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

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<th>Journal:</th>
<th>ChemComm</th>
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<tr>
<td>Manuscript ID</td>
<td>CC-COM-06-2018-004436.R1</td>
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A coupled chlorinase-fluorinase system with high efficiency of trans-halogenation and a shared substrate tolerance†

H. Sun, H. Zhao, b* and E. L. Ang a*

Received 00th January 20xx, Accepted 00th January 20xx
DOI: 10.1039/x0xx00000x

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 up to 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'-ClDA) and L-methionine (L-Met) (Scheme 1A). To date, only five fluorinases and one chlorinase 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.

Fluorinase-mediated trans-halogenation has emerged as a useful strategy for PET probe synthesis. 10-12 Fluorinases can catalyze the trans-halogenation of 5'-ClDA to 5'-FDA via two steps: 1) in situ SAM synthesis from 5'-ClDA and L-Met/L-seleno-methionine (L-SeMet); and 2) 5'-FDA generation from SAM and fluoride ion. Thus, cheap and stable 5'-ClDA can be converted to [18F]-5'-FDA, a potential PET probe that can also be further converted to a wide range of potentially useful probes such as [18F]-fluorooacetate, [18F]-fluororibose, and [18F]-fluoronucleosides. 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'-ClDEA) to 5'-fluorodeoxy-2-ethynyladenosine (5'-FDEA). 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 αvβ3 have been used in clinical trials for tumor detection and staging. Fluorinase-mediated trans-halogenation can even be employed for direct radiolabeling of RGD tethered to the C-2 position of the adenine ring. Due to the short half-life of F-18 (t1/2 = 109.7 min), it is important to develop rapid enzymatic protocols.
0.1 mM L-Met and 50 µM FIA4; 2) Fluorination reaction with 0.2 mM SAM, 80 mM NaCl and 50 µM FIA4. Yield = [product] detected / [product] expected at full conversion.

Our results showed that fluorinase-mediated trans-halogenation is slow even for the fluorinase from Streptomyces xinghaiensis (FIA4), the most efficient fluorinase 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. 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 subspp. wuyiensis CK-15 and four strains of Streptomyces albulus, all isolated from soil (Table S2). CIA2 (P077_11362) was identified via BLAST search of a collection of actinomycete genome sequences. 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 (FIAA 92-113) (Figure S1).

Unlike SalL discovered from high-chloride marine source, 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 trans-halogenation of 5’-CDA to 5’-FD. 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.

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 µM FIA4 alone produced 5’-FDA at only 3.2% yield. Addition of 30 µM chlorinase improved 5’-FDA yield by up to 25.6 fold. However, addition of 30 µM 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 as above.
conditions for the trans-halogenation of 5'-ClDA to 5'-FDA. In the presence of L-

**Table 2.** Comparison of 5'-FDA/5'-FDEA yields in the presence of L-Met/L-SeMet.

<table>
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<tr>
<th>Reaction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Conversion of 5'-ClDA to 5'-FDA</th>
<th>Conversion of 5'-ClDEA to 5'-FDEA</th>
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<tr>
<td></td>
<td>L-Met</td>
<td>L-SeMet</td>
</tr>
<tr>
<td>50 µM FIA4</td>
<td>3.20 ± 0.07</td>
<td>11.00 ± 0.06</td>
</tr>
<tr>
<td>80 µM FIA4</td>
<td>4.69 ± 0.10</td>
<td>16.23 ± 0.08</td>
</tr>
<tr>
<td>50 µM FIA4 + 30 µM SalL</td>
<td>69.89 ± 0.08</td>
<td>90.18 ± 0.40</td>
</tr>
<tr>
<td>50 µM FIA4 + 30 µM CIA1</td>
<td>81.77 ± 0.26</td>
<td>98.04 ± 0.27</td>
</tr>
<tr>
<td>50 µM FIA4 + 30 µM CIA2</td>
<td>73.47 ± 0.32</td>
<td>96.63 ± 0.18</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each reaction contains 0.2 mM 5'-ClDA/5'-ClDEA, 80 mM NaF and 0.1 mM L-Met/L-SeMet. Reactions were incubated at 37°C for 1 h.

Met, the trans-halogenation efficiencies decreased dramatically compared to the conversion of 5'-ClDA to 5'-FDA. L-SeMet<sup>10, 11</sup> was used instead of L-Met to improve the trans-halogenation efficiency on 5'-ClDEA. In the presence of L-SeMet, the reaction with 50 µM FIA4 alone produced 5'-FDEA at only 4.7% yield in 1h. Addition of 30 µM FIA4 only increased the yield by 1.7 fold. Impressively, addition of 30 µM CIA2 led to the highest 5'-FDEA yield (91.6%), with a 19.6-fold improvement. Comparison of the conversion of 5'-ClDEA and L-SeMet to the SAM derivative by the three chlorinases showed that CIA2 exhibited highest consumption rate of 5'-ClDEA 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'-ClDEA 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'-ClDEA to 5'-FDEA. The acetylene group will enable the linkage with an azide tethered peptide via a “click” reaction (“two step” strategy).<sup>11</sup> 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” strategy<sup>13</sup> or a “last step” protocol<sup>10, 12</sup> if the chlorinase can further tolerate a tethered peptide at the C-2 position of the adenine ring.

This work was funded by the GlaxoSmithKline - Singapore Economic Development Board partnership for Green and Sustainable Manufacturing (E.L.A), the A*STAR Visiting Investigator Program (H.Z.), and the National Institutes of Health (GM077596) (H.Z.). We thank Dr. Bin Wang and Dr. Ryan E Cobb from Department of Chemical and Biomolecular Engineering, UIUC, and Dr. James R. Doroghazi from Institute for Genomic Biology, UIUC for helpful bioinformatic assistance during identification of CIA2. We also thank members of MERL and Dr. Yee Hwee Lim from ICES for their comments.

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*ChemComm*, 2013, *00*, 1-3 | 3
Conflicts of interest

There are no conflicts to declare.

Notes and references

Graphical abstracts (table of contents entry):

SAM-dependent chlorinases exhibited tolerance to the modification at the C-2 position of the adenosine substrate and acted cooperatively with the fluorinase for rapid trans-halogenation.