

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

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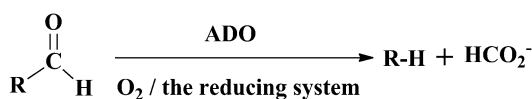
Self-sufficient aldehyde deformylating oxygenases (ADOs) from *Synechococcus elongatus* PCC7942 fused to alternative electron transfer systems were successfully designed, constructed, characterized and used for efficient conversion of aldehydes into alkanes for the first time.

Fatty alk(a/e)nes, 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 aldehyde and conversion of fatty aldehydes into alk(a/e)nes and formate, respectively (Scheme 1).^{1,2a} 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 dimetal-carboxylate enzymes.^{1,2g} 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).^{1,2b-d} The protein-based reducing system is composed of ferredoxin (Fd) and ferredoxin-NADP⁺ reductase (FNR),^{1,2c} whereas the chemical reducing system consists of phenazine methosulfate (PMS) or 1-methoxy-5-methylphenazinium methylsulfate (MeOPMS)

and NADH.^{2c,d} Very recently, we found that the homologous electron transfer system supported greater ADO activity than the heterologous and chemical ones.³ However, the fact that ADOs require separate redox partner(s) might significantly limit their applications in biotechnology.^{1,2c,d,3}

P450BM3 from *Bacillus megaterium*, in which the P450 domain is naturally fused to its redox partners, 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.⁵ 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.^{6,7} The reductase domain RhFRED has been artificially fused to some P450s to generate self-sufficient enzymes.⁸ Moreover, self-sufficient proteins of P450s with putidaredoxin (Pd) or Fd and putidaredoxin reductase (PDR) or FNR have been constructed and investigated.^{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 aldehydes 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).^{3,6} 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 flexibility of the linker, 10 additional amino acids (a highly flexible linker) were inserted into Fusa between 1593 and the 16-aa linker in FusaB, which were from the iGEM Registry (Registry of Standard Biological Parts) ([http://parts.igem.org/Part:BBa_J18922]). FusaC was



Scheme 1 ADO-catalysed reaction.^{2a-c}

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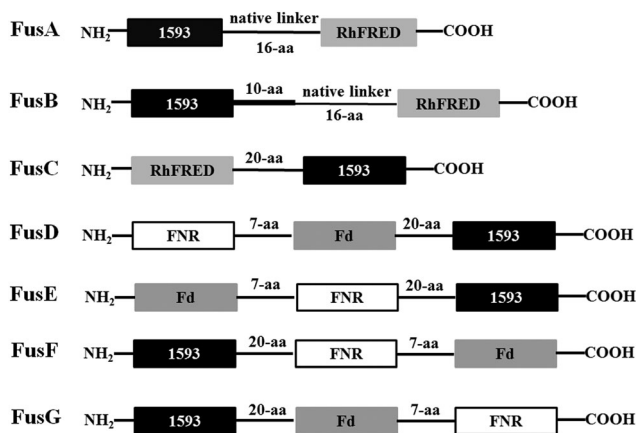


Fig. 1 Schematic structures of fusion ADOs. Native 16-aa linker, VLHRHQPTIGPEAAR; 10-aa linker, GSGSGSGSGS; 20-aa linker, ASGAGGSEGGGSEGGTSGAT; 7-aa linker, TDGASSS.

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

	$k_{\text{cat}}^{\text{app}}$ (min^{-1})		Ferricyanide ($\mu\text{M min}^{-1}$)	Cytochrome <i>c</i> ($\mu\text{M min}^{-1}$)
	PMS	Self-sufficient ^a		
FusA	0.46 ± 0.03	0.046 ± 0.001	36.6 ± 0.6	10.1 ± 1.1
FusB	1.16 ± 0.12	0.071 ± 0.001	48.6 ± 0.7	44.0 ± 1.3
FusC	0.74 ± 0.02	0.021 ± 0.001	29.1 ± 1.3	32.4 ± 0.2
FusD	0.60 ± 0.01	0.22 ± 0.01	40.4 ± 1.2	1.2 ± 0.04
FusE	0.75 ± 0.02	0.23 ± 0.03	49.2 ± 1.8	6.3 ± 0.6
FusF	0.50 ± 0.02	0.27 ± 0.02	35.4 ± 0.3	1.9 ± 0.06
FusG	1.45 ± 0.03	0.62 ± 0.03	31.1 ± 0.3	25.2 ± 0.3
Native 1593	0.45 ± 0.01 ^b	0.23 ± 0.01 ^{b,c}	ND ^e	ND ^e
FNR ^d	ND ^e	ND ^e	57.4 ± 0.4	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,³ since slightly different assay conditions, including catalase and the different ratio of Fd, FNR and 1593, were used in the current paper (in ESI). ^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.

