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4 **Directed evolution of thermotolerant malic enzyme**
5 **for improved malate production**
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7 Running title: Directed evolution of thermotolerant malic enzyme

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The directed evolution of the thermotolerant NADP(H)-dependent malic enzyme from *Thermococcus kodakarensis* was conducted to alter the cofactor preference of the enzyme from NADP(H) to NAD(H). The construction and screening of two generations of mutant libraries led to the isolation of a triple mutant that exhibited 6-fold higher $k_{\text{cat}}/K_{\text{m}}$ with NAD^+ than the wild type. We serendipitously found that, in addition to the change in the cofactor preference, the reaction specificity of the mutant enzyme was altered. The reductive carboxylation of pyruvate to malate catalyzed by the wild type enzyme is accompanied by HCO_3^- -independent reduction of pyruvate and gives lactate as a byproduct. The reaction specificity of the triple mutant was significantly shifted to malate production and the mutant gave a less amount of the byproduct than the wild type. When the triple mutant enzyme was used as a catalyst for pyruvate carboxylation with NADH, the enzyme gave 1.2 times higher concentration of malate than the wild type with NADPH. Single-point mutation analysis revealed that the substitution of Arg221 with Gly is responsible for the shift in reaction specificity. This finding may shed light on the catalytic mechanisms of malic enzymes and other related CO_2 - and/or HCO_3^- -fixing enzymes.

Key words: malic enzyme; directed evolution; cofactor preference; reaction specificity;

41 HCO_3^- fixation

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Thermophilic enzymes have been receiving increased attention as a promising tool for industrial biotechnology owing to their high operational stability, co-solvent compatibility, and low risk of contamination (1-3). Recently, we have developed a simple approach to design and construct an *in vitro* artificial metabolic pathway using thermophilic enzymes (4-6). In this approach, recombinant mesophiles (e.g., *Escherichia coli*) having heterologous thermophilic enzymes are used as biocatalytic modules to construct an *in vitro* pathway. The heat-treatment of recombinant cells results in the denaturation of indigenous enzymes and the elimination of undesired side reactions; therefore, biocatalytic modules that are as highly selective as purified enzymes can be prepared in one step. By rationally combining these modules, an *in vitro* synthetic pathway specialized for chemical manufacture can be constructed. Previously, we constructed a chimeric Embden-Meyerhof (EM) pathway using nine recombinant *E. coli* strains, each of which overproduces a thermophilic glycolytic enzyme (5). In this pathway, an enzyme couple of a phosphate-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and an ATP-generating phosphoglycerate kinase (PGK) of a bacterial/eukaryotic EM pathway was substituted with a non-phosphorylating GAPDH (GAPN) of the archaeal modified EM pathway. Consequently, the chimeric pathway with balanced ATP/ADP consumption and

regeneration rates could be constructed.

Malic enzymes (EC 1.1.1.39) can reversibly catalyze the NAD(P)H-dependent reductive carboxylation of pyruvate to malate. Ohno *et al.* reported the fixation of HCO_3^- to pyruvate using the malic enzyme from *Pseudomonas diminuta* coupled with glucose 6-phosphate dehydrogenase for NADH regeneration (7). The system is capable of converting 100 mM pyruvate to 38 mM malate within 24 h. A similar experiment using the *Brevundimonas diminuta* malic enzyme coupled with an electrode-mediated NADH regeneration system has resulted in a nearly 1.1 mmol of malate production from 12.5 mmol of pyruvate and 2.5 mmol of HCO_3^- (8). We have demonstrated that the integration of the thermotolerant NADPH-dependent malic enzyme from *Thermococcus kodakarensis* (TkME) (9) to the chimeric EM pathway enables the construction of a cofactor-balanced and HCO_3^- -fixing synthetic pathway, through which the direct conversion of glucose to malate can be achieved (6). However, the thermal degradation of the redox cofactors NADP^+ and NADPH tends to be a major obstacle to the long-term operation of the *in vitro* metabolic system because, unlike living biological systems, it is not equipped with the complete enzyme apparatus for the *de novo* synthesis of these cofactors. The decomposition of the redox cofactors leads to the decline in the catalytic abilities of GAPN and TkME. Consequently, the

conversion rate through the synthetic pathway gradually decreases. Furthermore, we found that the *Tk*ME-mediated malate production is accompanied by HCO_3^- -independent reduction of pyruvate to lactate (6). Owing to these facts, the final malate concentration is only modest (2.6 mM) and lactate was produced as the byproduct (6).

