Supporting information

Designed surrogate for high throughput screening of fatty aldehyde reductase engineering

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Materials

Chemicals were purchased from either Sigma-Aldrich or TCI Chemicals. Column chromatography purification was performed on a silica gel (Merck, Germany) as a stationary phase. Analytical thin layer chromatography (TLC) was performed on TLC Silica gel 60 F254 (Merck, Germany) and visualized in a UV cabinet (254 and 365 nm). ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker-500 MHz spectrometer at room temperature. Chemical shifts of ^{1}H-NMR spectra were reported in ppm and calibrated from the solvent ($CDCl_3$ 7.26 ppm; DMSO-d_6 2.50 ppm). ^{1}H-NMR data are reported as the following: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad), coupling constants, and number of protons. Proton decoupled ^{13}C-NMR spectra were reported from tetramethylsilane (TMS) resonance ($CDCl_3$ 77.0 ppm).

E. coli MG1655 was purchased from NBRP-E. coli at NIG in Japan. The genomic DNA were purified with a BioFact Genomic DNA Prep Kit. Oligonucleotides were purchased from Integrated DNA Technologies. Enzymes for molecular cloning were purchased from New England Biolabs. DNA purification kits were purchased from Vivantis. All plasmids made were constructed by Gibson assembly of PCR products. *E. coli* MG1655 RARE is a gift from Prof. Kristala Prather at the Massachusetts Institute of Technology. The overexpression plasmid of MAACR is a gift from Prof. Brian Pfleger at University of Wisconsin-Madison. The overexpression plasmids of *Pa*FDH-WT and *Pa*FDH-V9 are gifts from Prof. Arren Bar-Even at Max Planck Institute of Molecular Plant Physiology.

T6 and T10 synthesis

The mixture of formic acid (50.05 mmol) and acetic anhydride (45.93 mmol) was stirred at 60 ºC for 2 h. An alkanethiol (22.96 mmol) was then added and stirred for 12 h at room temperature. The excess of formic acid and acetic anhydride was evaporated under reduced pressure in the presence of hexane (20 mL) as an azeotropic mixture. After evaporation, crude product was purified by chromatography on column by using silica gel and the hexane as eluent. The ^{1}H- and ^{13}C-NMR spectra of **T6** and **T10** are shown in Fig. S1 and S2.

T6: *S*-butyl methanethioate. Colorless oil, 35% yield. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 10.07 (s, 1H), 2.91 (t, J = 7.5 Hz, 2H), 1.53 (q, J = 7.0 Hz, 2H), 1.34 (q, J = 7.5 Hz, 2H), 0.86 (t, J = 7.5 Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 187.8, 31.5, 26.4, 21.8, 13.5.

T10: *S*-octyl methanethioate. Colorless oil, 85% yield. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 10.07 (s, 1H), 2.91 (t, J = 7.5 Hz, 2H), 1.54 (q, J = 7.5 Hz, 2H), 1.30 (br, 2H), 1.21 (br, 8H), 0.81 (t, J = 6.0 Hz, 3H; ^{13}C-NMR (125 MHz, $CDCl_3$) δ 187.7, 31.7, 29.5, 29.1, 29.0, 28.7, 26.7, 22.6, 14.0.

Stability of T6 in lysate and buffer

The assays were initiated by adding 500 μM of **T6** to 200 μL of 100 mM potassium phosphate, pH 7.5 or *E. coli* MG1655 RARE lysate. All assays were incubated at room temperature for 10, 20, 40, and 60 min, respectively. 1 mM DTNB (5,5′-Dithiobis-2-nitrobenzoic acid) was added to quench the assays to determine 1-butanethiol generation from **T6** decomposition. The absorbance at 412 nm was immediately measured and used to calculate butanethiol generation according to the calibration curve of 1-butanethiol standard solution.

Overexpression plasmid construction

The genes encoding YahK, DkgA, and YbbO were amplified by PCR from the genomic DNA of *E. coli* MG1655. Every PCR product was assembled into pET28 or pET30 vectors by Gibson assembly.

Protein overexpression and purification (YahK, DkgA, YbbO, and MBP-MAACR)

Every protein was overexpressed as a fusion with an N-terminal His tag. Ten milliliters of overnight culture of *E. coli* BL21(DE3) harboring an overexpression plasmid were inoculated into 1 L of Luria-Bertani broth (LB) containing 50 μg/mL kanamycin. The culture was shaken at 200 rpm and 37 ºC until OD_{600} reached about 0.6. The protein expression was induced by adding isopropyl-β-*D*-1-thiogalactopyranoside (IPTG) with the final concentration of 200 μM. The culture mixture was shaken at 200 rpm and 20 ºC for an additional 16 hours. The cells were collected by centrifugation at 5000 rpm and 8 ºC for 25 min. The cells were resuspended in the lysis buffer (300 mM NaCl, 50 mM NaH_2PO_4, and 10 mM imidazole, pH 8.0). The cells were lysed for 30 seconds three times by sonication (1.5 s cycle, 50% duty) on ice, followed by the centrifugation at 12,000 rpm and 4 °C for 40 min. The supernatant was applied to Ni-NTA column (QIAGEN) and the proteins were purified by the manufacturer's instructions. After elution, the proteins were desalted using a 10-DG column (BioRad) pre-equilibrated with 100 mM potassium phosphate buffer, 20% glycerol, pH 7.5. Every purified protein was stored in aliquots at -80 °C.

***Pa*FDH-WT and *Pa*FDH-V9 overexpression and purification**

Ten milliliters of overnight culture of *E. coli* BL21(DE3) harboring pZ-ASL-*Pse*FDH-WT or pZ-ASL-*Pse*FDH-V9 were inoculated into 1 L of terrific broth containing 100 μg/mL streptomycin. The culture was shaken at 200 rpm and 30 ºC for an additional 16 hours. The cells were collected by centrifugation at 5000 rpm and 8 ºC for 25 min. The cells were resuspended in buffer A (20 mM Tris, 500 mM NaCl, and 5 mM imidazole, pH 7.9). The cells were lysed by sonication for 30 seconds three times (1.5 s cycle, 50% duty) on ice, followed by the centrifugation

at 12,000 rpm and 4 °C for 40 min. The supernatant was loaded onto Ni-NTA column (QIAGEN) and the protein was eluted by the manufacturer's instructions. After elution, the proteins were desalted using a 10-DG column (BioRad) pre-equilibrated with 100 mM Na_2HPO_4, pH 7.0. The purified proteins were stored in aliquots at -80 °C.

