Supplementary Information

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

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1.1 Materials

Horse-heart cytochrome *c*, BSA (Bovine Serum Albumin), catalase, NADPH, NADH, *n*-heptanal, potassium ferricyanide, phenazine methosulfate (PMS), ferrous ammonium sulfate, and NTA (Nitrilotriacetic acid) were obtained from Sigma (St. Louis, Missouri, USA). Oligonucleotides were synthesized by Shanghai Sangon Biotech Co. Ltd (Shanghai, China). *Taq* and *Pfu* DNA polymerases, and all restriction endonucleases were from Fermentas (Pittsburgh, Pennsylvania, USA) or Takara Biotechnology (Otsu, Japan). The kits used for molecular cloning were from Omega Bio-tek (Norcross, Georgia, USA) or Takara Biotechnology. Nickel column and the expression vector pET-28a(+) were from Novagen. Amicon YM10 membrane was purchased from Millipore (Frankfurter Straße, Darmstadt, Germany).

1.2 Bacterial strains, plasmids, and media

Escherichia coli DH5α was used for routine DNA transformation and plasmid isolation. *Escherichia coli* BL21(DE3) was utilized for protein overexpression. *E. coli* strains were routinely grown in Luria-Bertani broth or terrific broth at 37^{o}C with aeration or on LB supplemented with 1.5% (w/v) agar.[1] 50 μg/ml Kanamycin was added when required.

1.3 DNA manipulations

General molecular biology techniques were carried out following the standard procedures.[1] Restriction and modification enzymes were used following the recommendations of the manufacturers. DNA fragments were purified from agarose gels using the DNA gel extraction kit. Plasmid DNA was isolated using the plasmid

miniprep kit.

All fusion ADOs were constructed as follows:

FusA: The fragment 1593A without the stop codon was amplified by PCR using the plasmid pET28a-1593 as the template and the primers FusA-1593-F/FusA-1593-R,[2] and subsequently the PCR product was digested with *Nde*I and *EcoR*I, and re-cloned into *pikC*-RhFRED digested with the same restriction enzymes to give the plasmid pET28a-FusA.[3]

FusB: The fragment RhFRED (10aa-C-terminus) containing addition 10-aa at the N-terminus of RhFRED was amplified by PCR using the plasmid *pikC*-RhFRED as the template and the primers FusB-10aa-RhFRED-F/FusB-10aa-RhFRED-R,[3] and the PCR product was then digested with *Hind*III and *EcoR*I, and cloned into FusA digested with the same restriction enzymes to get the plasmid pET28a-FusB.

FusC: The fragment RhFRED (N-terminus) without the stop codon was amplified by PCR using the plasmid *pikC*-RhFRED as the template and the primers FusC-RhFRED-F/FusC-RhFRED-R.[3] The fragment 1593 (C-terminus) was obtained through PCR using pET28a-1593 as the template and the primers Fus-1593-F1/Fus-1593-R1.[2] These two fragments were fused through overlap extension PCR using the primers FusC-RhFRED-F/Fus-1593-R1, and the fused product was subsequently digested with *Nde*I and *Xho*I, and re-cloned into the vector pET-28a(+) digested with the same restriction enzymes to generate the plasmid pET28a-FusC.

FusD: The fragment FNR (N-terminus) without the stop codon was amplified using the plasmid pET28a-FNR as the template and the primers FusD-FNR-F/FusD-

FNR-R.[2] The fragment Fd (middle) without the stop codon was amplified using pET28a-Fd as the template and the primers FusD-Fd-F/FusD-Fd-R.[2] The fragments FNR (N-terminus), Fd (middle), and 1593 (C-terminus) were fused by overlap extension PCR using the primers FusD-FNR-F/Fus-1593-R1, and the fused product was then digested with *Nde*I and *Xho*I, and re-cloned into the vector pET-28a(+) digested with the same restriction enzymes to obtain the plasmid pET28a-FusD.