One of the possible approaches to achieve a long-term operation of the *in vitro* system and to obtain a higher titer of malate is to alter the cofactor preference of the NADPH-dependent malic enzyme from NADP(H) to NAD(H), because the thermal stabilities of NADP^+ and NADPH are significantly lower than those of NAD^+ and NADH, particularly under neutral and alkaline conditions (10) (Figure S1). We herein report the alteration of the cofactor preference of *Tk*ME, the bottleneck enzyme in the synthetic pathway for malate production, by a directed evolution approach. We serendipitously find that, in addition to the change in the cofactor preference, the reaction specificity of the mutant enzyme is significantly shifted to malate production.

MATERIALS AND METHODS

Plasmid construction *Escherichia coli* JM109 was used as the host strain for DNA manipulation. The gene coding for *Tk*ME was amplified by polymerase chain reaction

(PCR) from the chromosomal DNA of *T. kodakarensis* KOD1 using a primer set of ME-F and ME-R (Table 1). The amplicon was digested with *SacI* and *HindIII* and inserted into the corresponding restriction sites of pUC18 (Takara Bio, Ohtsu, Japan). The resulting plasmid was designated as pUC-ME. Expression vectors encoding a hexahistidine (His6)-tagged *TkME* and mutant enzymes were constructed using pET-21a (Novagen, Madison, WI, USA). Genes were PCR-amplified with a primer pair of His_ME-F and His_ME-R (Table 1), digested with *NdeI* and *HindIII*, and then inserted to the corresponding restriction sites of pET-21a. The resulting plasmids were transformed in *E. coli* Rosetta2 (DE3) pLysS (Novagen). The site-directed mutagenesis of *TkME* was conducted using a PrimeSTAR mutagenesis kit (Takara Bio) and adequate sets of oligonucleotide primers listed in Table 1.

Construction of mutant library Error-prone PCR was conducted using pUC-ME as the template. GoTaq DNA polymerase (Promega, Madison, WI, USA) and M13 primers (Table 1) were used for the amplification according to the manufacturer's instruction. Additionally, 0.1 mM MnCl₂ was added to the PCR mixture to increase error rate. Under the experimental conditions, 1-4 nucleotides of the gene were mutated on average ($n=4$). Amplified DNAs were introduced into the *SacI/HindIII*

restriction sites of pUC18 and then transformed in *E. coli* JM109 (Takara Bio). Transformants harboring the correct insertion were selected through the blue-white screening on a Luria-Bertani (LB) agar medium supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin, 100 $\mu\text{g ml}^{-1}$ 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal), and 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG).

High-throughput screening A colorimetric high-throughput assay was employed for the first-round screening of the mutant library. Transformants were cultivated in a 96-deep-well plate containing 500 μl per well of LB medium supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin and 0.2 mM IPTG. Cells were cultivated at 37°C for 15 h with orbital shaking, harvested by centrifugation (800 $\times g$, 10 min), and then resuspended in 50 mM Tris-HCl (pH 8.0). Cell concentration was adjusted to approximately 60 and 20 mg wet cells ml^{-1} for the screening of the first- and second-generation libraries, respectively. The cell suspension was incubated at 70°C for 30 min and mixed with an equal volume of a mixture comprising 20 mM L-malate, 2 mM NAD^+ , 10 mM MnCl_2 , 12 μM 1-methoxy-5-methylphenazinium methyl sulfate (1-methoxy-PMS, Dojindo, Kumamoto, Japan), 300 μM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Dojindo), and 50 mM Tris-HCl (pH 8.0). After incubation at room temperature for 1 h,

the accumulation of formazan crystals, which is formed by the reduction of MTT, was visually examined.

Enzyme preparation *E. coli* having COOH-terminal His6-tagged malic enzymes were cultivated in a 500-ml Erlenmeyer flask containing 200 ml of LB medium supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin and 30 $\mu\text{g ml}^{-1}$ chloramphenicol. Cells were cultivated at 37°C for 15 h with orbital shaking. Gene expression was induced by adding 0.2 mM IPTG at the late log phase. Cells were harvested by centrifugation, resuspended in 50 mM Tris-HCl (pH 8.0) containing 20 mM imidazole and 500 mM NaCl, and then disrupted with a UD-201 ultrasonicator (Kubota, Osaka, Japan). The crude lysate was heated at 70°C for 30 min. Cell debris and denatured proteins were removed by centrifugation at 12,000 $\times g$ and 4 °C for 10 min. The enzymes were homogenously purified by eluting the heat-treated lysates of recombinant cells through a HisTrap HP column (GE Healthcare, Piscataway, NJ) with a linear gradient of imidazol. The purified enzymes were dialyzed against 50 mM Tris-HCl (pH 8.0). Protein concentration was measured with the Bio-Rad assay system (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard.

