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A coupled chlorinase-fluorinase system with high efficiency of trans-halogenation and a shared substrate tolerance†

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Abstract

Enzymatic trans-halogenation enables radiolabeling under mild and aqueous conditions, but rapid reactions are desired. We develop a coupled chlorinase-fluorinase system for rapid trans-halogenation. Notably, the chlorinase shares a substrate tolerance with the fluorinase, enabling these two enzymes to cooperatively produce 5'-fluorodeoxy-2-ethynyladenosine (5'-FDEA) at upto 91.6% yield in 1 h.

S-adenosyl-L-methionine (SAM)-dependent nucleophilic halogenating enzymes are a newly discovered family of halogenases, which convert SAM and fluoride/chloride ion to 5'-fluoro-5'-deoxyadenosine (5'-FDA)/5'-chloro-5'-deoxyadenosine (5'-CIDA) and L-methionine (L-Met)¹ (Scheme 1A). To date, only five fluorinases^{2–7} and one chlorinase⁸ within this unique halogenase family have been discovered and characterized.

Fluorinases enable selective C-F bond formation under mild conditions in aqueous phase. The aqueous method of fluorination is especially desirable for positron emission tomography (PET) application. The aqueous [¹⁸F]-fluoride ion generated in the cyclotron from [¹⁸O]-water can be utilized directly by the fluorinase, and [¹⁸F]-labeling of soluble biomolecules can be readily achieved in a buffer solution near physiological pH.⁹

Fluorinase-mediated trans-halogenation has emerged as a useful strategy for PET probe synthesis.^{10–12} Fluorinases can catalyze the trans-halogenation of 5'-CIDA to 5'-FDA via two steps: 1) *in situ* SAM synthesis from 5'-CIDA and L-Met/L-seleno-methionine (L-SeMet); and 2) 5'-FDA generation from SAM and fluoride ion.¹³ Thus, cheap and stable 5'-CIDA can be converted to [¹⁸F]-5'-FDA,¹⁴ a potential PET probe that can also be further

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Conflicts of interest

There are no conflicts to declare.

converted to a wide range of potentially useful probes such as [^{18}F]-fluoroacetate,¹⁵ [^{18}F]-fluororibose¹⁶, and [^{18}F]-fluoronucleosides.¹⁷ The fluorinase was demonstrated to tolerate the acetylene functionality at the C-2 position of the adenine ring, leading to conversion of 5'-chlorodeoxy-2-ethynyladenosine (5'-CIDEA) to 5'-fluorodeoxy-2-ethynyladenosine (5'-FDEA).¹⁰ The acetylene moiety on FDEA enabled a "click" reaction to an azide-bearing arginylglycylaspartic acid (RGD) peptide. RGD-based PET tracers with high affinity and specificity for integrin $\alpha_v\beta_3$ have been used in clinical trials for tumor detection and staging.¹⁸ Fluorinase-mediated trans-halogenation can even be employed for direct radiolabeling of RGD tethered to the C-2 position of the adenine ring.^{10, 12} Due to the short half-life of F-18 ($t_{1/2} = 109.7$ min), it is important to develop rapid enzymatic protocols.

Our results showed that fluorinase-mediated trans-halogenation is slow even for the fluorinase from *Streptomyces xinghaiensis* (FIA4), the most efficient fluorinase⁷ among the five reported ones (Scheme 1A). Comparing the two separate reaction steps, we found that the SAM synthesis is the rate-limiting step (Scheme 1B; Table S1). Hence, fast *in situ* SAM synthesis will be the key to improving the overall trans-halogenation efficiency. The first SAM-dependent chlorinase SalL was reported to prefer the conversion of 5'-CIDA to SAM *in vitro*.⁸ Here, our newly discovered SAM-dependent chlorinases, CIA1 and CIA2, showed that they are significantly more efficient in SAM synthesis from 5'-CIDA than the fluorinase FIA4. Based on this, we developed a coupled chlorinase-fluorinase system for efficient trans-halogenation of 5'-CIDA to 5'-FDA. The chlorinase was for the first time found to exhibit substrate tolerance on the C-2 position of the substrate and to work together with the fluorinase, enabling improved trans-halogenation of 5'-CIDEA to 5'-FDEA.

