Double clicking for site-specific coupling of multiple enzymes

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Electronic Supplementary Information (ESI)

Materials

p-Azido-L-phenylalanine (AZF) was obtained from Chem-Impex International (Wood Dale, IL). DBCO-tetrazine, DBCO-PEG_4-carboxyrhodamine, and DBCO-PEG12-TCO (*trans*-cyclooctene) were purchased from Bioconjugate Technology Company (Scottsdale, AZ). Ni-nitrilotriacetic acid (NTA) agarose and pQE80 plasmid were purchased from Qiagen (Valencia, CA). ZipTip C18 and Vivaspin centrifugal concentrators with a MWCO of 50 kDa were purchased from Millipore Corporation (Billerica, MA) and Sartorius Corporation (Bohemia, NY), respectively. Sequencing grade-modified trypsin was purchased from Promega Corporation (Madison, WI). Biologic DuoFlow chromatography system as well as UNO Q1 anion exchange column was purchased from Bio-Rad (Hercules, CA). Superdex 200 10/300 GL size exclusion column, HiTrap SP HP cation exchange column, and PD-10 desalting columns were obtained from GE Healthcare (Piscataway, NJ). All chemicals were obtained from Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated.

Plasmid Construction and Strains

A plasmid pEVOL-pAzF encoding an AZF-specific engineered pair of tyrosyl-tRNA synthetase/amber suppressor tRNA derived from *Methanococcus jannaschii* (Plasmid ID: 31186) was obtained from Addgene (Cambridge, MA),[1] and used without modification. pQE-80 TsFDH plasmid encoding the *fdh* gene, originally obtained from *Thiobacillus sp.* KNK65MA, with an additional C-terminal histidine sequence, was prepared previously.[2] Site-directed mutagenic PCR was performed with pQE80-FDH as a template to replace valine codon at position 237 with amber codons (UAG), yielding pQE80-FDH-V237amb, respectively. *E. coli* TOP10 was transformed with pQE80-FDH for expression of the wild-type FDH (FDH-WT), affording TOP10 [FDH-WT]. As an expression host for AZF-incorporated FDH (FDH-V237AZF), genomically engineered *E. coli* C321.ΔA.exp was obtained from Addgene (ID: 49018),[3] and co-transformed with pEVOL-pAzF and pQE80-FDH-V237amb, affording C321.ΔA.exp [FDH-V237amb], respectively. The *mdh* gene, which encodes mannitol-2-dehydrogenase originating from *Pseudomonas fluorescens*,[4] with an additional C-terminal histidine sequence was synthesized by GenScript (Piscataway, NJ), and subcloned into pQE80 to generate pQE80-MDH. Site-directed mutagenic PCR was performed with pQE80-MDH as a template to replace valine codon at position 417 with an amber codon (UAG), yielding pQE80-MDH-V417amb. *E. coli*

TOP10 was transformed with pQE80-MDH for expression of the wild-type MDH (MDH-WT), affording TOP10 [MDH-WT]. As an expression host for AZF-incorporated MDH (MDH-AZF), genomically engineered *E. coli* C321.ΔA.exp was co-transformed with pEVOL-pAzF and pQE80-MDH-V417amb, affording C321.ΔA.exp [MDH-V417amb]. All DNA cloning in this study were performed by the restriction-free cloning technique.[5]

Site-specific Incorporation of AZF into FDH and MDH

The saturated culture of C321.ΔA.exp [FDH-V237amb or MDH-V417amb] was inoculated into fresh 2×YT medium containing 100 μg/mL ampicillin and 35 μg/mL chloramphenicol at 1:100 (v/v) dilution, and was subjected to vigorous shaking (220 rpm) at 37 °C. When the OD_{600} of 0.5 was reached, AZF solution was added to a final concentration of 1 mM. After 10 min, temperature was shifted to 30 °C, and protein expression was induced by 1 mM IPTG and 0.2% (w/v) *L*-(+)-arabinose. Cells were harvested after 12 h, and pelleted by centrifugation at 5,000 rpm for 10 min before storage at -20 °C. To extract and purify FDH or MDH containing AZF, cell pellets were resuspended with the lysis buffer consisting of 50 mM sodium phosphate (pH 7.5), 0.3 M NaCl, 10 mM imidazole, 1 mg/mL lysozyme, DNase, RNase, and protease inhibitor cocktail, and mixed by rotation at 37 °C for 1 h followed by at 4 °C for 2 h. After centrifugation at 11,000 rpm for 30 min, the clear supernatant was recovered, mixed with Ni-NTA agarose for 1 h, and then washed with the washing buffer consisting of 50 mM sodium phosphate (pH 7.5), 0.3 M NaCl, and 20 mM imidazole on a gravity-flow column to remove impurities. Proteins were eluted by the elution buffer consisting of 50 mM sodium phosphate (pH 7.5), 0.3 M NaCl, and 250 mM imidazole, and then buffer-exchanged to a storage buffer (PBS, pH 7.2) by a PD-10 column. Expression and purification of FDH-WT or MDH-WT were performed similarly except that TOP10 [FDH-WT or MDH-WT] was used as an expression host without adding AZF and *L*-(+)-arabinose.