Enzyme assay Malate decarboxylating activity was spectrophotometrically determined by monitoring the concomitant reduction of NAD⁺. The standard reaction mixture was composed of 50 mM Tris-HCl (pH 8.0), 5 mM MnCl₂, 5 mM NAD⁺, and an appropriate amount of enzyme. Alternatively, 0.5 mM NADP⁺ was used instead of NAD⁺ when needed. The mixture was preincubated at 70°C for 2 min, and then the reaction was initiated by adding L-malate at a final concentration of 30 mM. The absorbance at 340 nm was monitored using a UV-VIS spectrophotometer (Model UV-2450, Shimadzu, Kyoto, Japan). Molar extinction coefficients of 6200 and 6300 M⁻¹ cm⁻¹ for NADH and NADPH, respectively, were used to calculate the reaction rate.

Malate production The reaction mixture (1 ml) contained 500 mM HEPES-NaOH (pH 7.0), 5 mM NH₄Cl, 0.5 mM MnCl₂, 30 mM pyruvate, 30 mM glucose, 85 mM NaHCO₃, 2 µg of *TkME*, 2 U of glucose 1-dehydrogenase (GDH, Thermostable Enzyme Laboratory, Kobe, Japan), and 1 mM NADH or NADPH. The reaction mixture was stirred in a sealed container (φ15 × 50 mm) kept at 50°C, and the headspace of the container was equilibrated with CO₂ gas for 5 min before the addition of the substrates (pyruvate and glucose). After the reaction at 50°C, the mixture was ultrafiltered using Amicon 3K (Millipore, Bedford, MA) and subjected to

high-performance liquid chromatography (HPLC), performed as described elsewhere (6).

RESULTS

Screening of mutant library In total, 1016 transformants were screened with the MTT colorimetric assay, and the positive clones were further evaluated by determining their malate decarboxylating activities under standard assay conditions. Consequently,

16 transformants were found to exhibit higher specific activities with NAD^+ than that of the transformant having the wild type *TkME* (data not shown). Among them, the transformant exhibiting the highest activity with NAD^+ was selected for further study.

Sequence analysis of the encoding gene revealed that there were three substitutions at nucleotide positions of 661 (AGG→GGG, resulting in an amino acid substitution of R221G), 683 (AAG→AGG, resulting in K228R), and 1179 (GTC→GTT, resulting in a synonymous mutation of V393V). The plasmid encoding the R221G/K228R mutant

of *TkME* was then used as the template for the second round of error-prone PCR. After screening the second-generation library, which consisted of 1384 mutants, the triple mutant having an additional amino acid substitutions of I310V, which is caused by a nucleotide substitution at a position of 928 (ATA→GTA), was found to show the

highest activity with NAD^+ . For the determination of the specific activities of *TkME* and the mutant enzymes, these enzymes were fused with a His6 tag on their COOH terminals and homogeneously purified by Ni-affinity column chromatography. The specific malate decarboxylating activities of R221G/K228R and R221G/K228R/I310V enzymes with NAD^+ were 1.6 and 3.9 times higher than that of the wild type, respectively (Table 2). No significant change was observed in the thermal stability of the wild type and mutant enzymes (data not shown).

Kinetic analysis

Since both the oxidative carboxylation of pyruvate ($\text{pyruvate} + \text{HCO}_3^- + \text{NAD(P)H} + 2 \text{H}^+ \rightarrow \text{malate} + \text{NAD(P)}^+ + \text{H}_2\text{O}$; $\Delta G^{0'} = +7.3 \text{ kJ mol}^{-1}$) and the lactate oxidation ($\text{lactate} + \text{NAD}^+ \rightarrow \text{pyruvate} + \text{NADH} + \text{H}^+$; $\Delta G^{0'} = +14.8 \text{ kJ mol}^{-1}$) catalyzed by *TkME* are thermodynamically unfavorable (11, 12), these reactions hardly proceed under the standard assay conditions.

