Supplementary Information for

Engineering Cofactor Specificity of a Thermostable Phosphite Dehydrogenase for a Highly-efficient and Robust NADPH Regeneration System

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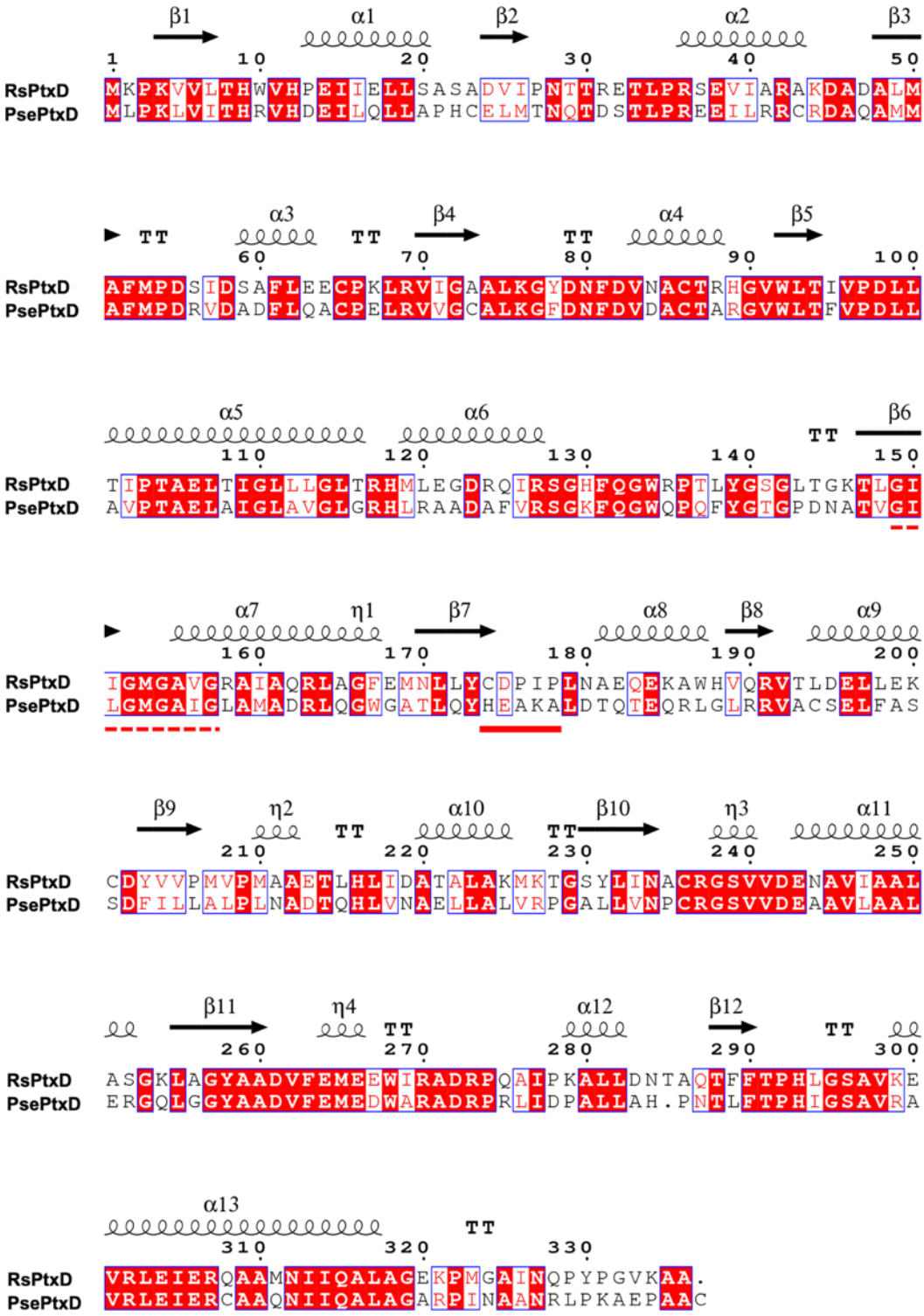
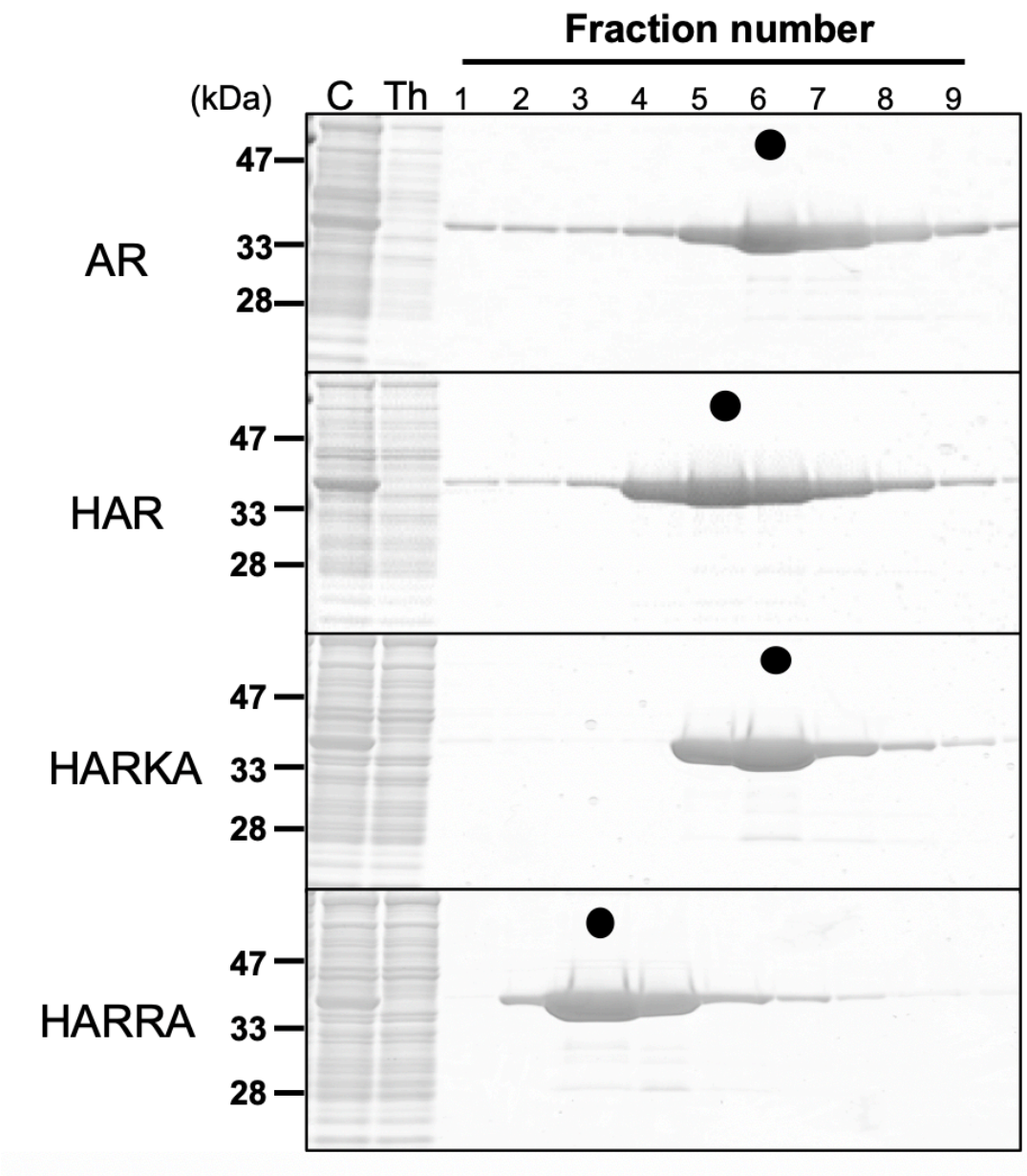
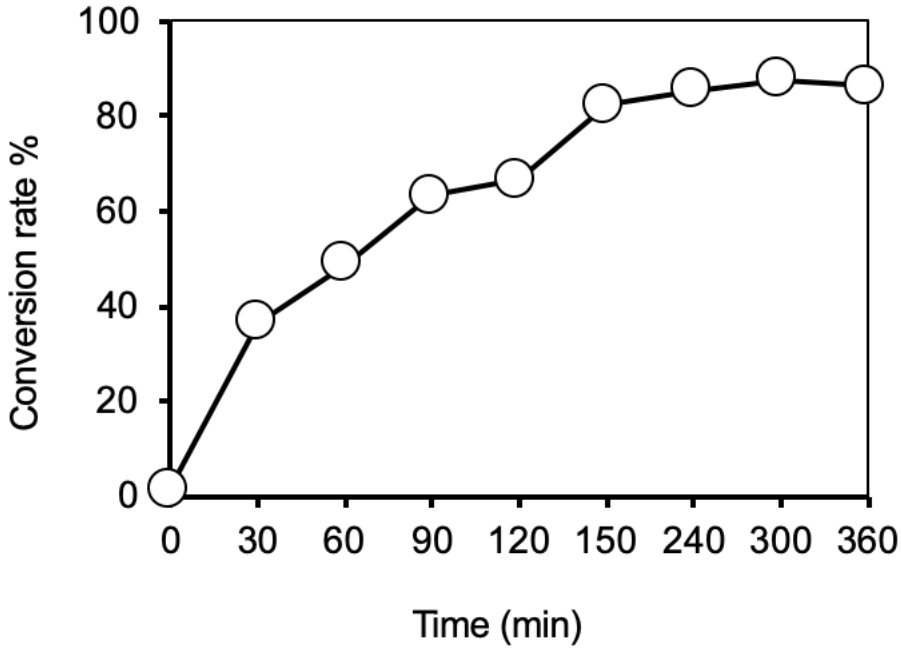