MALDI-TOF Mass Spectrometry

Proteins in the storage buffer at 0.5 mg/mL were digested with trypsin at 37°C overnight, and then desalted on a ZipTip C18 according to the manufacturer's protocol. Purified tryptic digests mixed with DHB matrix (20 mg/mL of 2,5-dihydroxybenzoic acid and 2 mg/mL of *L*-

(−)-fucose dissolved in 10% ethanol) at 1:1 (v/v) were subjected to mass characterization by Microflex MALDI-TOF M/S (Bruker Corporation, Billerica, MA).

Dye Labeling by SPAAC

FDH-WT, MDH-WT and their variants at 30 μM in the storage buffer were separately reacted with DBCO-PEG_4-carboxyrhodamine at 100 μM at RT for 2 h, and then loaded onto SDS-PAGE to measure in-gel fluorescence in a BioSpectrum imaging system (UVP, Upland, CA). Upon illumination at λ_{ex} = 480 nm, the emitted light above 510 nm was captured.

Enzymatic Activity Assay

Enzymatic activity of FDH-WT and its variants was measured by formate oxidation to CO_2. The reaction was initiated by mixing 5 μL of 1,600 nM FDH-WT or its variant with 195 μL of the assay buffer consisting of 50 mM formate and 300 μM of NAD^+ in PBS, and then monitored at $A_{340\ nm}$. Enzymatic activity of MDH-WT and its variants was measured by D-fructose reduction to D-mannitol. The reaction was initiated by mixing 5 μL of 160 nM MDH-WT or its variant with 195 μL of the assay buffer consisting of 50 mM D-fructose and 200 μM of NADH in PBS and then monitored at $A_{340\ nm}$. All measurements were made in triplicate at 25 ˚C in a standard 96-well plate on the Synergy™ four multimode microplate reader (BioTek, Winooski, VT). The change in absorbance after 1 min was taken as a measure of catalytic activity.

Synthesis of FDH-MDH Conjugates

First, hetero-bifunctional linkers DBCO-tetrazine and DBCO-PEG12-TCO were conjugated to FDH-AZF and MDH-AZF by SPAAC to generate FDH-TET and MDH-TCO, respectively. Second, the FDH-TET was conjugated to a MDH-TCO by IEDDA reaction. Lastly, the FDH-MDH conjugate was purified by ion exchange liquid chromatography. Detailed conditions are as follows. FDH-AZF was mixed with 4 molar excess of DBCO-tetrazine in PBS containing 5% (v/v) DMSO, and reacted at RT for 7 hrs. To remove residual DBCO-tetrazine, the reaction mixture was desalted on a PD-10 column, and buffered-exchanged to 20 mM bis-tris buffered at pH 6.0. MDH-AZF was similarly treated except that DBCO-PEG12-TCO was used instead of DBCO-tetrazine. FDH-TET and MDH-TCO thus obtained were mixed at 1:1 molar

stoichiometry, concentrated to a total protein concentration of 5 mg/mL, and reacted at RT for 1 hr. The reaction mixture was directly loaded onto an anion exchange column, UNO Q1, pre-equilibrated with 20 mM bis-tris (pH 6.0), and resolved by applying a NaCl gradient. A fraction containing the FDH-MDH conjugate was collected and characterized on a size exclusion column, Superdex 200, to estimate its size and purity.

Determination of the molar composition of the FDH-MDH Conjugate

Calibration curves for determining a molar composition of the FDH-MDH conjugate based upon respective catalytic activities were constructed by plotting the absorbance change at $A_{340\ nm}$ for 1 min, i.e. slope, upon initiation of enzymatic reactions as described above except that enzyme concentrations were varied: 100, 200, and 400 nM for FDH; 5, 10, 20 nM for MDH. Linear regression was applied to express enzyme concentrations as a linear function of $A_{340\ nm}$ slopes. An appropriate volume of the FDH-MDH conjugate solution was individually subjected to both enzymatic activities under the same conditions to obtain $A_{340\ nm}$ slopes, which was then fit to linear functions to estimate molar concentrations of FDH and MDH.

