Engineering self-sufficient aldehyde deformylating oxygenases fused to alternative electron transfer systems for efficient conversion of alkanes into alkanes†

Qing Wang,‡ab Xuenian Huang,‡a Jingjing Zhang,‡a Xuefeng Lu,a Shengying Lia and Jian-Jun Lia*

Self-sufficient aldehyde deformylating oxygenases (ADOs) from Synechococcus elongatus PCC7942 fused to alternative electron transfer systems were successfully designed, constructed, characterized and used for efficient conversion of alkanes into alkanes for the first time.

Fatty alkanes, which can be biologically produced by plants, insects, green algae, cyanobacteria, among others, are the main components of conventional fuels and could be the ideal replacement for fossil-based fuels.1 Recently, Schirmer et al. identified two key enzymes involved in alk(a/e)ne biosynthesis in cyanobacteria: acyl-ACP reductase and aldehyde deformylating oxygenase (ADO), which catalyze reduction of fatty acyl-ACP into the corresponding fatty aldehydes into alk(a/e)nes and formate, respectively (Scheme 1).1,2 Since the identification of ADO, it has attracted particular interest due to the chemically difficult and unusual reactions it catalyzes.1,2 Based on the crystal structure of ADO from Prochlorococcus marinus MIT9313, ADO belongs to the ferritin-like non-heme dinitro-carboxylate enzymes.1,2 Though the ADO-catalyzed reaction is seemingly hydrolytic, it has been proved that oxygen and an auxiliary reducing system (protein-based or chemical) are absolutely required for ADO activity (Scheme 1).1,2,4 Since the identification of ADO-catalysed reaction, it has been shown to be the first self-sufficient P450 enzyme.4 Its fusion nature greatly improved electron transfer efficiency, enabling it to be one of the most efficient P450 enzymes.3 A new class of self-sufficient P450 has been recently discovered: P450RhF from Rhodococcus sp. strain NCIMB 9784, comprising an N-terminal P450 domain fused to an FMN (flavin mononucleotide)- and Fe/S-containing reductase domain.5,6 The reductase domain RhFRED has been artificially fused to some P450s to generate self-sufficient enzymes.7 Moreover, self-sufficient proteins of P450s with putidaredoxin (Pd) or Fd and putidaredoxin reductase (PdR) or FNR have been constructed and investigated.8,9,10

For practical purposes, the self-sufficient ADO systems could be as useful as self-sufficient P450s to better harness the catalytic power of ADOs. Inspired by the observation that self-sufficient P450s showed improved catalytic activity and could be used more conveniently and cost-effectively, for the first time we report self-sufficient ADOs fused to alternative electron transfer systems for efficient conversion of alkanes into alkanes. Since the native redox partners of ADOs remain unknown, the reductase domain of P450RhF (RhFRED) from Rhodococcus sp. and the recently identified cognate reducing system (Fd/FNR) from S. elongatus PCC7942 were fused to ADO 1593 (Synpcc7942_1593) in different orders, respectively (Fig. 1).1,2,4 ADO 1593 was first linked to RhFRED by the natural 16-aa (amino acid) linker between the RhFRED and P450 domains in P450RhF to generate FusA.6,8 The preliminary results demonstrated that FusA showed relatively low self-sufficient ADO activity and electron transfer efficiency (reduction of cytochrome c) (Table 1), which could presumably result from the rigidity of the natural linker. To increase the stiffness of the linker, 10 additional amino acids (a highly flexible linker) were inserted into FusA between 1593 and the 16-aa linker in FusB, which were from the iGEM Registry (Registry of Standard Biolog-ical Parts) ([http://parts.igem.org/Part:BBa_J18922]). FusA was

![Scheme 1](attachment:image.png)

O
R
C
H
O₂ / the reducing system

ADO
R-H + HCO₂⁻

**Key Laboratory of Biofuels, Shandong Provincial Key Laboratory of Energy Genetics, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, No. 189 Songling Road, Qingdao 266101, China. E-mail: lij@qibebt.ac.cn; Fax: +86(0)532 80662712; Tel: +86(0)532 80662712