Enzymatic assay

All assays were carried out in a total volume of 1 ml. The assays were initiated by the addition of purified enzyme to the reaction mixture containing 100 μM of NAD(P)H, and 150 or 500 μM of **A6** (hexanal), **A10** (decanal), **T6**, or **T10** in 100 mM potassium phosphate buffer (pH 7.5). All assays were performed in triplicate at room temperature. The NAD(P)H consumption was measured at 340 nm using NanoDrop 2000c spectrophotometer.

Kinetic constant determination

For determining apparent kinetic parameters on coenzymes, the reactions were carried out in 1 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 0-300 μM of NAD(P)H, and 500 μM of **A6**. For the apparent kinetic parameters for NADPH, the enzyme concentrations of wild-type YahK, E3, and C8 were 5 nM. For the apparent kinetic parameters for NADH, the enzyme concentrations of wild-type YahK, E3, and C8 were 75 nM, 20 nM, and 50 nM, respectively. For the apparent kinetic parameters for the substrates (**A6** and **T6**), the reactions were performed in 1 mL of 100 mM potassium phosphate buffer (pH 7.5) with the final concentration of 100 μM of NADPH and 0-100 μM of substrate. The enzyme concentrations were 10 nM for **A6** and 100 nM for **T6**. NAD(P)H consumptions were measured at 340 nm in 2 s interval for 10 min using NanoDrop 2000c spectrophotometer. Initial rates were calculated using extinction coefficient of NAD(P)H ($\varepsilon = 6.22\ mM^{-1}cm^{-1}$) with the linear initial slope of reaction. The apparent kinetic parameters, K_m and V_{max}, were calculated by fitting the reaction rate and substrate concentration in nonlinear regression of the Michaelis–Menten curves with GraFit 5.0 (Erithacus Software, Horley, Survey, UK). The apparent k_{cat} was obtained by dividing V_{max} by the final enzyme concentration.

Purpald assay

For enzymatic experiment, the reactions were initiated by adding purified enzyme to the reaction mixture containing 100 μM of NADPH, 150 or 500 μM of **T6** or **T10** in 100 mM potassium phosphate buffer (pH 7.5) with a final volume of 200 μL. For cell lysate experiment, the harvested cells were lysed by 0.2 mg/mL lysozyme and the mixture was incubated at 37 ºC for 30 min. The cell lysate was centrifuged at 4000 rpm at 4 ºC for 20 min to obtain the supernatant.

The assays were initiated by the adding 100 μL of the supernatant to the reaction mixture containing 100 μM of NADPH, 500 μM of **T6**, and 100 mM potassium phosphate buffer (pH 7.5) to bring the final volume to 200 μL. All assays were incubated at room temperature for 10 min, followed by adding 50 μL of 100 mM Purpald solution (100 mM Purpald was prepared in 1 M NaOH). After incubating at room temperature aerobically for 15 min, the absorbance of solution showing purple was measured at 550 nm using microplate spectrophotometer (Thermo Scientific™ Multiskan™ GO). The formaldehyde amount was calculated by the calibration curve of formaldehyde standard solution.

DTNB assay

All assays were carried out in a total volume of 200 μL. The reactions were initiated by adding 0.1 μM purified *Ec*YahK to the reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5), 100 μM of NADPH, 500 μM of **T6**, and 1 mM of DTNB. The absorbance at 412 nm was measured at 0 min and after 10 min using microplate spectrophotometer (Thermo Scientific™ Multiskan™ GO). The amount of produced thiol was calculated by the calibration curve of 1-butanethiol standard solution.

Mutant library construction

The saturation mutagenesis library of T205/T206 was constructed by the amplification of pET30a-*Ec*YahK using the primers of RYL185 and RYL186 by Q5® High-Fidelity DNA polymerase. The resulted PCR product was treated with *Dpn*I, followed by the purification. The purified PCR product was phosphorylated by T4 DNA kinase followed by ligation by T4 DNA ligase to complete the library construction. The library was transformed in *E. coli* MG1655 RARE for the high throughput screening of YahK cofactor engineering.
RYL185: 5'-NNKTCTGAGGCAAAACGCGAAGC-3'
RYL186: 5'-MNNAAATGCCACCACATGTGCCC-3'

High-throughput screening of YahK variants

For high-throughput screening assay, the colonies were grown in 100 μL of LB in 96-well plates at 30 ºC and induced by 200 μM IPTG. The plates were shaken at 150 rpm and 20 ºC for an additional 16 hours. The cell culture was then centrifuged at 4000 rpm at 8 ºC for 20 min to remove the supernatant. The cell pellet was lysed with 0.2 mg/mL lysozyme, followed by incubation at 37 ºC for 30 min. The lysed cells were centrifuged at 4 ºC and 4000 rpm for 20 min to obtain the supernatant. 100 μL of the resulting supernatant were transferred to a new 96-well plate. Subsequently 100 μL of reaction mixture containing 100 mM potassium phosphate buffer, pH 7.5, 10 μM of NAD^+, 5 μM of purified *Pa*FDH-WT, 10 mM of ammonium formate, and 500 μM of

T6 were added to each sample well. The 96-well plate was shaken for 10 min at room temperature, followed by adding 50 μL of Purpald solution (100 mM Purpald in 1 M NaOH). After incubating for 15 min at room temperature aerobically, the intense of purple product in each sample well was measured at 550 nm using microplate spectrophotometer (Thermo Scientific™ Multiskan™ GO).

Structural analysis

The structure of YahK[1] (PDB ID: 1UUF) and ligand NADPH (NDP) were downloaded from PDB database (https://www.rcsb.org/). The ligand and receptor models were prepared using AutoDockTools (Scripps Research Institute, La Jolla, CA). AutoDock vina[2] was used to perform docking of ligands to wild-type YahK. Protein pictures were created using PyMOL (http://www.pymol.org).

