Journal of Bioscience and Bioengineering
VOL. 117 No. 2, 147–152, 2014

www.elsevier.com/locate/jbiosc

Directed evolution of thermotolerant malic enzyme for improved malate production

Yumi Morimoto,[1] Kohsuke Honda,[1,2,*] Xiaoting Ye,[1] Kenji Okano,[1] and Hisao Ohtake[1]

Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan[1] and PRESTO, Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan[2]

Received 4 June 2013; accepted 11 July 2013
Available online 8 August 2013

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_{cat}/K_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; HCO_3^- fixation]

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* (*Tk*ME) (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 *Tk*ME. 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

* Corresponding author at: Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 7438; fax: +81 6 6879 7439.
E-mail address: honda@bio.eng.osaka-u.ac.jp (K. Honda).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2013.07.005

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) (Fig. 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 *E. 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 *Sac*I and *Hin*dIII 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 *Tk*ME 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 *Nde*I and *Hin*dIII, 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 *Tk*ME 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 $MnCl_2$ 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 *Sac*I/*Hin*dIII 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 μg ml^{-1} ampicillin, 100 μg 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 μg ml^{-1} ampicillin and 0.2 mM IPTG. Cells were cultivated at 37°C for 15 h with orbital shaking, harvested by centrifugation (800 ×*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 μg ml^{-1} ampicillin and 30 μg 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 ×*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, USA) 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 $MnCl_2$, 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 NH_4Cl, 0.5 mM $MnCl_2$, 30 mM pyruvate, 30 mM glucose, 85 mM $NaHCO_3$, 2 μg of *Tk*ME, 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 (ϕ 15 × 50 mm) kept at 50°C, and the headspace of the container was equilibrated with CO_2 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, USA) 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 *Tk*ME (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 *Tk*ME was then used as the template for the second round of error-prone PCR. After screening

TABLE 1. Oligonucleotide primers used in this study.

Primer	Sequence (5′ → 3′)	Purpose
ME-F	TTGAGCTC[a]GAATCCCCTCGAATTCCATAGGGACAAC	Construction of pUC-ME
ME-R	TTAAGCTT[b]CTAGGGGGAACTCCCTCTACCG	
His_ME-F	TTCATATG[c]AATCCCCTCGAATTCCATAGGGA	Construction of the expression vectors encoding His6-tagged malic enzymes
His_ME-R	TTAAGCTT[b]GGGGGAACTCCCTCTACCGCTG	
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	GTTGTTCACCTGGTTCGGGAAGTCGCT	

[a] The *Sac*I restriction site is underlined.
[b] The *Hin*dIII restriction site is underlined.
[c] The *Nde*I restriction site is underlined.