FusE: The fragment Fd (N-terminus) without the stop codon was amplified using pET28a-Fd as the template and the primers FusE-Fd-F/FusE-Fd-R.[2] The fragment FNR (middle) without the stop codon was amplified using pET28a-FNR as the template and the primers FusE-FNR-F/FusE-FNR-R.[2] The fragments Fd (N-terminus), FNR (middle), and 1593 (C-terminus) were fused by overlap extension PCR using the primers FusE-Fd-F/Fus-1593-R1. The fused product was then digested with *Nde*I and *Xho*I, and re-cloned into pET-28a(+) digested with the same restriction enzymes to give the plasmid pET28a-FusE.

FusF: The fragment 1593B (N-terminus) without the stop codon was amplified using pET28a-1593 as the template and the primers Fus-1593B-F2/Fus-1593B-R2.[2] The fragment FNR-Fd (C-terminus) was amplified using FusD as the template and the primers FusF-FNR-Fd-F/FusF-FNR-Fd-R. These two fragments were fused through overlap extension PCR using the primers Fus-1593B-F2/FusF-FNR-Fd-R. The fused product was subsequently digested with *Nde*I and *Xho*I, and re-cloned into the vector pET-28a(+) digested with the same restriction enzymes to obtain the plasmid pET28a-FusF.

FusG: The fragment Fd-FNR (C-terminus) was amplified using FusE as the template and the primers FusG-Fd-FNR-F/FusG-Fd-FNR-R. The fragments 1593B (N-terminus) and Fd-FNR (C-terminus) were fused through overlap extension PCR using the primers Fus-1593B-F2/FusG-Fd-FNR-R. The fused product was then digested with *Nde*I and *Xho*I, and re-cloned into the vector pET-28a(+) digested with the same restriction enzymes to give the plasmid pET28a-FusG.

All fusion constructs were confirmed by DNA sequencing.

1.4 Overexpression and purification of proteins

Fd, FNR, and 1593 were overexpressed as previously.[2]

All fusion proteins were transformed into competent *E. coli* BL21(DE3). Protein expression was carried out at 37°C in terrific broth media supplemented with 50 μg/mL kanamycin. The cultures were induced with 0.1 mM IPTG in the presence of 50 μM ferrous ammonium sulfate when OD_{600} reached 0.6, and were shaken at 18°C for additional 24 hours. The his-tagged proteins were purified using Nickel chelating resin according to the manufacturer's protocol. Apo-1593 was prepared by dialyzing the protein against the buffer containing 1 mM EDTA and 1 mM NTA, and the diferrous form of ADO 1593 was reconstituted by the addition of the stoichiometric amounts of ferrous ammonium sulfate to the apo-1593 prior to assay. Proteins were concentrated with Amicon YM10 membrane (10 kDa cut-off). Protein concentration was determined by the Bradford method using bovine serum albumin as a standard.[4]

SDS-PAGE was performed in 12% polyacrylamide gels, using Coomassie Blue R-250 staining.[1]

1.5 Enzyme assay

Enzymatic assays for reduction of potassium ferricyanide and cytochrome *c* were done on the Beckman Coulter DU 800 UV/Vis Spectrophotometer at 1 ml scale at 25 °C. All experiments were done at least in duplicate.

a) For reduction of potassium ferricyanide

Assays were performed in 50 mM Tris-HCl (pH 8.0) containing 0.7 mM potassium ferricyanide and 500 μM NADPH concentrations.[2,5,6] The reactions were initiated by the addition of 0.01 μM FNR or fusion ADOs. The absorption decrease at 420 nm (reduction of ferricyanide, ε_{420} = 1000 $M^{-1}cm^{-1}$) was recorded to determine the reduction rates of fusion ADOs.

