chain are unlikely to form any polar interactions or hydrogen bonds with the bulky iodine substituent.

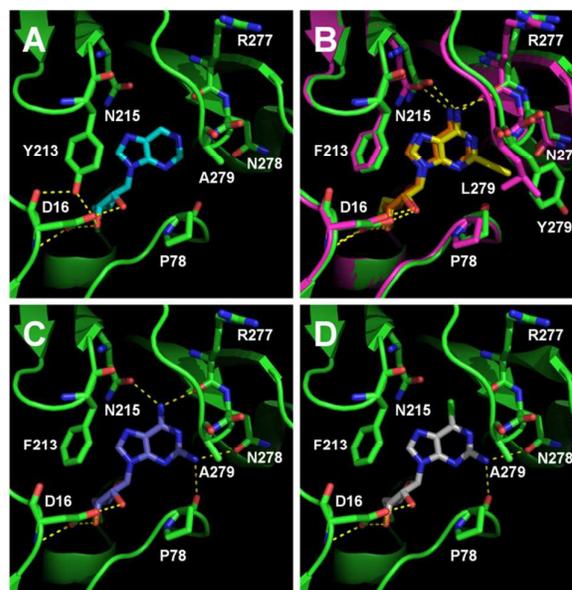


Figure 2. Novel substrate binding to wild-type FIA1 or FIA1 variants. A. Substrate **9** (in light blue) bound to fah2019, showing the interaction between Y213 and D16 residues. B. Substrate **2** (in orange and yellow) bound to fah2047 (in magenta) and fah2081 (in green), showing the interaction of R¹ substituent of ethynyl group with L279 and Y279 residues respectively. C. Substrate **5** (in blue) bound to wild-type FIA1, with hydrogen bonds shown between substrate and enzyme. D. Substrate **6** (in white) bound to FIA1 showing the loss of hydrogen bonds interaction with the removal of R² substituent of amino group.

The FIA1-5'-CIDA enzyme complex model when compared with the bound models for **6** – **11** identifies the main reason for the diminished or loss of activity. The R² substituent of amino group is hydrogen-bonded to the enzyme through the amide side-chain of N215, and the peptide carbonyl of R277 (Figure 2C). The loss of this group removes this interaction, which would lower the affinity of the enzyme for the substrate, and substituting with chlorine (**6** and **7**), hydrogen (**8** and **9**) or oxygen (**11**) will not form any stabilizing hydrogen bonds with N215 (Figure 2D and Figure S1A-B). In the case of **10**, the substituted methoxy group could be too bulky to fit into this pocket, and thus cannot form any stabilizing hydrogen bonds (Figure S1C). We tried to mutate N215 in order to explore potential alterations in specificity at the C-6 position, but so far the mutations we tested (N215LQ) have rendered the protein insoluble (data not shown). The addition of an amino group at the C-2 position helps to compensate the loss of key interactions at C-6 position and improve the binding of some substrates (e.g. **6** and **8**) due to the additional hydrogen bonding (*vide supra*), as well as the favourable van der Waals interactions from A279L and Y.

In conclusion, we found that substitutions at the C-2 position of the adenine group are sensitive to steric bulk whereas an amino group at the C-2 position led to enhanced fluorination yield. Additionally, our evolved FIA1 fluorinase variants are active against substrates that are modified at the C-2 and/or C-6 positions of the adenine ring despite being evolved against 5'-CIDA **1** and L-Met substrates. Modifications at F213 and/or A279 residues showed improved activity over FIA1 on the eight new substrates tested including novel activity for two of the substrates, **4** and **7**. Based on our previous kinetic studies,¹⁸ it is likely that the improvement in activity shown by our enzyme variants over FIA1 for these substrates is due to the enhancement of the first step, i.e. conversion of the substrate and L-Met to a SAM-analog in this two-step fluorination process. This work has shed light on the specificity of the FIA1 fluorinase with respect to modifications at the adenine moiety of the 5'-CIDA substrate and the mutations that can improve the promiscuity of the enzyme against non-native substrates. We believe that our work can serve as starting points for engineering of the enzyme to perform fluorination on more structurally diverse small molecules.

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