CIA1 (WP_078486934) was identified via BLAST (Basic Local Alignment Search Tool) search in the NCBI (National Center for Biotechnology Information) server. It shares 59.6% amino acid identity with SalL. Coding sequences (CDSs) for the CIA1 protein are present in the genomes of *Streptomyces ahygroscopicus* subsp. *wuyiensis* CK-15¹⁹ and four strains of *Streptomyces albulus*,^{20–23} all isolated from soil (Table S2). CIA2 (P077_11362) was identified via BLAST search of a collection of actinomycete genome sequences^{24, 25} (PRJNA238534). It shares 52.7% amino acid identity with SalL. CDS for CIA2 is present in the genome of *Umezawaea tangerine* NRRL B-24463 isolated from soil. Multiple sequence alignment showed that CIA1, CIA2 and SalL do not have the 22-residue loop region, which can be found in all the five known fluorinases (FIA 92-113) (Figure S1).

Unlike SalL discovered from high-chloride marine source,⁸ the two new chlorinases were unveiled from soil bacteria. To find if terrestrial bacteria could evolve more efficient chlorinases than SalL, kinetic studies of SalL, CIA1 and CIA2 were carried out for the chlorination reaction with varying concentrations of Cl^- (Table S3; Figure S2). The relative catalytic efficiencies ($k_{\text{cat}}/K_{\text{M}}$) of CIA1 and CIA2 are quite similar to that of SalL. The affinity of CIA1 for Cl^- is lower than that of SalL. These two new chlorinases may rely on subsequent steps in the biosynthetic pathway to pull the reaction forward.

Prediction of secondary metabolite clusters by antiSMASH (antibiotics & Secondary Metabolite Analysis Shell)²⁶ showed that *cIA1* is within the putative biosynthetic gene cluster for γ -butyrolactone (GBL) biosynthesis. GBLs are small signaling molecules (also

known as autoregulators) that trigger antibiotic biosynthesis and morphological development in *Streptomyces* species at nanomolar concentrations.²⁷ The gene encoding CIA1 is close to the genes coding for putative NAD(P)-dependent oxidoreductase, γ -butyrolactone biosynthesis enzyme and γ -butyrolactone receptor protein (Figure S3). Though 14 GBLs with shared GBL core and varied fatty acid side chains are known,²⁸ no halogenated GBL has ever been discovered.

The conversion of 5'-CIDA to SAM is more efficient than the conversion of SAM to 5'-CIDA for both CIA1 and CIA2 (Table 1; Figure S4 and S5). The k_{cat}/K_M also showed that the two chlorinases are significantly more efficient in SAM formation from 5'-CIDA than the fluorinase FIA4 (Table 1). Thus, the rate-limiting step of fluorinase-mediated trans-halogenation can be accelerated by the chlorinases. This led us to couple the chlorinases, the robust SAM synthesis enzymes, to the fluorinase for improved overall trans-halogenation.

The chlorinases were coupled to FIA4 for one-pot conversion of 5'-CIDA to 5'-FDA in the presence of L-Met or L-SeMet at 37°C for 1 h (Table 2; Figure S6A). The trans-halogenation reactions with chlorinase coupled to FIA4 were compared with the reactions without chlorinase. In the presence of L-Met, the trans-halogenation reaction with 50 μ M FIA4 alone produced 5'-FDA at only 3.2% yield. Addition of 30 μ M chlorinase improved 5'-FDA yield by up to 25.6 fold. However, addition of 30 μ M FIA4 just increased the yield by 1.5 fold. Although higher concentration of the fluorinase is often believed to increase product yields, the cocktail of chlorinase and fluorinase achieved significantly higher yields than the fluorinase alone with the same total amount of enzyme. The trans-halogenation reactions were further improved in the presence of L-SeMet, leading to >90% 5'-FDA yields obtained by the coupling of FIA4 and the chlorinase. Thus, the chlorinase-fluorinase system is able to accelerate enzymatic trans-halogenation of 5'-CIDA to 5'-FDA.

To probe if the chlorinase can tolerate the C-2 position of the adenine ring of the substrate 5'-CIDA, we coupled the chlorinase to the fluorinase for the trans-halogenation of 5'-CIDEA to 5'-FDEA (Table 2; Figure S6B). The reactions were run under same conditions for the trans-halogenation of 5'-CIDA to 5'-FDA. In the presence of L-Met, the trans-halogenation efficiencies decreased dramatically compared to the conversion of 5'-CIDA to 5'-FDA. L-SeMet^{10, 11, 13} was used instead of L-Met to improve the trans-halogenation efficiency on 5'-CIDEA. In the presence of L-SeMet, the reaction with 50 μ M FIA4 alone produced 5'-FDEA at only 4.7% yield in 1h. Addition of 30 μ M FIA4 only increased the yield by 1.7 fold. Impressively, addition of 30 μ M CIA2 led to the highest 5'-FDEA yield (91.6%), with a 19.6-fold improvement. Comparison of the conversion of 5'-CIDEA and L-SeMet to the SAM derivative by the three chlorinases showed that CIA2 exhibited highest consumption rate of 5'-CIDEA to produce the SAM derivative (Figure S7). Thus, the chlorinase is able to tolerate the linear acetylene moiety at the C-2 position of the adenine ring and work together with the fluorinase for highly efficient trans-halogenation of 5'-CIDEA to 5'-FDEA.