b) For reduction of cytochrome *c*

Assays were carried out in 50 mM Tris-HCl (pH 7.8) containing 500 μM NADPH and 50 μM cytochrome *c*.[2,5,6] The reactions were started by the addition 0.1 μM FNR/Fd or fusion ADOs. The reduction rates were determined by monitoring the resulting absorption increases at 550 nm (reduction of cytochrome *c*, ε_{550} = 19,100 $M^{-1}cm^{-1}$).

c) For fusion ADOs

(1) Using the chemical reducing system

Assays were performed in 1.5 mL gastight vials with a total volume of 500 μL. The reactions contained 2 mM *n*-heptanal in 100 mM HEPES buffer (pH 7.2), including 100 mM KCl, 10% glycerol, 4% DMSO, 5 μM fusion ADOs, 20 μM ferrous ammonium sulphate, 75 μM PMS, 750 μM NADH, and 1 mg/ml catalase. The

other procedures were carried out as before.[2]

(2) Self-sufficient ADOs

For enzymatic assays of self-sufficient ADOs, 2 mM NADPH was used to substitute for 75 μM PMS and 750 μM NADH. The others were same as above. When the kinetic parameters were determined, different *n*-heptanal concentrations were used. To determine whether the reactions catalyzed by fusion ADOs are intramolecular or intermolecular, 1, 2.5, 5, 7.5, and 10 μM FusG and 2 mM *n*-heptanal were used for the assay.

d) For native (or non-fusion) ADO 1593

When the chemical reducing system was used, the assays were carried out as above. While the cognate reducing system was used, the assays were done with equal molar 1593, Fd and FNR, and the other conditions were same as before. To determine the kinetic parameters of 1593 in the presence of the chemical and cognate reducing systems respectively, different *n*-heptanal concentrations were used.

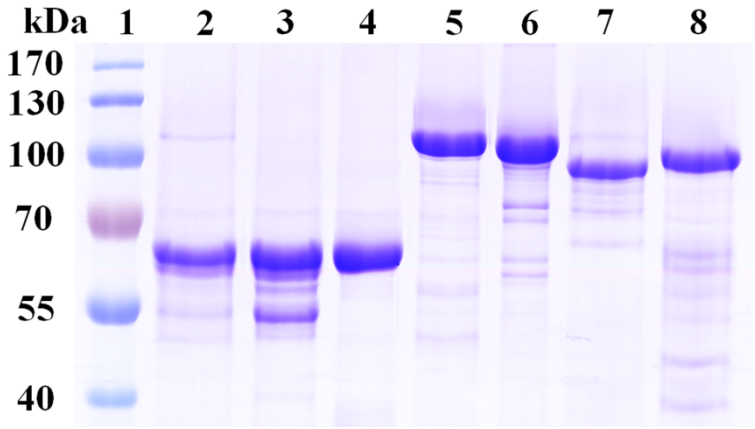
1.6 Table S1 Primers used for construction of fusion ADOs

Fragments	Primer names	Sequence 5→3
1593A (N-terminus)[a]	FusA-1593A-F	ACTCAACATATGCCGCAGCTGGAGG[b]
	FusA-1593A-R	CATCGTGAATTCTACCGCCGCCAGCCA[b]
RhFRED (10aa-C-terminus)	FusB-10aa-RhFRED-F	GGGAATTCGGTAGCGGCAGCGGTAGCGGTAGCGG
		CAGCGTGCTGCACCGCCATCAACCG[b]
	FusB-10aa-RhFRED-R	ACATCAAGCTTTCAGAGGCGCAGGGCCAG[b]
1593 (C-terminus)[a]	Fus-1593-F1	GGCTCTGAAGGCGGTGGCTCTGAAGGTGGTACCTC
		TGGTGCGACC ATGCCGCAGCTGGAGGCGTC
	Fus-1593-R1	CGATCTCGAGTTATACCGCCGCCAGACCATAG[b]
RhFRED (N-terminus)[a]	FusC-RhFRed-F	CGCGGCCATATGGCGGTGTCCCGCACCGTCAC[b]
	FusC-RhFRED-R	ACCACCTTCAGAGCCACCGCCTTCAGAGCCGCCCG
		CACCAGACGCCAGAGGCGCAGGGCCAGGCG
Fd (middle)[a]	FusD-Fd-F	ACCTACACGGATGGTGCATCCAGCTCGTGGCAACC
		TACAAGGTTAC
	FusD-Fd-R	ACCACCTTCAGAGCCACCGCCTTCAGAGCCGCCCG
		CACCAGACGCGTAGAGGTCTTCTTCTTTGTG
FNR (N-terminus) [a]	FusD-FNR-F	CGCGGCCATATGTTGAATGCGAGTGTGGC[b]
	FusD-FNR-R	CATCGAGCTGGATGCACCATCCGTGTAGGTTTCAA
		CATGCCAAC