University of Chinese Academy of Sciences, Beijing 100049, China

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c4cc00591k

‡ Both authors contributed equally to this work.
constructed by attaching the RhFRED domain to the N-terminus of 1593 through a flexible 20-aa linker, which has been used for fusing catalase to ADO from Prochlorococcus marinus MIT9313 and covalent connection of the subunits of the ATPase ClpX to form hexamers.\textsuperscript{2c,11} For FusD, FNR was first fused to Fd through a 7-aa linker, which has been used to connect Fd and PdR,\textsuperscript{9} and the obtained FNR-Fd fragment was then linked to 1593 through the 20-aa linker to get FusD. FusE was made similarly to FusD, except for the opposite order of Fd and FNR. FusF and FusG, which were the reverse orientations of FusD and FusE, respectively, were also constructed. All constructs were cloned into the pET-28a(+) vector, overexpressed in E. coli and purified, respectively.

To test whether the activity of 1593 could be affected by fusion to the reducing systems, fusion ADOs were assayed in the presence of non-fused Fd/FNR, FusF and FusG, which were the reverse orientations of FusD and FusE, respectively, were also constructed. All constructs were cloned into the pET-28a(+) vector, overexpressed in E. coli and purified, respectively.

To test whether the activity of 1593 could be affected by fusion to the reducing systems, fusion ADOs were assayed in the presence of the chemical reducing system (PMS) using NADPH as the electron donor. FusA, FusB, FusC, FusD, FusE, FusF, FusG, and native 1593 were allowed to react with non-fused Fd/FNR. Table 1 shows the results from these experiments.

![Table 1](https://example.com/table1.png)

Table 1: Comparison of the apparent $k_{cat}$ values and the electron transfer efficiency of fusion ADOs

| ADO   | $k_{cat}^{app}$ (min$^{-1}$) | Ferricyanide $c$ (µM min$^{-1}$) | Cytochrome $c$ (µM min$^{-1}$) |
|-------|-----------------------------|---------------------------------|-------------------------------|
| FusA  | 0.46 ± 0.03                 | 0.046 ± 0.001                   | 36.6 ± 0.6                     |
| FusB  | 1.16 ± 0.12                 | 0.071 ± 0.001                   | 48.6 ± 0.7                     |
| FusC  | 0.74 ± 0.02                 | 0.021 ± 0.001                   | 29.1 ± 1.3                     |
| FusD  | 0.60 ± 0.01                 | 0.22 ± 0.001                    | 40.4 ± 1.2                     |
| FusE  | 0.75 ± 0.02                 | 0.23 ± 0.003                    | 49.2 ± 1.8                     |
| FusF  | 0.50 ± 0.02                 | 0.27 ± 0.002                    | 35.4 ± 0.3                     |
| FusG  | 1.45 ± 0.03                 | 0.62 ± 0.003                    | 31.1 ± 0.3                     |
| Native 1593 | 0.45 ± 0.01$^b$ | 0.23 ± 0.01$^b$ | 57.4 ± 0.4 $^d$ |
| FNR    | ND$^e$                     | ND$^e$                          | 1.6 ± 0.1                      |

$^a$ NADPH was used as the electron donor. $^b$ Different apparent $k_{cat}$ values for native 1593 from the published ones in the presence of the chemical reducing system and the cognate one were obtained, since slightly different assay conditions, including catalase and the different ratio of Fd, FNR and 1593, were used in the current paper (in ESIL). $^c$ 1593 was reconstituted in a molar ratio of 1 : 1 : 1 with Fd and FNR. $^d$ Reduction of cytochrome $c$ by Fd/FNR was carried out with equal molar of Fd and FNR. $^e$ ND, not determined.

Presumably, the fusion of domains via the covalent linkage stabilizes the interaction between ADO and the redox partners, thus enhancing electron transfer efficiency and ADO activity.
Unexpectedly, the reduction rate of cytochrome $c$ of native 1593 in the presence of PMS (Table 2) was greater than that of native 1593 in the presence of the chemical reducing system, consistent with the findings of previous studies. This inconsistency was also observed for FusD and FusE (Tables 1 and 2), which might imply that Fd–FNR mediated electron transfer to ADO occurs through an intramolecular pathway rather than by intermolecular transfer of electron from one fusion protein to another.$^9$