Therefore, the kinetic parameters of the enzymes were obtained from their specific malate decarboxylating activities using various concentrations of NAD^+ and NADP^+ (Table 2). Although the K_m of the R221G/K228R mutant for NAD^+ was less than half that of the wild type, that of the mutant for NADP^+ was 30 times higher than that of the wild type. Similarly, more than 1.5-fold increase was observed in the k_{cat} of the mutant with NAD^+ although that

with NADP⁺ was markedly lowered. These facts indicate that the increased specific activity of the R221G/K228R mutant with NAD⁺ was attributed to the shift in the cofactor preference of the enzyme to NAD⁺. By contrast, the increased specific activity of the triple mutant (R221G/K228R/I310V) was more likely due to the improvement in the catalytic ability of the enzyme rather than to the shift in the cofactor preference. In fact, although the R221G/K228R mutant exhibited a significantly lower specific activity with NADP⁺ than the wild type, the additional mutation of I310V restored the specific activity regardless of the cofactor used.

To further investigate the impact of each mutation (R221G, K228R, or I310V) on cofactor preference, site-directed mutations were introduced into the His6-tagged *TkME*. Among the resulting single-point mutants, only the K228R mutant showed a significantly low K_m for NAD⁺. The other two mutations (R221G and I310V) mainly affected k_{cat} but not the affinity to NAD⁺. Although the single-point mutation of R221G had no apparent effect on the K_m for NAD⁺, our observation that the K_m of the R221G/K228R mutant for NAD⁺ was significantly lower than that of the K228R mutant implied that these two mutations had a synergistic effect on the affinity of the enzyme to NAD⁺.

Malate production

The malate production assay was carried out by coupling the wild type or triple mutant of *TkME* with the thermotolerant GDH, which can accept both NAD^+ and NADP^+ , to regenerate the redox cofactor. Furthermore, the oxidation of glucose to gluconolactone catalyzed by GDH ($\text{glucose} + \text{NAD(P)}^+ \rightarrow \text{gluconolactone} + \text{NAD(P)H} + \text{H}^+$; $\Delta G^{0'} = -4.0 \text{ kJ mol}^{-1}$) gives a negative free energy change and can shift the overall chemical equilibrium to the direction of malate production. Previously, we demonstrated that *TkME* utilizes HCO_3^- but not dissolved CO_2 as the active carboxylation species for pyruvate carboxylation (6). Moreover, the reductive carboxylation of pyruvate catalyzed by *TkME* is accompanied by lactate formation (HCO_3^- -independent reduction of pyruvate) and carboxylation rate is dependent on HCO_3^- concentration in the solution phase (6). At certain temperatures, HCO_3^- concentration is dependent on pH and the partial pressure of CO_2 . Thus, a relatively high concentration of the buffer was employed and the headspace of the reaction vessel was equilibrated with CO_2 gas to maintain a certain HCO_3^- concentration. When the R221G/K228R/I310V mutant was used with NADH, the mutant gave 1.2 and 2.7 times higher malate concentration than the wild type with NADPH and NADH, respectively (Fig. 1). These results can be partly explained by the alteration of the cofactor preference of the mutant enzyme, since the half-life of NADH is approximately

241 1.3 times longer than that of NADPH under our experimental conditions at 50°C (Figure
242 S1). However, the K_m of the triple mutant for NAD^+ remained 190 times higher than
243 that of the wild type for NADP^+ . This indicates that the wild type can have a
244 comparatively higher catalytic ability at much lower NADPH concentrations than the
245 triple mutant used with NADH. On the other hand, our results demonstrated that, in
246 addition to the change in cofactor preference, the reaction specificity of the mutant
247 enzyme was significantly shifted to malate formation. Regardless of the enzyme used,
248 the malate production rate decreased time-dependently probably owing to HCO_3^-
249 consumption, whereas the lactate production rate was almost constant for 6 h.
250 Consequently, when the wild type was used, the final lactate concentration was higher
251 than the final malate concentration. By contrast, malate was mainly produced when
252 the R221G/K228R/I310V mutant was employed as the catalyst. The total
253 concentration of malate and lactate produced by the wild type with NADPH was 1.3
254 times higher than that by the triple mutant with NADH, implying that the improved
255 malate production by the triple mutant was more likely attributed to the redirection of
256 the reaction specificity rather than to the alteration of cofactor preference. When the
257 R221G/K228R/I310V mutant was used with NADPH, the mutant gave lower
258 concentration of malate than when used with NADH; however, the ratio of malate in the

total product concentration (malate + lactate) remained higher than that of lactate (Fig. 1C). The effect of each mutation point on the reaction specificity of *Tk*ME was further investigated using the single-point mutants. The results showed that K228R (ratio of malate in overall yield: 58%) and I310V (61%) mutants gave almost the same levels of malate as the wild type (66%) while the reaction specificity of R221G (78%) was significantly shifted to the direction of malate production. The triple mutant gave slightly higher ratio of malate (86%) than R221G, implying that the other two mutations of K228R and I310V might have a synergistic effect on the reaction specificity of *Tk*ME (Fig. 2).