In conclusion, we discovered two new SAM-dependent chlorinases from soil bacteria and developed a coupled chlorinase-fluorinase system for highly improved trans-halogenation reactions. The chlorinase was for the first time demonstrated to tolerate the modification at

the C-2 position of the adenine ring and act cooperatively with the fluorinase to accelerate the trans-halogenation of 5'-CIDEA to 5'-FDEA. The acetylene group will enable the linkage with an azide tethered peptide via a "click" reaction ("two step" strategy).¹¹ The coupled chlorinase-fluorinase system offers the prospect of developing rapid radiolabeling protocols under mild and aqueous conditions. Future work will be focused on exploitation of the coupled chlorinase-fluorinase system for radiolabeling of cancer relevant peptides either by a "two step" strategy¹¹ or a "last step" protocol^{10, 12} if the chlorinase can further tolerate a tethered peptide at the C-2 position of the adenine ring.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Scheme 1.

Fluorinase-mediated trans-halogenation and its rate-limiting step. A) Trans-halogenation by FIA4: 0.2 mM 5'-CIDA, 80 mM NaF, 0.1 mM L-Met, 50 μ M FIA4 at 47°C. B) Two separate reactions incubated at 47°C: 1) SAM synthesis with 0.2 mM 5'-CIDA, 0.1 mM L-Met and 50 μ M FIA4; 2) Fluorination reaction with 0.2 mM SAM, 80 mM NaF and 50 μ M FIA4. Yield = [product] detected / [product] expected at full conversion.

Table 1.

Comparative kinetic data of CIA1, CIA2 and FIA4.

Enzyme	K_M (μM)	k_{cat} (min^{-1})	$[k_{\text{cat}}/K_M]$ ($\text{mM}^{-1} \text{min}^{-1}$)
<i>Conversion of SAM to 5'-CIDA: [SAM]^[a]</i>			
CIA1	15.69	4.68 ± 0.27	298.09
CIA2	2.48	0.67 ± 0.02	270.14
<i>Conversion of 5'-CIDA to SAM: [5'-CIDA]^[b]</i>			
CIA1	13.98	12.49 ± 0.99	893.42
CIA2	9.45	14.91 ± 0.92	1577.44
FIA4	29.87	0.69 ± 0.01	22.96

^[a] Assays contain 200 mM NaCl and various concentrations of SAM. K_M refers to SAM K_M .

^[b] Assays contain 20 mM L-Met and various concentrations of 5'-CIDA. K_M refers to 5'-CIDA K_M .

Table 2.

Comparison of 5'-FDA/5'-FDEA yields in the presence of L-Met/L-SeMet.

Reaction ^[a]	Conversion of 5'-CIDA to 5'-FDA 5'-FDA Yield (%)		Conversion of 5'-CIDEA to 5'-FDEA 5'-FDEA Yield (%)	
	L-Met	L-SeMet	L-Met	L-SeMet
50 μM FIA4	3.20 $\pm$ 0.07	11.00 $\pm$ 0.06	0.87 $\pm$ 0.05	4.68 $\pm$ 0.06
80 μM FIA4	4.69 $\pm$ 0.10	16.23 $\pm$ 0.08	1.54 $\pm$ 0.03	7.79 $\pm$ 0.08
50 μM FIA4 + 30 μM SalL	69.89 $\pm$ 0.08	90.18 $\pm$ 0.40	6.47 $\pm$ 0.38	58.31 $\pm$ 0.08
50 μM FIA4 + 30 μM CIA1	81.77 $\pm$ 0.26	98.04 $\pm$ 0.27	5.76 $\pm$ 0.16	59.70 $\pm$ 0.18
50 μM FIA4 + 30 μM CIA2	73.47 $\pm$ 0.32	96.63 $\pm$ 0.18	12.37 $\pm$ 0.29	91.60 $\pm$ 0.13

^[a] Each reaction contains 0.2 mM 5'-CIDA/5'-CIDEA, 80 mM NaF and 0.1 mM L-Met/L-SeMet. Reactions were incubated at 37°C for 1 h.