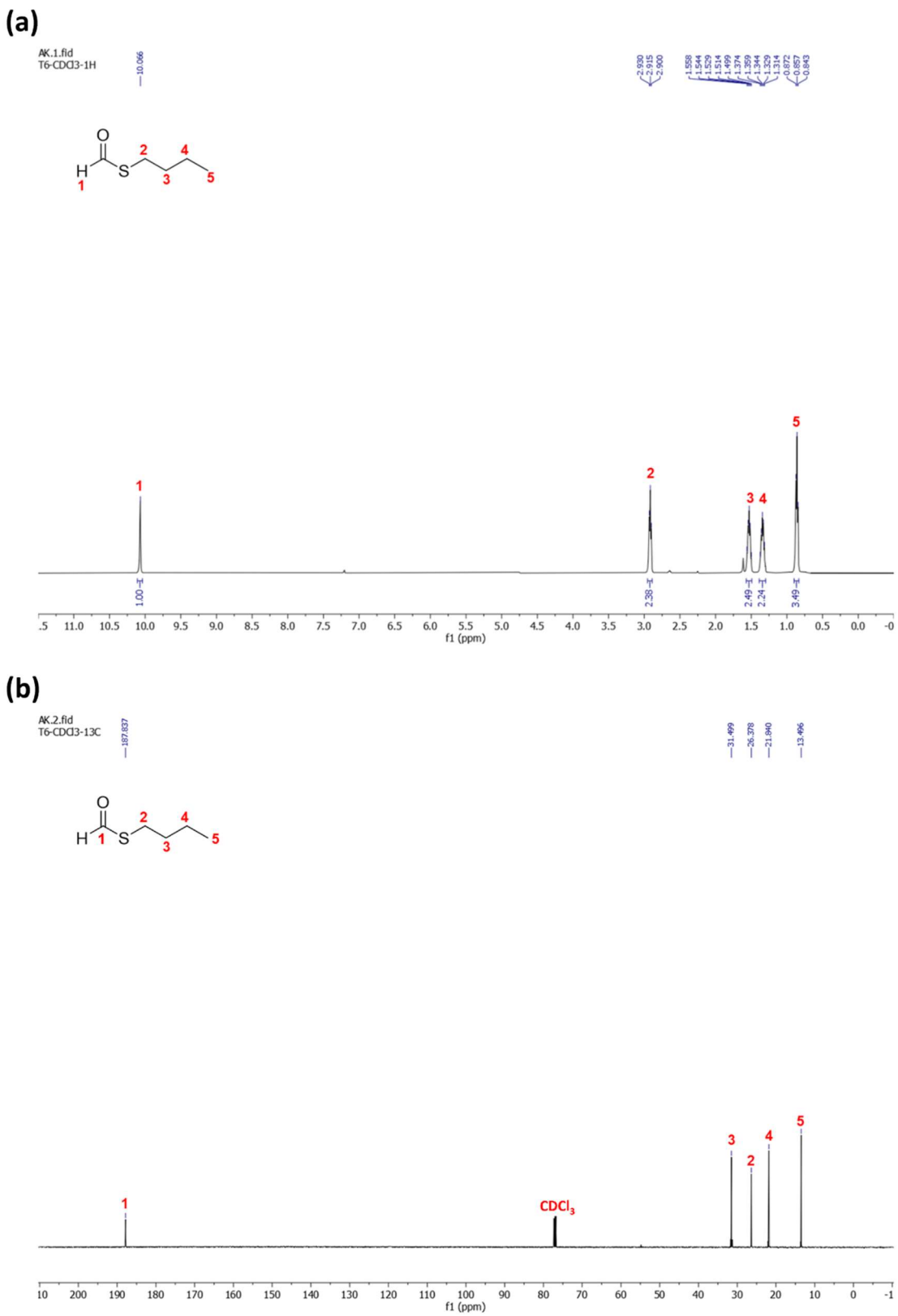


Figure S1. ^{1}H- (a) and ^{13}C- (b) NMR of **T6** in $CDCl_3$.