Fd (N-terminus)[a]	FusE-Fd-F	CGCGGCCATATGGCAACCTACAAGGTTAC[b]
	FusE-Fd-R	CATCGAGCTGGATGCACCATCCGTGTAGAGGTCTT CTTCTTTGTG
FNR (middle)[a]	FusE-FNR-F	CTCTACACGGATGGTGCATCCAGCTCGATGTTGAA TGCGAGTGTGGC
	FusE-FNR-R	ACCACCTTCAGAGCCACCGCCTTCAGAGCCGCCCG CACCAGACGCGTAGGTTTCAACATGCCAAC
1593B (N-terminus)[a]	Fus-1593B-F2	CGCGGCCATATGCCGCAGCTGGAGGCGTC[b]
	Fus-1593B-R2	ACCACCTTCAGAGCCACCGCCTTCAGAGCCGCCCG CACCAGACGCTACCGCCGCCAGACCATAG
FNR-Fd (C-terminus)[a]	FusF-FNR-Fd-F	GGCTCTGAAGGCGGTGGCTCTGAAGGTGGTACCTC TGGTGCGACCATGTTGAATGCGAGTGTGGC
	FusF-FNR-Fd-R	CGATCTCGAGTTAGTAGAGGTCTTCTTCTTTGTG[b]
Fd-FNR (C-terminus)[a]	FusG-Fd-FNR-F	GGCTCTGAAGGCGGTGGCTCTGAAGGTGGTACCTC TGGTGCGACCATGGCAACCTACAAGGTTAC
	FusG-Fd-FNR-R	CGATCTCGAGTTAGTAGGTTTCAACATGCCAAC[b]

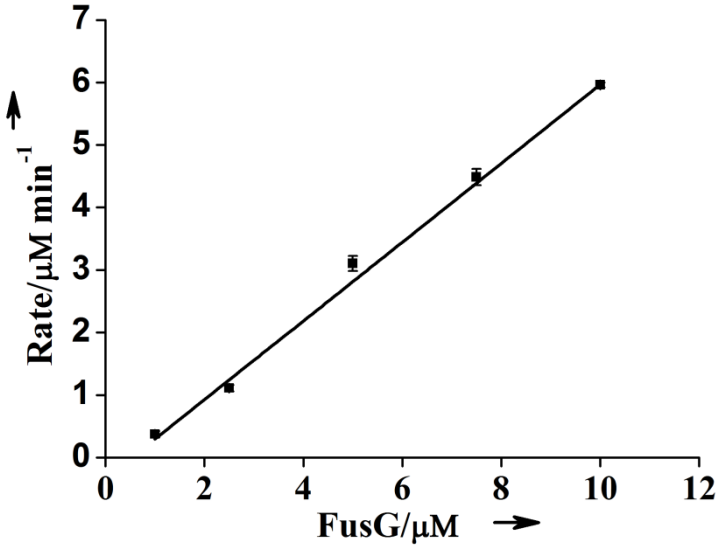
[a] N-terminus, C-terminus, and middle indicate the relative positions of the fragments in fusion ADOs. [b] The restrictions sites of *Nde*I, *EcoR*I, *Hind*III, and *Xho*I were underlined.