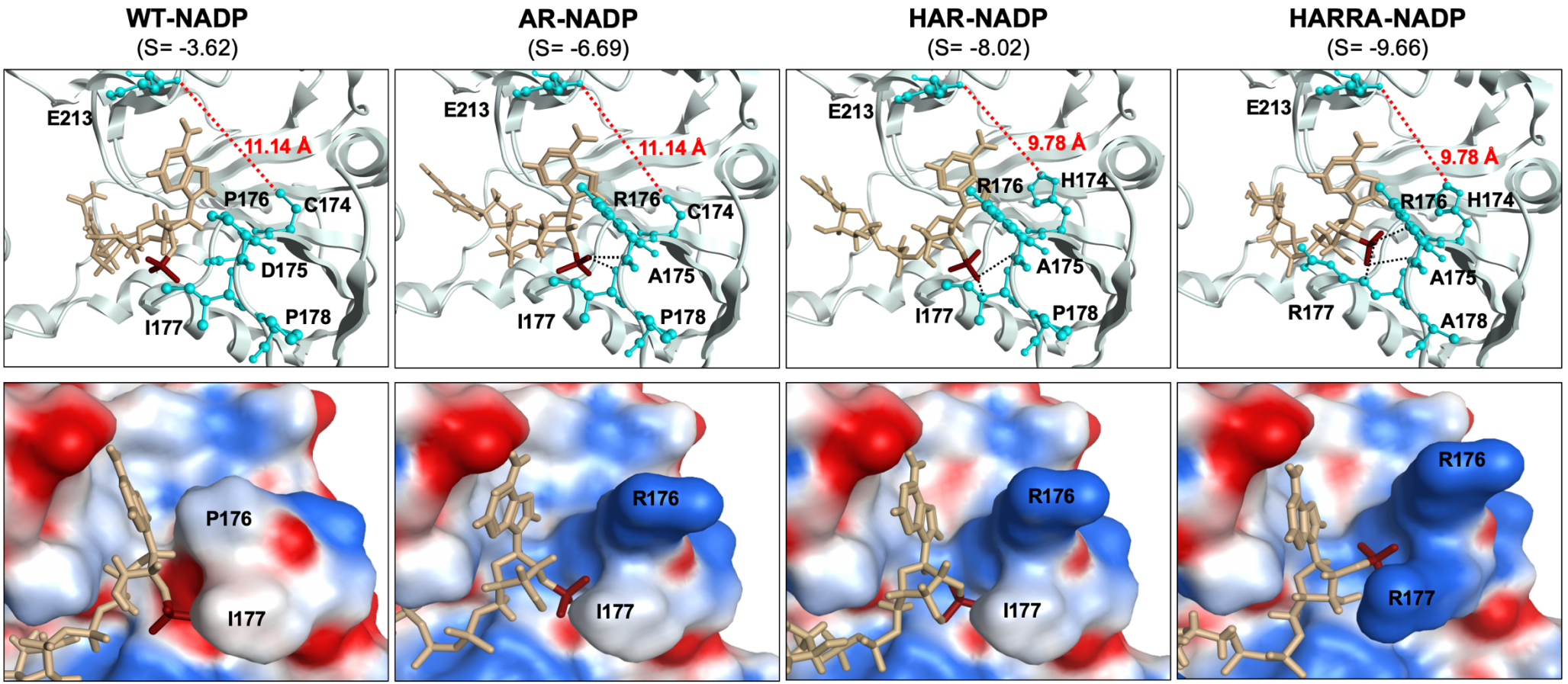
Supplementary Figure 1. Sequence alignment of phosphite dehydrogenases from *Ralstonia* sp. 4506 (RsPtxD) and *P. stutzeri* WM88 (PsePtxD). The alignment was generated using ESPrint 3.0 (Robert and Gouet 2014; http://espri pt.ibcp.fr/ESPri pt/cgi-bin/ESPri pt.cgi). The α-helixes (coiled lines) and β-sheets (solid arrows) are shown at the top of the alignment sequence. The GxxGxGxxG motif and the target site for the amino acid substitutions are shown below the alignment by a red dashed line and solid red line, respectively.