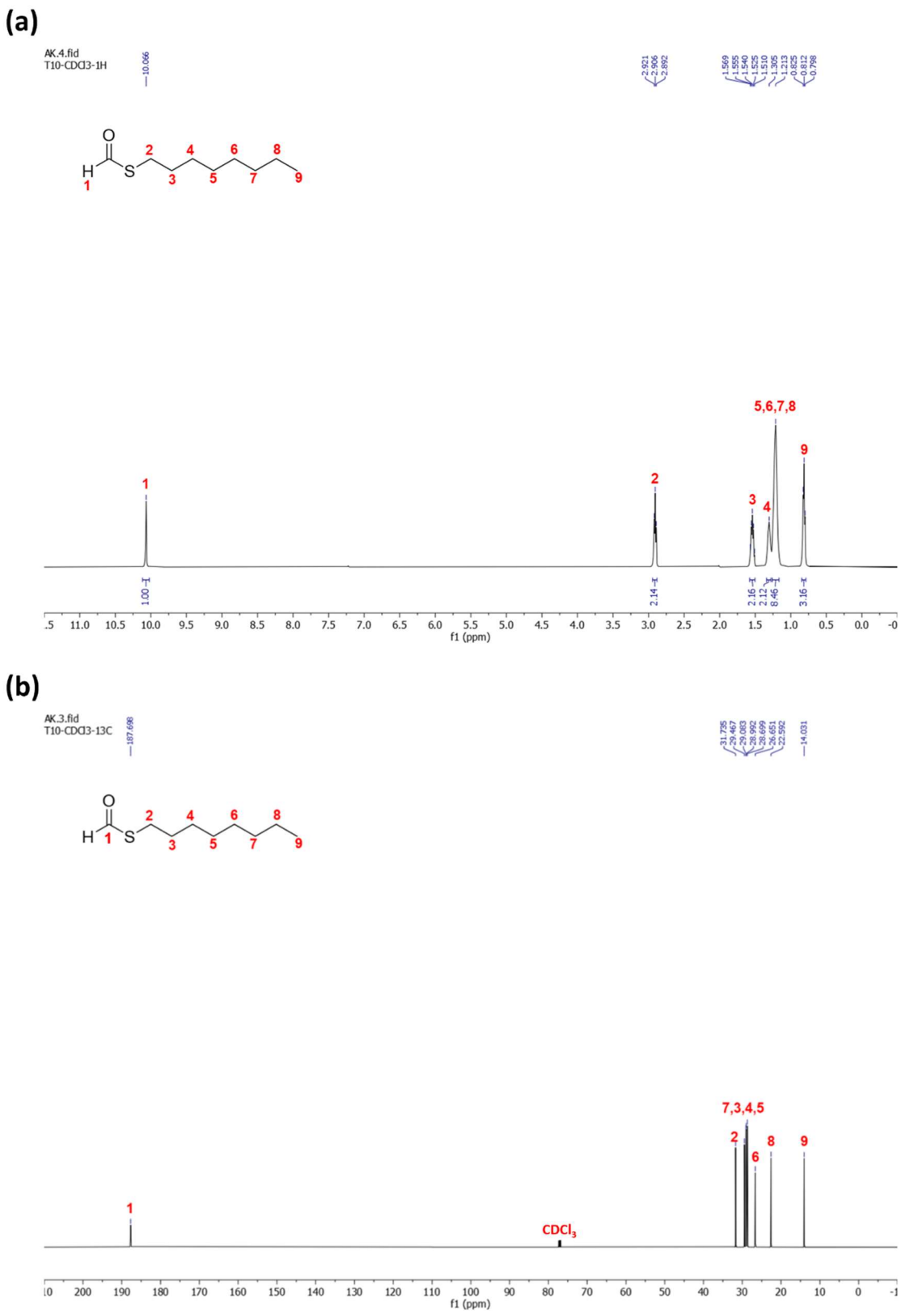


Figure S2. ^{1}H- (a) and ^{13}C- (b) NMR of **T10** in $CDCl_3$.

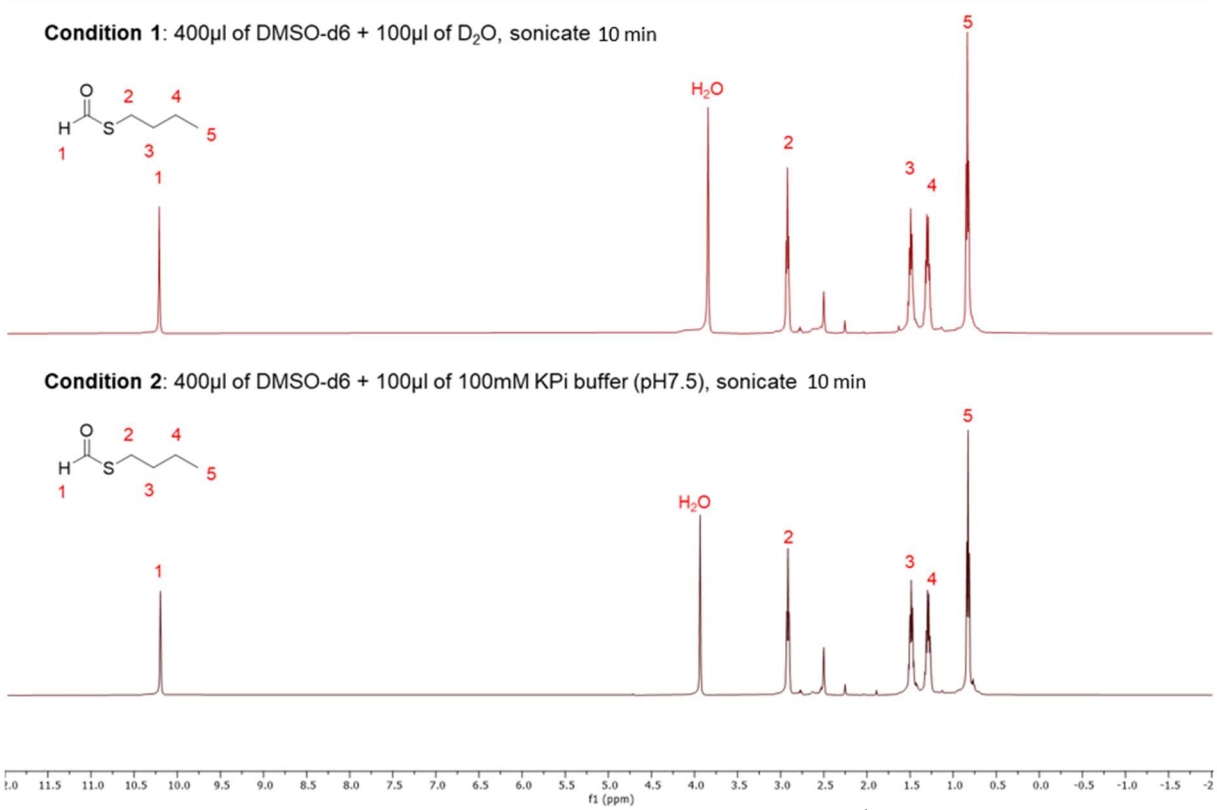


Figure S3. Stability of **T6** in potassium buffer (pH 7.5) studied by ^{1}H-NMR.

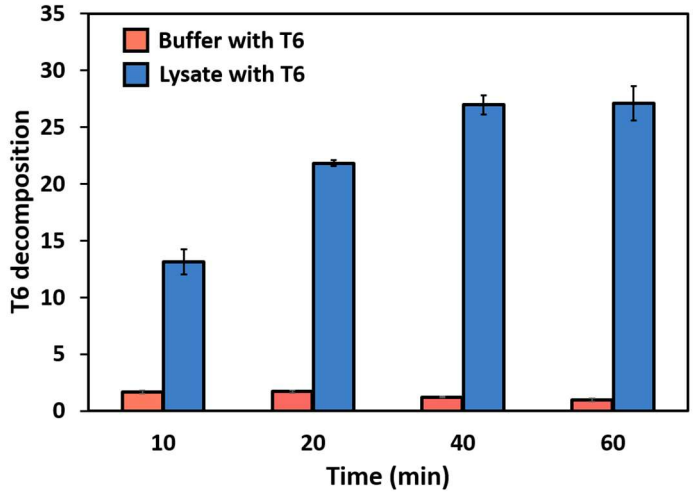


Figure S4. T6 stability in 100 mM potassium phosphate (pH 7.5) and *E. coli* MG1655 RARE lysate. 500 µM **T6** was in the buffer or lysate for different time points. **T6** decomposition was quantified by detection of the hydrolyzed product, 1-butanethiol, by DTNB assay.