DISCUSSION

The X-ray structural analysis of the pigeon (*Columba livia*) cytosolic malic enzyme (*C/ME*) cocrystalized with NADP⁺ has revealed that the 2'-phosphate group of NADP⁺ interacts with Ser346 and the side chain amino group of Lys362 of the enzyme (13). In *C/ME*, the substitution of Lys362 with Ala leads to a 70-fold increase in the apparent K_m for NADP⁺, without significantly changing the affinity of the enzyme to malate and Mn²⁺ (14). Similarly, the substitution of Lys362 of the human cytosolic malic enzyme with Gln leads to a 144-fold increase in the K_m of the enzyme for NADP⁺ and a 1.5-fold

277 decrease in that for NAD⁺ (15). Amino acid sequence alignment analysis showed that
278 these Lys residues are highly conserved among *Tk*ME, *C*ME, and other
279 NADPH-dependent malic enzymes (Fig. 3), in spite of the fact that the similarity
280 between the whole amino acid sequences of *Tk*ME and the eukaryotic malic enzymes is
281 modest (< 20%). In this study, we confirmed that the substitution of the Lys228 of
282 *Tk*ME, which corresponds to the Lys362 of *C*ME, also led to a shift in cofactor
283 preference (Table 2). These observations strongly indicate that these Lys residues play
284 a crucial role in the interaction of malic enzymes with NADP(H).

285 Regardless of the cofactor used (NADP⁺ or NAD⁺), the substitution of the Arg221 of
286 *Tk*ME with Gly resulted in an increased k_{cat} of the enzyme (Table 2). This residue
287 corresponds to the Arg354 of *C*ME and is well conserved particularly in
288 NADP(H)-dependent malic enzymes (Fig. 3). The crystal structural analysis of *C*ME
289 suggested that the Arg354 forms an ion pair with Asp345 (13). These facts imply that
290 R221G mutation in *Tk*ME causes the partial disintegration of the cofactor binding site
291 and the loss of cofactor selectivity. The structural modeling analyses indicated that
292 Ile310, which was changed to Val in the triple mutant, is also located nearby the
293 putative cofactor binding pocket of *Tk*ME (Fig. 4). However, this residue is not
294 conserved in *C*ME and other malic enzymes with structural information in complex

with redox cofactors, and likely is not involved in the direct interaction with the cofactors. This assumption is also supported by the fact that increased k_{cat}/K_m of the I310V mutant was mainly attributed to the increased k_{cat} (Table 2).

Under physiological conditions, malic enzymes are involved in lipogenesis (16) and energy production (17) through NAD(P)H production concomitant with malate decarboxylation. Therefore, most studies so far have been focused on the catalytic mechanisms of the oxidative decarboxylation of malate to pyruvate (18, 19). On the other hand, the mechanisms of malic-enzyme-mediated pyruvate carboxylation have rarely been studied, and, to our knowledge, the key amino acid residues involved in the interaction with HCO_3^- have not yet been identified. Recently, the utilization of carbon dioxide as a building block for synthetic chemistry has emerged as one of the most outstanding research topics for establishing independence from fossil reserves, and thus the applications of CO_2 - and/or HCO_3^- -fixing enzymes and microorganisms to the production of biofuels and other useful chemicals have been intensively studied (20-23). Our serendipitous finding of the possible key residue of *TkME* for pyruvate carboxylation may shed light on the catalytic mechanisms of malic enzymes and other related CO_2 - and/or HCO_3^- -fixing enzymes.

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Legends of Figures

FIG. 1 Malate productions by the wild type (A, B) and R221G/K228R/I310V mutant (C, D). Reactions were performed using either NADPH (A, C) or NADH (B, D) as a redox cofactor. After reaction for the indicated time periods, the concentrations of malate (closed square) and lactate (open square) were determined by HPLC.

FIG. 2 Reaction specificities of the wild type and mutant enzymes. Reactions were performed with 2 μ g each of the enzymes using NADH as the cofactor. After the reaction for 1 h, the ratios of malate (black bar) and lactate (gray bar) to the total product concentration (malate and lactate) were determined. The total product concentrations are shown in parenthesis.