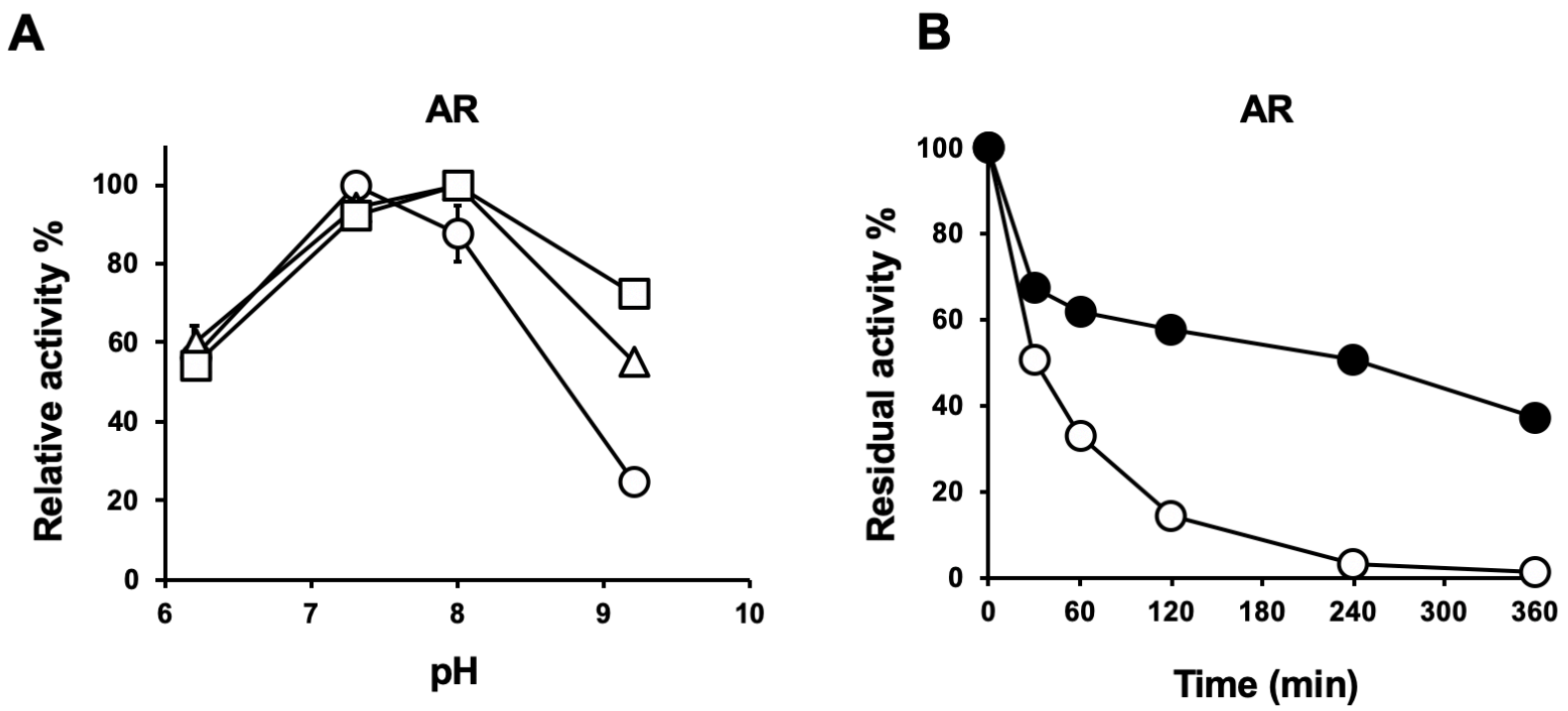
Supplementary Figure 2. Purification of the recombinant RsPtxD proteins using a nickel column. Indicated fractions by black dots were collected, buffer exchanged, and concentrated for the analysis. C: crude extract, Th: through fraction. AR, $RsPtxD_{AR}$. HAR, $RsPtxD_{HAR}$. HARKA, $RsPtxD_{HARKA}$. HARRA, $RsPtxD_{HARRA}$.