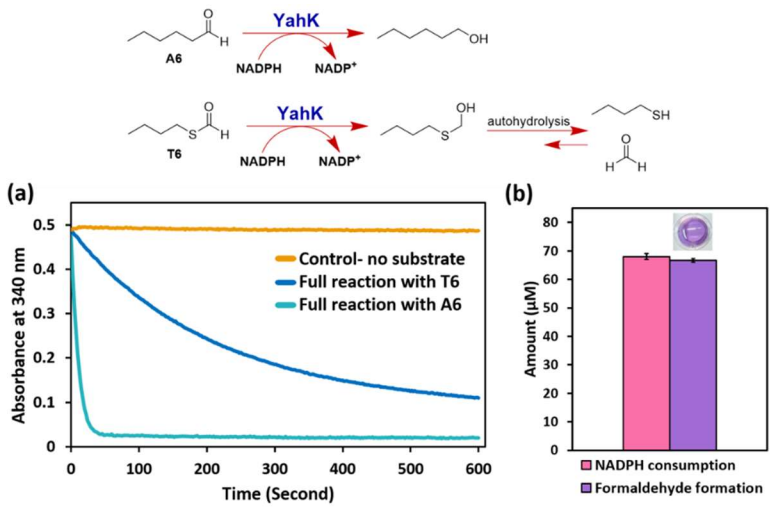


Figure S5. (a) 150 μM of **A6** and **T6** reduction catalyzed by 0.1 μM YahK in the presence of 100 μM NADPH. The reactions were monitored at 340 nm for NADPH consumption. (b) The full reaction with **T6** showed that NADPH consumption is stoichiometric with formaldehyde formation quantified by Purpald assay.

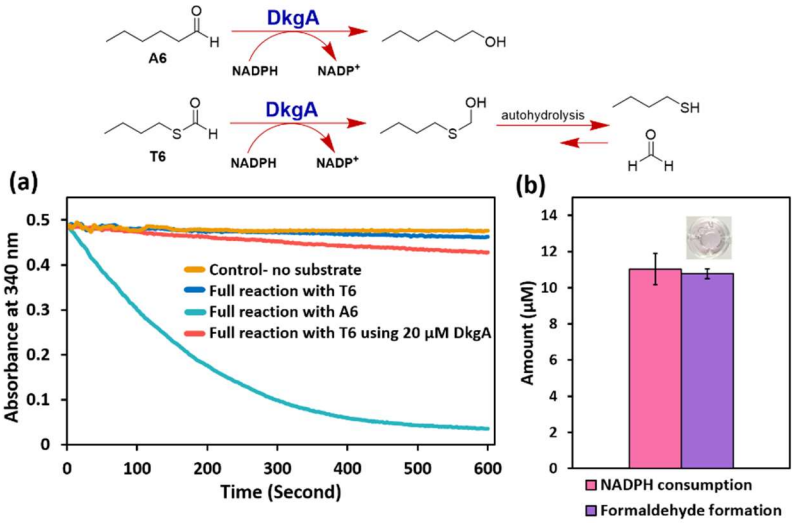


Figure S6. (a) 150 μM of **A6** and **T6** reduction catalyzed by 2.5 μM DkgA in the presence of 100 μM NADPH. The reactions were monitored at 340 nm for NADPH consumption. To confirm that DkgA could reduce **T6**, 20 μM DkgA was used to increase the amount of **T6** reduction in the same period of reaction time. (b) The full reaction with **T6** using 20 μM DkgA showed that NADPH consumption is stoichiometric with formaldehyde formation quantified by Purpald assay.

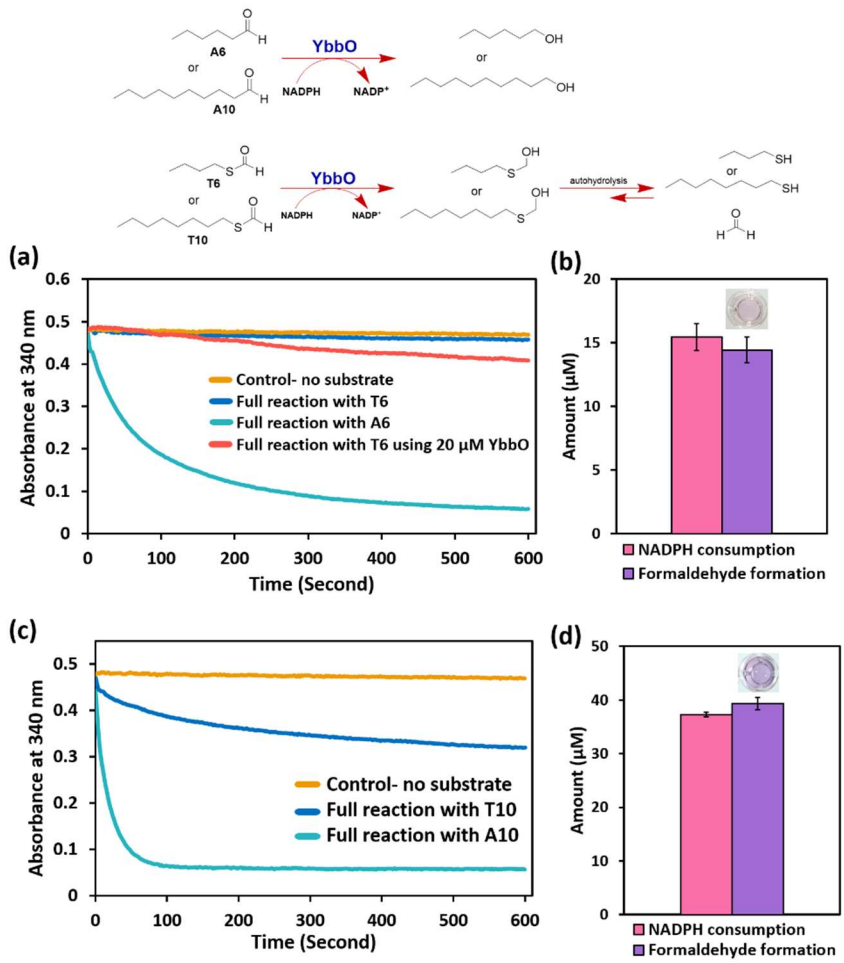


Figure S7. (a) 150 µM of **A6** and **T6** reduction catalyzed by 2.5 µM YbbO in the presence of 100 µM NADPH. The reactions were monitored at 340 nm for NADPH consumption. To confirm that YbbO could reduce **T6**, 20 µM YbbO was used to increase the amount of **T6** reduction in the same period of reaction time. (b) The full reaction with **T6** using 20 µM YbbO showed that NADPH consumption is stoichiometric with formaldehyde formation quantified by Purpald assay. (c) 150 µM of **A10** and **T10** reduction catalyzed by 2.5 µM YbbO in the presence of 100 µM NADPH. The reactions were monitored at 340 nm for NADPH consumption. (d) The full reaction with **T10** showed that NADPH consumption is stoichiometric with formaldehyde formation quantified by Purpald assay.