Finally, the kinetic parameters of self-sufficient FusD, FusE, FusF and FusG were determined using $n$-heptanal as the substrate, and those of native 1593 in the presence of the chemical and non-fused Fd/FNR reducing systems were also measured (Table 2). The $K_m$ values of fusion ADOs and the native one in the presence of non-fused Fd/FNR were very close, but considerably lower than that of native 1593 in the presence of the chemical reducing system (Table 2), suggesting that ADO binds more tightly to the substrate in the presence of the cognate reducing system than the chemical one, which could possibly arise from the different interactions between ADO and the different reducing systems, and attachment of the reducing system has no big impact on substrate binding to ADO. This might be one of the reasons why fusion ADOs showed higher activity than the native one too when the chemical reducing system was used (Table 1). FusG exhibited the greatest $k_{cat}$ value (2-fold higher than those of FusD, FusE, FusF, and native 1593 in the presence of non-fused Fd/FNR), consistent with the findings that FusG showed the highest activity in the presence of PMS and the highest reduction rate toward cytochrome $c$, whereas the $k_{cat}/K_m$ values of FusD, FusE and FusF were close to each other (Table 2). Just like reduction of cytochrome $c$, these results confirm the importance of the order of the three components on the activities of fusion ADOs once again (Table 2 and Fig. 1).

What’s more, FusG showed the highest catalytic efficiency ($k_{cat}/K_m$), which is 2 to 3-fold greater than those of FusD, FusE, FusF, and native 1593 in the presence of non-fused Fd/FNR. FusG exhibited lower $k_{cat}$ values than native 1593 in the presence of PMS, their catalytic efficiencies were 2.5 to 3.7-fold greater than that of native 1593 in the presence of PMS (Table 2). Unexpectedly, the reduction rate of cytochrome $c$ of FusG was 12.3-fold higher than that of FusF, but its $k_{cat}$ value and catalytic efficiency towards $n$-heptanal were only about 2-fold greater than those of FusF (Tables 1 and 2). This inconsistency was also observed for FusD and FusE (Tables 1 and 2), which might imply that Fd-mediated electron transfer to 1593 in FusG and FusE is not very effective or the electron transfer potential of the Fd–FNR unit is not fully released in FusG and FusE (Tables 1 and 2), and electron transfer from the reducing systems to ADO is a limiting factor for the activities of self-sufficient ADOs. Inefficient electron transfer from the reducing systems to ADO in the fusion proteins is possibly due to structural constraints that prevent optimal intramolecular interaction between ADO and the reducing systems, in particular between ADO and Fd. Comparison of the $k_{cat}$ values and catalytic efficiencies of FusE and FusG demonstrates the importance of the order of the ADO and Fd–FNR domains on the activity of self-sufficient ADOs (Table 2 and Fig. 1).

Considering that the electron transport pathway in self-sufficient ADOs is NADPH → FNR or FMN → Fd or Fe$\text{S}_2$ → ADO, our results demonstrate that the match between ADO and the electron transfer systems and the order of the components are important for efficient electron transfer to ADO (Tables 1 and 2, Fig. 1), which is critical for the catalytic activities of self-sufficient ADOs.

In conclusion, self-sufficient ADOs were successfully designed, constructed and used for efficient conversion of aldehydes into alkanes. Our studies confirmed the feasibility of developing self-sufficient ADOs. Importantly, we have identified efficient electron transfer from the reducing systems to ADO as a key factor affecting ADO activity. Our results shed light on a general strategy for designing more active ADOs and establishing an efficient cell factory for production of fatty alka(e)nes.

The authors thank Prof. David H. Sherman at the University of Michigan for kindly providing the pET28b-pikC-RhFRED plasmid. We are grateful for financial support from the National Science Foundation of China (31170765) and the National Basic Research Program of China (973: 2011CBAA0907).