Supplementary Figure 3. Batch production of SA from 3-DHS with regeneration of NADPH using $RsPtxD_{HARRA}$ mutant. The reaction mixture contained 100 mM 3-DHS, 0.1 mM NADP, 150 mM phosphite, 40 μg mL^{-1} SDH, and 160 μg mL^{-1} $RsPtxD_{HARRA}$.



Supplementary Figure 4. Proposed model structures of the NAD-binding pocket of RsPtxD (WT), RsPtxD$_{AR}$ (AR), RsPtxD$_{HAR}$ (HAR), and RsPtxD$_{HARRA}$ (HARRA) (Top panels). The side chains of amino acid residues at 174, 175, 176, 177, 178, and 213 are shown in cyan. The protein structures were modeled by the Molecular Operating Environment software (MOE; Chemical Computing Group Inc., Montreal, Canada) using the PDB 6IH3 structure (Liu et al., ACS Catalysis 9: 1893-1887, 2019) as a template. The distance between the residues at 174 and 213 was shown with a red dotted line. The NAD moiety was shown in light brown and the 2'-phosphate group was shown in dark red. Note that the distances between the residues 174 and 213 in RsPtxD$_{HAR}$ and RsPtxD$_{HARRA}$ are shorter than that in RsPtxD and RsPtxD$_{AR}$. Hydrogen bonding between residues and cofactor were shown as black dotted lines. The numbers (S values) in the parenthesis are binding energy (kcal/mol) of NADP and RsPtxDs calculated by the GBVI/WSA dG scoring function of the MOE software. The electrostatic surface potentials of the proposed model structures of the NAD-binding pocket of RsPtxD and its mutants (Bottom panels). Positively charged region is indicated in blue and negatively charged region in red.