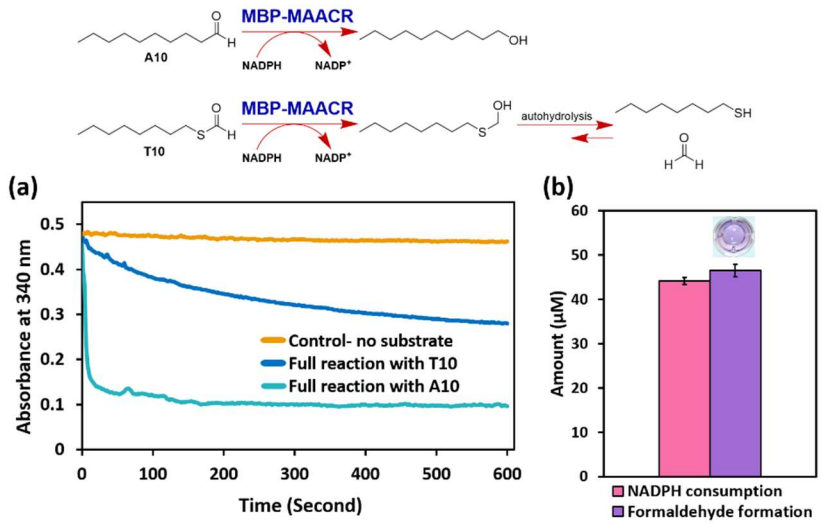


Figure S8. (a) 150 μM of **A10** and **T10** reduction catalyzed by 2.5 μM MBP-MAACR in the presence of 100 μM NADPH. The reactions were monitored at 340 nm for NADPH consumption. (b) The full reaction with **T10** showed that NADPH consumption is stoichiometric with formaldehyde formation quantified by Purpald assay.

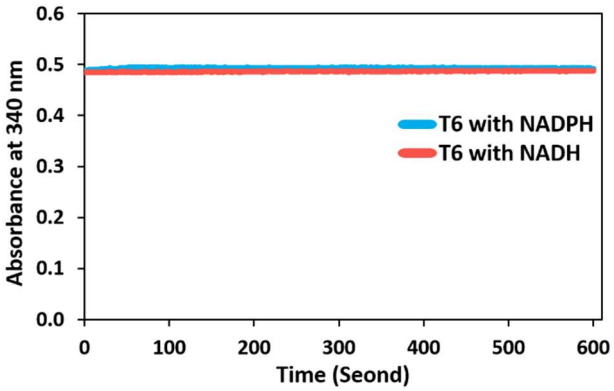
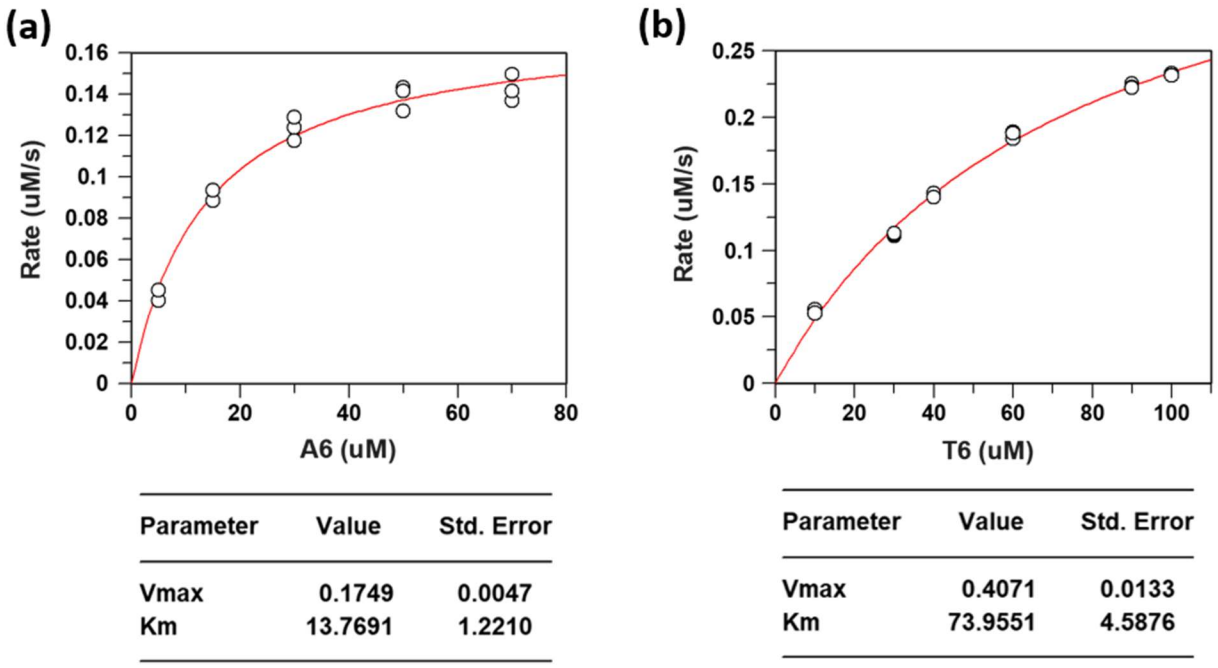


Figure S9. NADPH and NADH could not reduce **T6**. 100 μM T6 was incubated with 100 μM of NADPH or NADH in 100 mM potassium phosphate buffer (pH 7.5).



Parameter	Value	Std. Error
Vmax	0.1749	0.0047
Km	13.7691	1.2210

Parameter	Value	Std. Error
Vmax	0.4071	0.0133
Km	73.9551	4.5876

Figure S10. The apparent kinetic constants were determined at the fixed concentration of 100 μM NADPH with varied **A6** by 0.01 μM YahK (a) or varied **T6** by 0.1 μM YahK (b).