Measurement of D-Mannitol Production in Enzymatic Cascade Reaction

The cascade reaction was initiated by mixing 10 µL of FDH-MDH conjugate (50 × working concentration) or a free enzyme mix of FDH-WT and MDH-WT with 490 µL of the assay solution consisting of 50 mM formate, 50 mM D-fructose and 500 µM NAD^+ in PBS. At appropriate time points, 150 µL of the reaction mixture were sampled in separate tubes. After lowering pH to 3.0 by adding HCl, the sample was heated at 80 ˚C for 40 min to inactivate enzymes and residual cofactors.[6] The enzymatic D-mannitol assay was conducted to measure the amount of D-mannitol in the sample by mixing 40 µL with 160 µL of the assay solution consisting of 50 nM MDH-WT and 600 µM NAD^+ in sodium bicarbonate buffered at pH 9.5 and monitoring increase in $A_{340\ nm}$ attributed by enzymatic oxidation of D-mannitol to D-fructose. Absorbance change for 5 min was used to calculate the concentration of D-mannitol in the sample by fitting to a D-mannitol calibration curve which was obtained in advance by performing the D-mannitol assay using a known amount of D-mannitol (1.0, 2.5, 5.0 and 10 µM) and by relating the absorbance change for 5 min to D-mannitol concentrations by linear regression (Fig. S3). All measurements were made in triplicate.

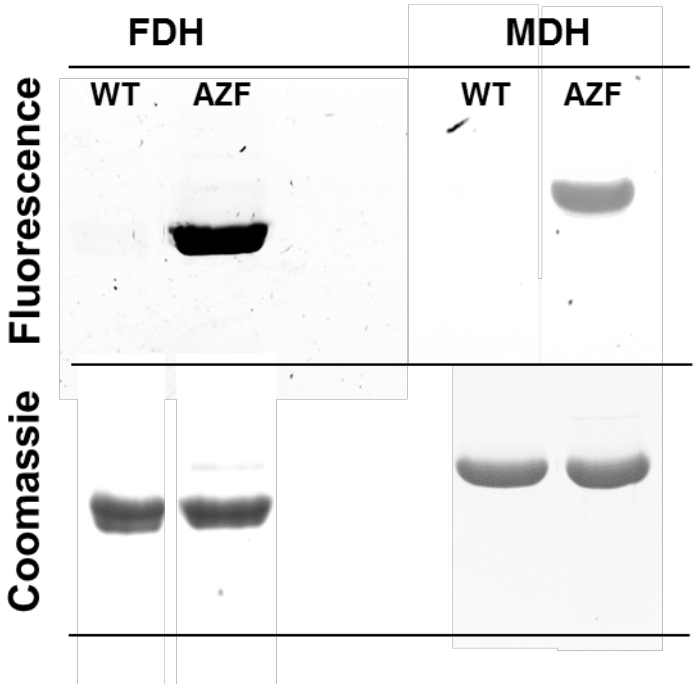

Fig. S1. In-gel fluorescence analyses of FDH-WT, MDH-WT, and their variants bearing AZF upon completion of reactions with DBCO-PEG_4-carboxyrhodamine. The gel was subjected to UV (390 nm) irradiation to excite the fluorophore (Fluorescence panel), and then stained with Coomassie blue (Coomassie panel) to visualize proteins.