Supplementary Figure 5. pH dependent profile (A) and thermal stability (B) of $RsPtxD_{AR}$ mutant (AR). (A) PtxD activity was measured using 0.5 mM (circles), 1.0 mM (triangles), and 1.5 mM (squares) of NADP. The data are shown as means ± standard deviation obtained from three independent experiments. (B) Thermal inactivation of $RsPtxD_{AR}$ was performed with 0.5 mM (open circles) and 1.5 mM (closed circles). The data was representative of two independent experiments, with essentially the same results.

Supplementary Table 1. The oligonucleotides and plasmids used in this study

Primers	Sequence 5'-3'*
D175A/P176R_fw	TTGC**GCACGT**ATTCCGCTCAATGCCGAA
D175A/P176R_rv	GGAAT**ACGTGC**GCAATACCAGAGATTCA
C174H/D175A/P176R _fw	TGTAT**CAC**GCACGTATTCCGCTCAATG
C174H/D175A/P176R _rv	ACGTGC**GTG**ATACAAGAGATTCATTTC
C174H/D175A/P176R/ I177K/P178A_fw	GCACGT**AAAGCG**CTCAATGCCGAACAAG
C174H/D175A/P176R/ I177K/P178A_rv	TGAG**CGCTTT**ACGTGCGTGATACAAGAG
C174H/D175A/P176R/ I177R/P178A_fw	GCACGT**CGCGCG**CTCAATGCCGAACAAG
C174H/D175A/P176R/ I177R/P178A_rv	TGAG**CGCGCG**ACGTGCGTGATACAAGAG
Plasmids	**Descriptions**
RsptxD/pET21b	pET21b (Novagen) containing *RsptxD* (Hirota et al., 2012)
$RsptxD_{(D175A/P176R)}$/pET21b	pET21b containing a mutant *RsptxD* gene for $RsPtxD_{AR}$ expression
$RsptxD_{(C174H/D175A/P176R)}$/pET21b	pET21b containing a mutant *RsptxD* gene for $RsPtxD_{HAR}$ expression
$RsptxD_{(C174H/D175A/P176R/I177K/P178A)}$/pET21b	pET21b containing a mutant *RsptxD* gene for $RsPtxD_{HARKA}$ expression
$RsptxD_{(C174H/D175A/P176R/I177R/P178A)}$/pET21b	pET21b containing a mutant *RsptxD* gene for $RsPtxD_{HARRA}$ expression
sdh/pET11a	pET11a plasmid containing shikimate dehydrogenase gene from *T. thermophilus* (Yokoyama et al., 2000)

*Boldface nucleotide sequences indicate mutation positions.

Supplementary Table 2. Comparison of kinetic parameters of NADP-dependent enzymes previously reported

Enzyme	NADP				Assay conditions	References
	K_M (μM, NADP)	K_{cat} (min^{-1})	K_{cat}/K_M (μM^{-1} min^{-1})	K_M (μM, Pt or formate)		
$PsePtxD_{E175A/A176R}$	3.5 ± 0.5	114 ± 33.0	32.5	21 ± 3.0	25 °C, pH 7.25	Woodyer et al., 2003
$PsePtxD_{12x\text{-}A176R}$	5.5 ± 0.7	82 ± 4.0	14.9	36 ± 14.0	25 °C, pH 7.25	Johannes et al., 2007.
mut PseFDH	150 ± 25	150 ± 9.0	1.0	9000 ± 3000	30 °C, pH 7.0	Serov et al., 2002.
$BstFDH_{G146M/A287G}$	90 ± 0.0	529 ± 1.8	5.9	31700 ± 3700	30 °C, pH 7.0	Jiang et al., 2020.
PseFDH-V9	26 ± 1.0	221 ± 1.8	8.5	24000 ± 2400	30 °C, pH 7.0	Calzadiaz-Ramirez et al., 2020.