Table S1. Apparent kinetic constants of YahK for **A6** and **T6**

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}/s^{-1}$)
A6	17.49 ± 0.47	13.77 ± 1.22	1.27 ± 0.15
T6	4.07 ± 0.13	73.96 ± 4.59	0.06 ± 0.01

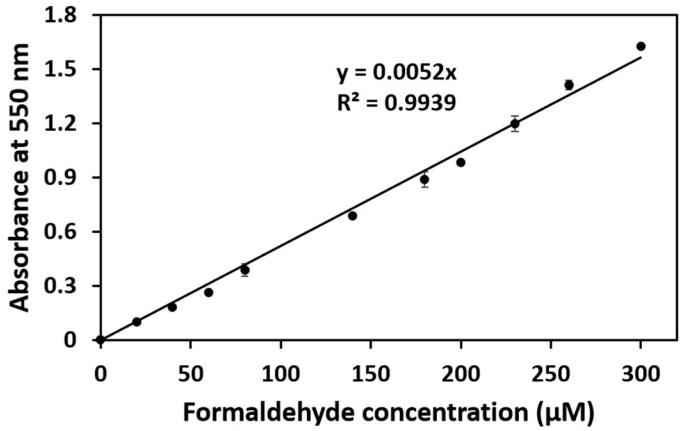


Figure S11. Calibration curve of formaldehyde standard solutions determined by Purpald assay.

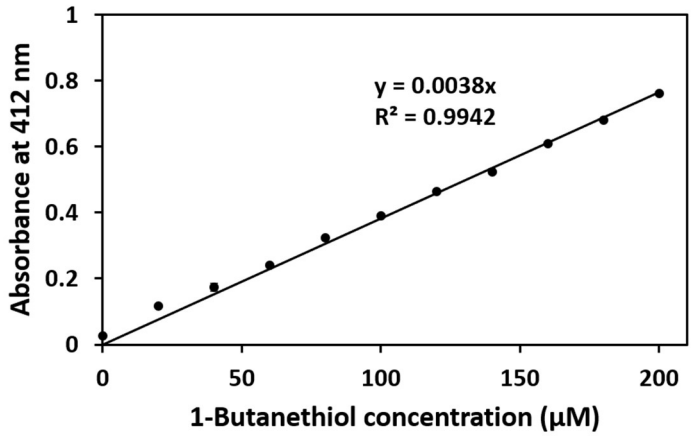


Figure S12. Calibration curve of 1-butanethiol standard solutions determined by DTNB assay.

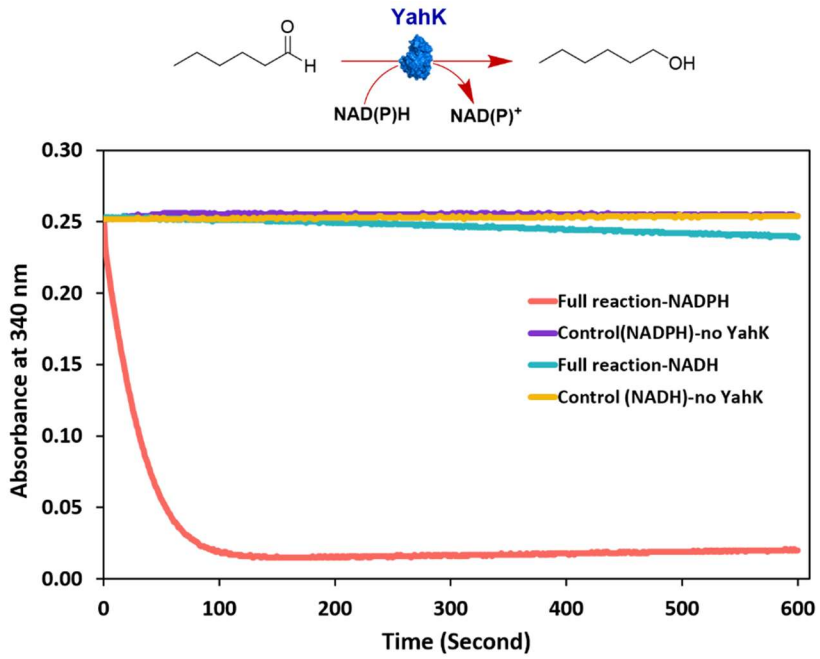


Figure S13. YahK characterization. The results show that it highly prefers NADPH, but can use NADH with very low preference.

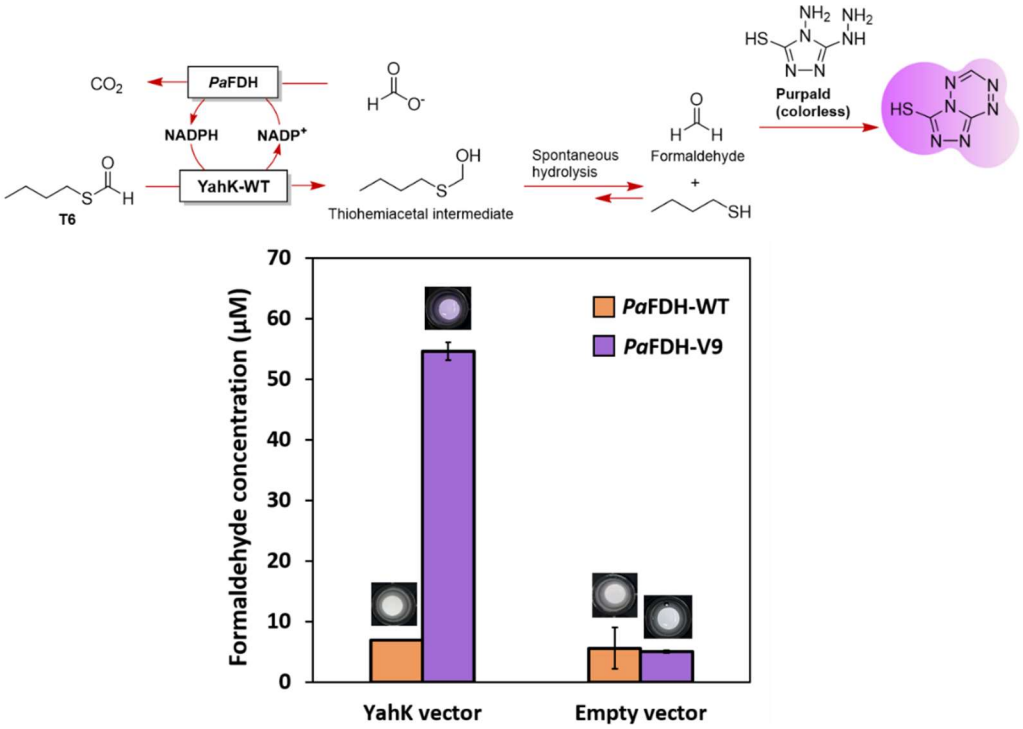


Figure S14. Cofactor regeneration tests by *Pa*FDH-WT and V9 in YahK-WT assay. The results showed that *Pa*FDH-V9 could specifically regenerate NADPH for **T6** reduction catalyzed by YahK-WT.