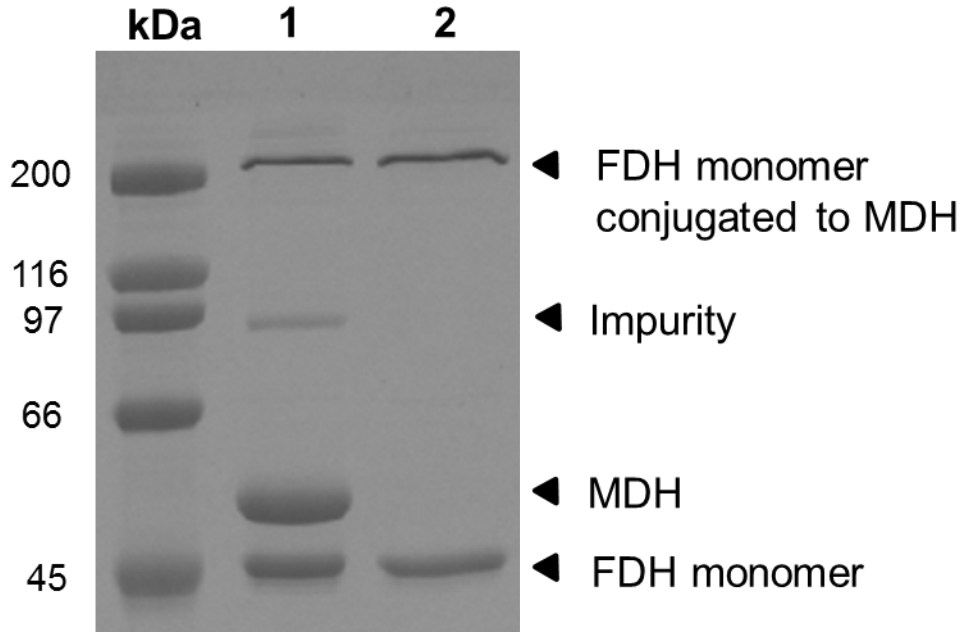

Fig. S2. SDS-PAGE analysis of the conjugation reaction mixture (lane 1) and the purified FDH-MDH conjugate (lane 2).

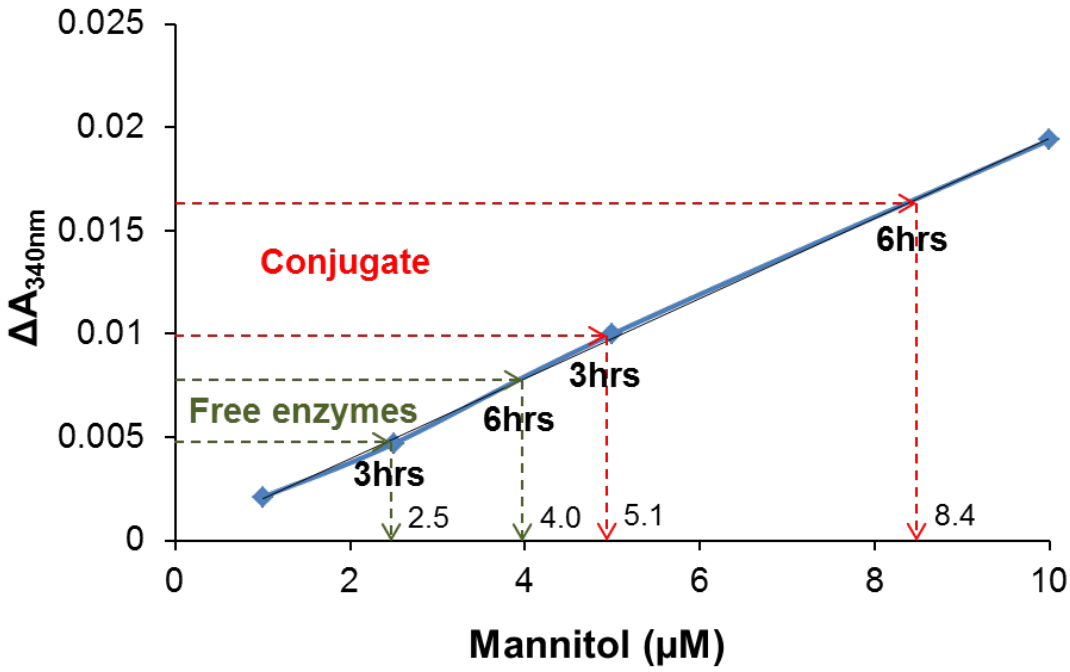

Fig. S3. D-mannitol calibration curve ranging from 1 to 10 μM of D-mannitol. Samples taken at 3 and 6 hrs after initiation of the multi-enzyme reaction in the presence of the FDH-MDH conjugate (Conjugate) or free FDH and MDH (Free enzymes) were subjected to the enzymatic mannitol assay, and absorbance changes at 340 nm were used to estimate the mannitol concentration by interpolation. Actual mannitol concentrations in samples were obtained by multiplying the dilution factor, 5 to yield Fig. 4.