FIG. 3 Alignment of amino acid sequences of malic enzymes near cofactor binding site. Sequences were aligned with the Genetyx program (ver. 11, GENETYX Co., Tokyo, Japan). Mutation points identified in this study are indicated by black stars.

FIG. 4 Structural model for the three-dimensional structure of the wild type (upper

panel) and R221G/K228R/I310V mutant (lower panel). The model was built by SWISS-MODEL-automated protein homology-modeling server (Swiss Institute of Bioinformatics) using the structure of malic enzyme derived from *Pyrococcus horikoshii* OT3 (pdb: 1WW8) as a template. These proteins share the amino acid sequence identity of 61%. The model was viewed and edited with PyMOL (www.pymol.org). The mutated residues in the triple mutant (green) and the corresponding residues in the wild type (orange) are shown as stick models, in which the nitrogen atoms are blue-colored.

412 TABLE 1 Oligonucleotide primers used in this study

Primer	Sequence (5' → 3')	Purpose
ME-F	TT <u>GAGCTC</u> _a GAATCCCCTCGAATTCCATAGGGACAAC	Construction of pUC-ME
ME-R	TTAAGCTT _b CTAGGGGGAAGTCCCTCTACCG	
His_ME-F	TT <u>CATATG</u> _c AATCCCCTCGAATTCCATAGGGA	Construction of the expression vectors encoding His6-tagged malic enzymes
His_ME-R	TTAAGCTT _b GGGGGAAGTCCCTCTACCGCTG	
M13-F	GTAAAACGACGGCCAGT	Error-prone PCR
M13-R	CAGGAAACAGCTATGAC	
R221G-F	GAGGGCGGCGAGGACCTCAACCCGTAC	Construction of the expression vector encoding R221G mutant
R221G-R	GTCCTCGCCGCCCTCGTGTATTATCCC	
K228R-F	AACCAGGTGAACAACGTGCTCGGCTTT	Construction of the expression vector encoding K228R mutant
K228R-R	TTCGCGACGGTACGGGTTGAGGTCCTC	
I310V-F	AACCAGGTGAACAACGTGCTCGGCTTT	Construction of the expression vector encoding I310V mutant
I310V-R	GTTGTTACCTGGTTCGGGAAGTCGCT	

413 ^a The *Sac*I restriction site is underlined.

414 ^bThe *Hind*III restriction site is underlined.

415 ^cThe *Nde*I restriction site is underlined.

416

417 TABLE 2 Specific activities and kinetic parameters of the wild type and mutant enzymes

Enzyme	Specific activity ^a		K_m ^b		k_{cat} ^b		k_{cat}/K_m	
	$(\mu\text{mol min}^{-1} \text{mg}^{-1})$		(mM)		(s^{-1})		$(\text{mM}^{-1} \text{s}^{-1})$	
	NAD ⁺	NADP ⁺	NAD ⁺	NADP ⁺	NAD ⁺	NADP ⁺	NAD ⁺	NADP ⁺
wild type	5.77 ± 0.31	38.4 ± 1.2	2.06 ± 0.03	0.008 ± 0.001	4.69 ± 0.33	31.1 ± 0.59	2.27	3760
R221G/K228R	9.32 ± 0.15	4.89 ± 0.28	0.96 ± 0.07	0.244 ± 0.007	7.44 ± 0.49	6.48 ± 0.25	7.72	27.5
R221G/K228R/I310V	22.9 ± 0.19	27.4 ± 2.4	1.54 ± 0.09	0.384 ± 0.06	21.3 ± 0.89	40.1 ± 6.3	13.8	104
R221G	13.0 ± 0.41	52.1 ± 3.3	2.29 ± 0.28	0.025 ± 0.007	12.7 ± 0.32	42.6 ± 2.9	5.57	1700
K228R	4.47 ± 0.09	3.57 ± 0.08	1.28 ± 0.09	0.019 ± 0.004	3.37 ± 0.20	2.97 ± 0.20	2.64	156
I310V	22.7 ± 0.38	5.23 ± 0.08	6.55 ± 0.25	0.021 ± 0.003	37.7 ± 1.5	43.4 ± 1.8	5.76	2070

418 ^a Enzyme assays were performed with either 5 mM NAD⁺ or 0.5 mM NADP⁺.

419 ^b The kinetic parameters were obtained by Lineweaver-Burk analysis.

Morimoto et al., FIG. 1