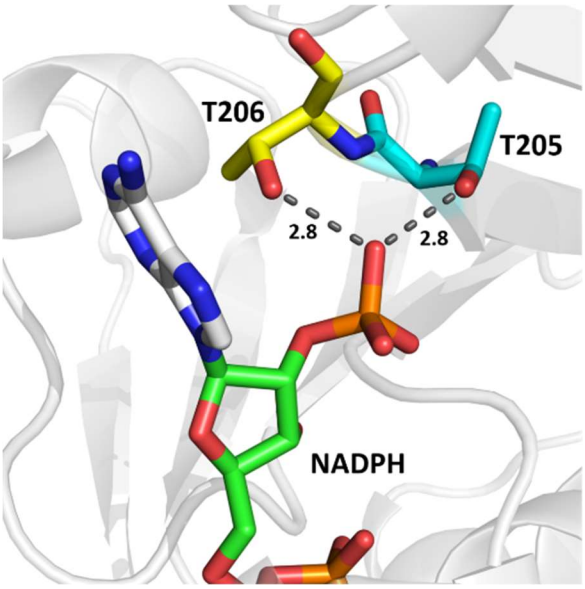
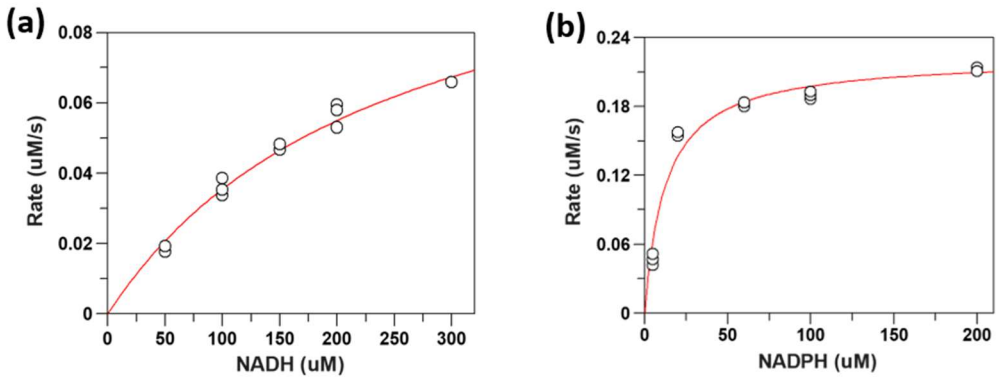
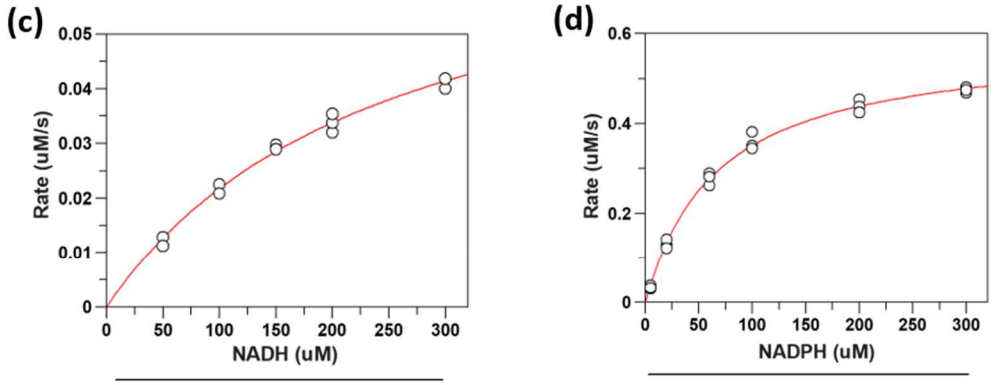


Figure S15. Active site of YahK with NADPH docking showing that T205 and T206 have hydrogen bonding interaction with the phosphate of NADPH.



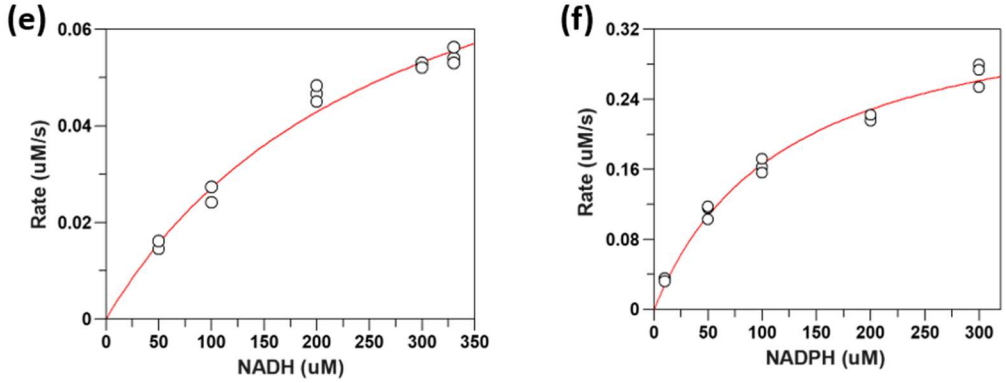
Parameter	Value	Std. Error
Vmax	0.1224	0.0087
Km	245.3673	30.8992

Parameter	Value	Std. Error
Vmax	0.2225	0.0069
Km	12.5008	1.8040



Parameter	Value	Std. Error
Vmax	0.0754	0.0039
Km	245.7143	22.5574

Parameter	Value	Std. Error
Vmax	0.5831	0.0117
Km	66.0626	3.9508



Parameter	Value	Std. Error
Vmax	0.1023	0.0081
Km	276.3693	40.8559

Parameter	Value	Std. Error
Vmax	0.3668	0.0165
Km	121.0289	13.0720

Figure S16. Apparent kinetic constants of YahK WT (a and b), E3 (c and d), and C8 (e and f) for NADH and NADPH.

References

1. A. Pick, W. Ott, T. Howe, J. Schmid and V. Sieber, *Journal of biotechnology*, 2014, **189**, 157–165.
2. O. Trott and A. J. Olson, *J Comput Chem*, 2010, **31**, 455–461.