1.7 Fig. S1 SDS-PAGE analysis of purified fusion ADOs*. Lane 1, protein molecular weight marker; Lane 2, FusA; Lane 3, FusB; Lane 4, FusC; Lane 5, FusD; Lane 6, FusE; Lane 7, FusF; Lane 8, FusG.

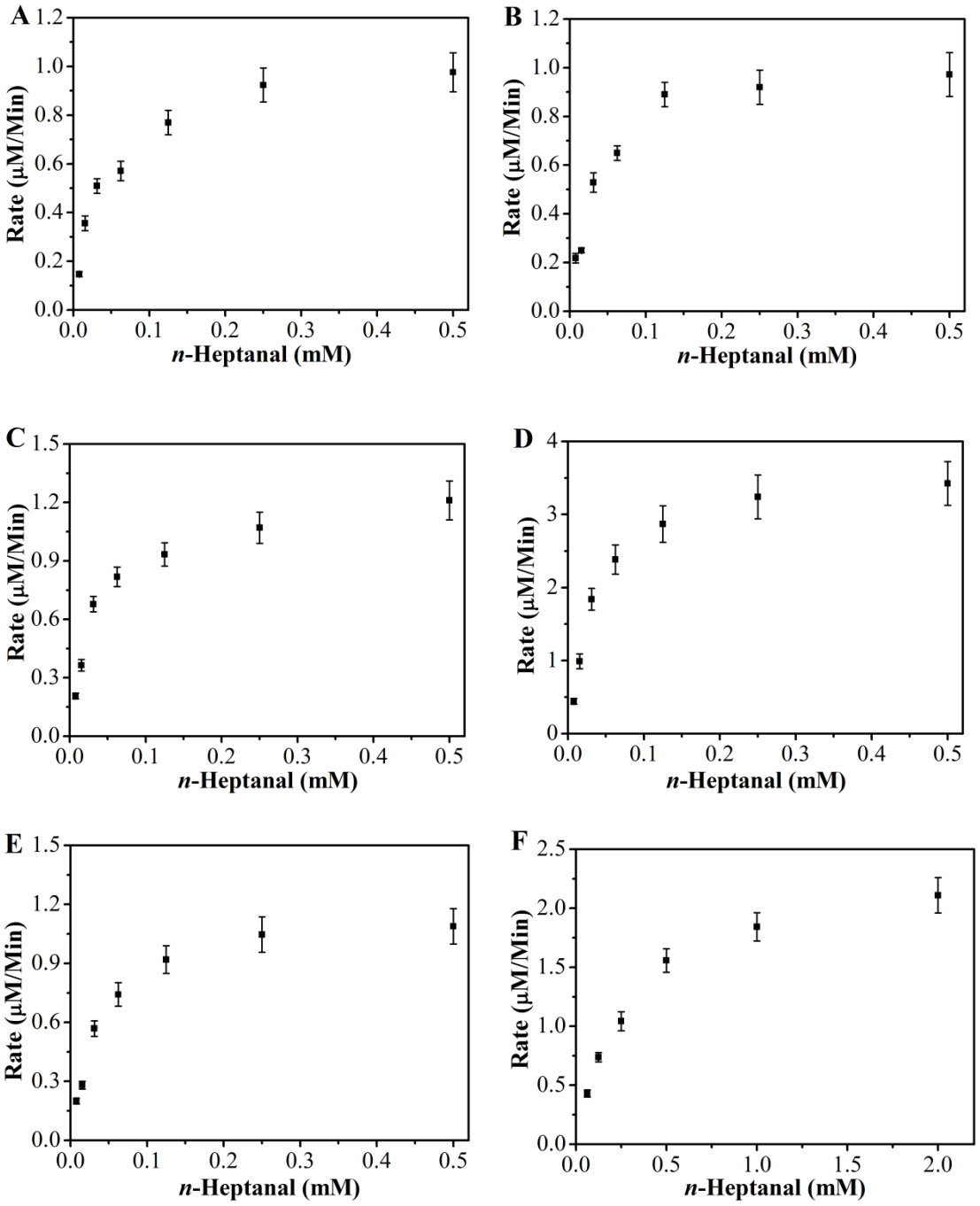


* It is worth pointing out that the predicted molecular weight (around 85.6 kDa) of FusD, FusE, FusF and FusG from the amino acid sequences are smaller than the estimated ones by SDS-PAGE. This is probably due to the acidic character of Fd, which prevents proper binding of SDS.[2]