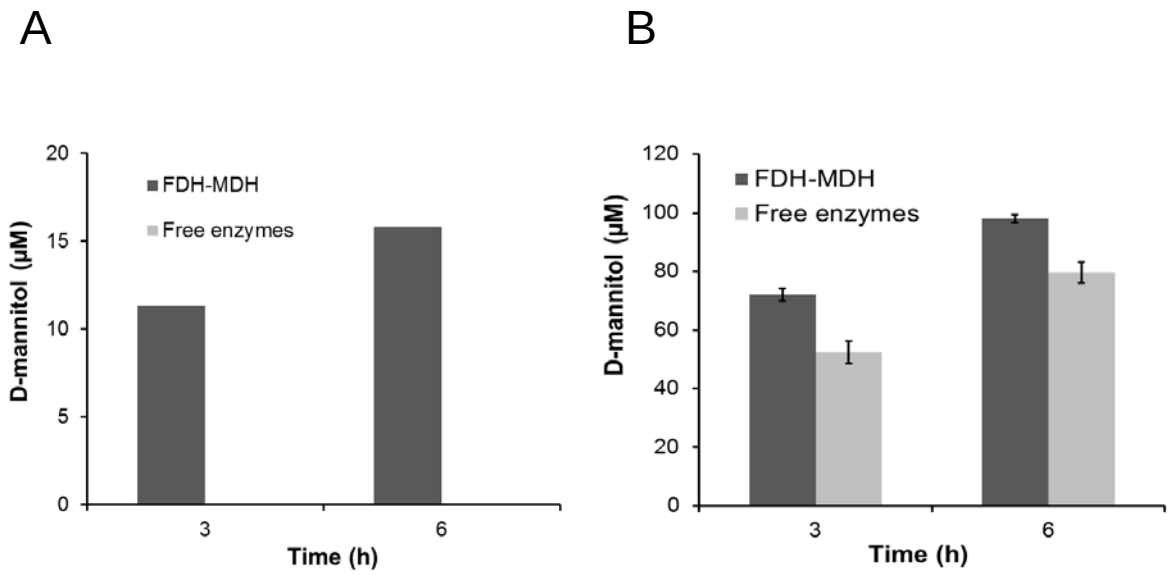

Fig. S4. D-mannitol production by the FDH-MDH conjugate or free enzymes in the multi-enzyme reaction system. (A) The FDH-MDH conjugate corresponding to 3 nM MDH activity or a comparable amount of unconjugated FDH and MDH (3.3 nM dimeric FDH and 3 nM MDH) was subjected to the multi-enzyme cascade reaction in the presence of 500 μM NAD^+ and 50 mM of formate and D-fructose. Concentrations of the product, D-mannitol, were enzymatically measured at 3 and 6 hrs after initiation. (B) The same multi-enzyme cascade reactions with the FDH-MDH conjugate corresponding to 10 nM MDH activity or a comparable amount of unconjugated FDH and MDH (11 nM dimeric FDH and 10 nM MDH).

Table S1. Protein recovery during the synthesis of the FDH-MDH conjugate

Step	Purification	FDH (mg)	Yield (%)	MDH (mg)	Yield (%)
Bacterial expression	Ni-NTA affinity	2.0[a]	100	2.0[b]	100
Linker conjugation by SPAAC	Desalting	1.9[c]	95	1.8[d]	90
Protein conjugation by IEDDA reaction	Anion exchange	0.54[e]	27	0.33[f]	17

[a]FDH-AZF produced from 0.40 L of culture

[b]MDH-AZF from 0.25 L of culture

[c]Total amount of FDH-TET and unconjugated FDH-AZF recovered after desalting

[d]Total amount of MDH-TCO and unconjugated MDH-AZF recovered after desalting

[e]Based upon 2:1 molar composition (dimeric FDH conjugated a single MDH) of the FDH-MDH conjugate and its amount isolated from the anion exchange chromatography. The purified amount of the FDH-MDH was measured by using a molecular weight of 145 kDa (2 × 45 kDa of dimeric FDH plus 55 kDa of MDH) and an extinction coefficient of 180,030 $M^{-1}cm^{-1}$ at 280 nm.

Supplementary references

1. J. W. Chin, S. W. Santoro, A. B. Martin, D. S. King, L. Wang and P. G. Schultz, *J Am Chem Soc*, 2002, **124**, 9026-9027.
2. S. I. Lim, S. Yoon, Y. H. Kim and I. Kwon, *Molecules*, 2015, **20**, 5975-5986.
3. M. J. Lajoie, A. J. Rovner, D. B. Goodman, H. R. Aerni, A. D. Haimovich, G. Kuznetsov, J. A. Mercer, H. H. Wang, P. A. Carr, J. A. Mosberg, N. Rohland, P. G. Schultz, J. M. Jacobson, J. Rinehart, G. M. Church and F. J. Isaacs, *Science*, 2013, **342**, 357-360.
4. P. Brunker, J. Altenbuchner, K. D. Kulbe and R. Mattes, *Biochim Biophys Acta*, 1997, **1351**, 157-167.
5. S. R. Bond and C. C. Naus, *Nucleic Acids Res*, 2012, **40**, W209-213.
6. J. T. Wu, L. H. Wu and J. A. Knight, *Clin Chem*, 1986, **32**, 314-319.