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.^{2e,11} For FusD, FNR was first fused to Fd through a 7-aa linker, which has been used to connect Pd and PdR,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 the chemical reducing system (PMS) using *n*-heptanal as the substrate. Nearly all fusion proteins exhibited higher activities than native non-fusion 1593 (Table 1), demonstrating that attachment of the reducing systems to the N-terminus or C-terminus of 1593 had no deleterious effects on the catalytic activity of 1593, and somehow enhanced the

activity instead. FusG showed the highest activity, 2.2-fold higher than native 1593 (Table 1). Comparison of the apparent k_{cat} values of FusA and FusB suggests that the flexible 10-aa linker between 1593 and RhFRED in FusB is beneficial for the activity of 1593 (Table 1 and Fig. 1). These results indicate that the linkers between 1593 and the reducing proteins as well as the order of the components have some effects on ADO activity (Table 1 and Fig. 1).

Considering that the catalytic activities of fusion ADOs are closely related to the electron transfer efficiency of the reducing systems, potassium ferricyanide and cytochrome *c* were used as the alternative electron acceptors to investigate whether the electron transfer abilities of the reducing systems (RhFRED and Fd/FNR) in fusion ADOs could be impacted by fusion of domains and the order of the components. Ferricyanide could be reduced by FNR as well as the FMN domain of RhFRED, and cytochrome *c* by Fd/FNR as well as RhFRED. As shown in Table 1, the reduction rates of ferricyanide by fusion ADOs were not greatly influenced compared with native (or non-fusion) FNR. However, reduction of cytochrome *c* by fusion ADOs showed significantly different results (Table 1). FusB and FusC exhibited greater reduction rates than FusD, FusE, FusF and FusG, indicating that the electron transfer ability of RhFRED is stronger than that of the Fd/FNR system (Table 1). The reduction rate of FusA was much lower than those of FusB and FusC, which could be a consequence of the rigidity of the natural 16-aa linker, suggesting that the flexible 10-aa linker in FusB is a positive factor for the improved electron transfer efficiency (Table 1 and Fig. 1). Notably, among fusion ADOs containing Fd/FNR, FusG (1593-Fd-FNR) showed the highest reduction rate toward cytochrome *c*, three-fold higher than that of FusE (Fd-FNR-1593), whereas reduction of cytochrome *c* by non-fused Fd/FNR was much slower than that by FusE and FusG, comparable to that by FusD (FNR-Fd-1593) and FusF (1593-FNR-Fd) (Table 1). These results demonstrate that the Fd-FNR unit in FusE and FusG shows higher electron transfer efficiency than the FNR-Fd unit in FusD and FusF, and fusion and the order of three components Fd, FNR and 1593 have a significant impact on the electron transfer efficiency of the Fd/FNR reducing system (Table 1 and Fig. 1).

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. To prove this, fusion proteins were assayed using *n*-heptanal as the substrate without the participation of the exogenous reducing systems. Native 1593 was also assayed in the presence of Fd and FNR from *S. elongatus* PCC7942 at a molar ratio of 1 : 1 : 1. FusD, FusE, FusF, FusG, and native 1593 in the presence of non-fused Fd/FNR exhibited much greater apparent k_{cat} values than FusA, FusB and FusC (Table 1). Since the ADO activities and the electron transfer abilities of FusA, FusB and FusC were not greatly impaired due to fusion (Table 1), these results proved again that the homologous electron transfer system matched better with ADO and supported greater ADO activity.³ Self-sufficient ADOs for efficient conversion of aldehydes into alkanes have been successfully engineered (Table 1).

Given that electron transfer from NADPH *via* FNR/Fd or RhFRED to ADO might occur intermolecularly and/or intramolecularly, the relationship between the activities of fusion ADOs and the protein concentration was investigated. The linear relationship between

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.

them, as exemplified by FusG (Fig. S2 in ESI[†]), indicates that electron transfer from NADPH *via* FNR/Fd or RhFRED 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} 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, 11.3-fold greater than that of native 1593 in the presence of the chemical reducing system (Table 2). Though FusD, FusE and FusF 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 Fe₂S₂ → 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 alk(a/e)nes.

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