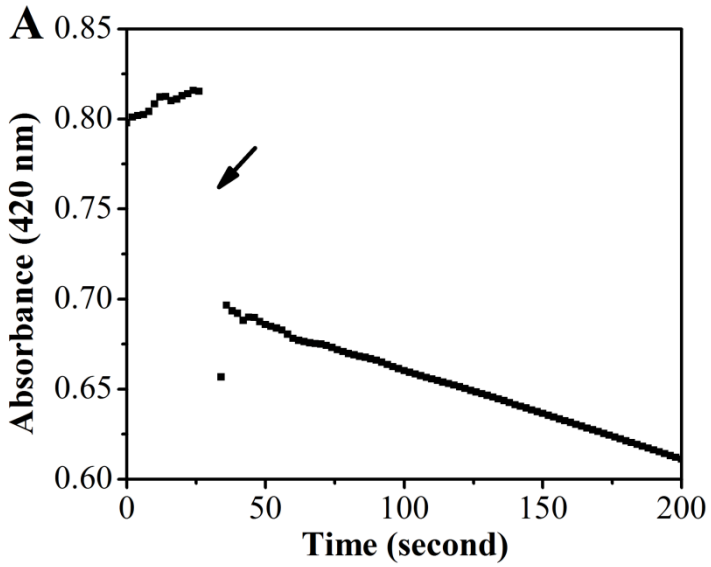
1.8 Fig. S2 The relationship between the catalytic rate and the concentration of FusG

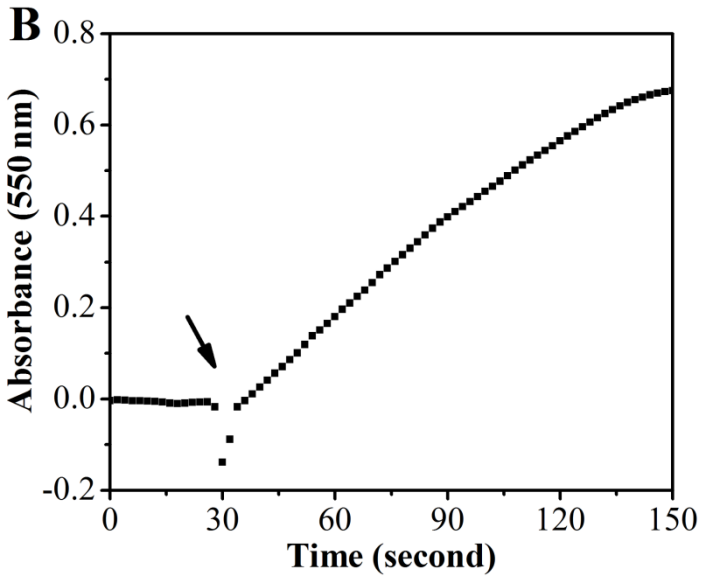


1.9 Fig. S3 The Michaelis–Menten plots of self-sufficient ADOs and native ADO. **A**, FusD; **B**, FusE, **C**, FusF; **D**, FusG; **E**, native ADO in the presence of Fd/FNR/NADPH; **F**, native ADO in the presence of PMS and NADH.



1.10 Fig. S3 The progress curves of reduction of potassium ferricyanide (**A**) and cytochrome *c* (**B**) by FusG. The arrow indicates FusG was added at that time point.





1.11 References

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