**Notes and references**

1. A. Schirmer, M. A. Rude, X. Li, E. Popova and S. B. del Cardayre, Science, 2010, 329, 559.
2. D. M. Warui, N. Li, H. Norgaard, C. Krebs, J. M. Bollinger and S. J. Booker Jr, J. Am. Chem. Soc., 2011, 133, 3316; (b) N. Li, H. Norgaard, D. M. Warui, S. J. Booker, C. Krebs and J. M. Bollinger Jr, J. Am. Chem. Soc., 2011, 133, 6158; (c) N. Li, W. Chang, D. M. Warui, S. J. Booker, C. Krebs and J. M. Bollinger Jr, Biochemistry, 2012, 51, 7908; (d) D. Das, B. E. Eser, J. Han, A. Sciore and E. N. G. Marsh, Angew. Chem., Int. Ed., 2011, 50, 7148; (e) C. Andre, S. W. Kim, X.-H. Yu and J. Shanklin, Proc. Natl. Acad. Sci. U. S. A., 2013, 110, 3191; (f) B. Paul, D. Das, B. Ellington and E. N. G. Marsh, J. Am. Chem. Soc., 2013, 135, 5234; (g) B. Khara, N. Menon, C. Levy, D. Mansell, D. Das, E. N. G. Marsh, D. Leys and N. S. Scrutton, ChemBioChem, 2013, 14, 1204; (h) M. E. Pandelia, N. Li, H. Norgaard, D. M. Warui, L. J. Rajalovich, W.-C. Chang, S. J. Booker, C. Krebs and J. M. Bollinger Jr, J. Am. Chem. Soc., 2013, 135, 13801; (i) K. G. Aukema, T. M. Makris, S. A. Stoian, J. E. Richman, E. Münck, J. D. Lipscomb and L. P. Wackett, ACS Catal., 2013, 3, 2228; (j) D. Das, B. Ellington, B. Paul and E. N. Marsh, ACS Chem. Biol., 2014, 9, 570; (k) E. N. Marsh and M. W. Waugh, ACS Catal., 2013, 3, 2515.
3. J. Zhang, X. Lu and J.-J. Li, Biotechnol. Biofuels, 2013, 6, 86.
4. R. T. Rettigter and A. J. Fulco, J. Biol. Chem., 1981, 256, 5728.
5. E. O’Reilly, M. Corbett, S. Hussain, P. P. Kelly, D. Richardson, S. L. Flitsch and N. J. Turner, Catal. Sci. Technol., 2013, 3, 1490.
6. G. A. Roberts, G. Grogan, A. Greter, S. L. Flitsch and N. J. Turner, J. Bacteriol., 2002, 184, 3898.
7. A. Çelik, G. A. Roberts, J. H. White, S. K. Chapman, N. J. Turner and S. L. Flitsch, Chem. Commun., 2006, 4492.
8. (a) A. Robin, G. A. Roberts, J. Kisch, F. Sabbadin, G. Grogan, N. Bruce, N. J. Turner and S. L. Flitsch, Chem. Commun., 2009, 2478; (b) M. Nodate, M. Kubota and N. Misawa, Appl. Microbiol. Biotechnol., 2006, 71, 455; (c) S. Li, L. M. Podust and D. H. Sherman, J. Am. Chem. Soc., 2007, 129, 12940.
9. O. Sibbesen, J. J. De Voss and P. R. Montellano, J. Biol. Chem., 1996, 271, 22462.
10. T. Lacour and H. Okhawa, Biochim. Biophys. Acta, 1999, 1433, 87.
11. A. Martin, T. A. Baker and R. T. Sauer, Nature, 2005, 437, 1115.

---

**Table 2** Kinetic parameters of self-sufficient ADOs and native ADO

|  | $K_m$ (mM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (min$^{-1}$ mM$^{-1}$) |
|---|---|---|---|
| FusD | 0.036 ± 0.008 | 0.2 ± 0.01 | 5.6 ± 0.2 |
| FusE | 0.039 ± 0.008 | 0.22 ± 0.01 | 5.6 ± 0.3 |
| FusF | 0.033 ± 0.005 | 0.25 ± 0.01 | 7.6 ± 0.2 |
| FusG | 0.038 ± 0.004 | 0.75 ± 0.02 | 19.7 ± 1.1 |
| 1593 (Fd/FNR)$^a$ | 0.039 ± 0.004 | 0.24 ± 0.0 | 6.2 ± 0.3 |
| 1593 (PMS) | 0.3 ± 0.02 | 0.48 ± 0.01 | 1.6 ± 0.1 |

$^a$ 1593 was reconstituted in a molar ratio of 1:1:1 with Fd and FNR.