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Site-saturation mutagenesis of formate dehydrogenase from *Candida bodinii* creating effective NADP$^+$-dependent FDH enzymes

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ABSTRACT

The analysis of previous reported results envisioned that residues Asp195, Tyr196 and Gln197 of formate dehydrogenase from *Candida bodinii* (CboFDH) might play critical roles in determining the enzyme's cofactor specificity. With the aim to develop novel NADP$^+$-dependent formate dehydrogenase enzymes, simultaneous site-saturation mutagenesis of residues Asp195 and Tyr196 of CboFDH coupled with screening resulted in two mutant enzymes, D195Q/Y196R and D195S/Y196P, which showed significant NADP$^+$ specificity. The overall catalytic efficiencies (k_{cat}/K_m) toward NADP$^+$ were 1.14×10^4 and 2.9×10^3 M^{-1} s^{-1}, respectively, which are higher than the reported mutant CboFDHs obtained by sequential mutagenesis. The ratio of catalytic efficiencies $(k_{cat}/K_m)^{NADP+}/(k_{cat}/K_m)^{NAD+}$ of D195Q/Y196R and D195S/Y196P were 2.1 and 0.2, respectively. Mutation of residue Gln197 of D195Q/Y196R to Asn further increased the enzyme's overall catalytic efficiencies (k_{cat}/K_m) toward NADP$^+$ to 29.1×10^3 M^{-1} s^{-1}, with $(k_{cat}/K_m)^{NADP+}/(k_{cat}/K_m)^{NAD+}$ being 17.1, which are much higher than the reported data for a mutant enzyme of formate dehydrogenase from *Pseudomanas* sp. 101 (PseFDH). This study demonstrates that residues 195, 196 and 197 really play critical roles in determining the enzyme's cofactor specificity.

1. Introduction

Formate dehydrogenase (FDH, EC 1.2.1.2) catalyzes the oxidation of formate to carbon dioxide with concomitant reduction of NAD$^+$ to NADH. Since the reaction is almost irreversible and the gaseous carbon dioxide can be easily removed from the reaction system, FDH is deemed as a very valuable catalyst for the cofactor (NADH) regeneration in organic synthesis employing other dehydrogenases, in which NADH is required [1,2]. For example, a yeast FDH from *Candida bodinii* (CboFDH) has been used to regenerate cofactor NADH in the production of *tert*-L-leucine at industrial scale [3]. Recently, dehydrogenases have been widely explored for the application to the synthesis of important pharmaceutical and agricultural chemicals [4,5]. However, many synthetically useful dehydrogenases utilize NADPH as the cofactor [6–8]. Since wild-type formate dehydrogenases are highly specific enzymes for NAD$^+$ [9], protein engineering of formate dehydrogenases with aim to switch their cofactor specificity to NADP$^+$ is of great practical importance [10–13]. In this context, single mutation of Asp195 residue of the yeast *Candida methylica* FDH (CmeFDH) to serine (Asp195Ser) removes the absolute requirement for NAD$^+$ over NADP$^+$, but it still have 40 times higher catalytic efficiencies for NAD$^+$ than for NADP$^+$ [12]. Serov et al. have introduced double mutations Asp196Ala and Tyr197Arg into a formate dehydrogenase from *Saccharomyces cerevisiae* (SceFDH), which shifts the coenzyme preference of SceFDH from NAD$^+$ to NADP$^+$ with the ratio of catalytic efficiencies $(k_{cat}/K_m)^{NADP+}/(k_{cat}/K_m)^{NAD+}$ being 1.5 at 0.50 M formate, respectively [11]. In addition, mutation of formate dehydrogenase from *Pseudomanas* sp. 101 (PseFDH) has been reported to relax the coenzyme specificity from NAD$^+$ to NADP$^+$ [13], although no details about amino acid substitutions have been described.

It has been known that the cofactor binding domain of formate dehydrogenases shares same motif G/AXGXXG which belongs to a classic Rossmann fold [6,14]. At the downstream 18 amino acids from the end (Gly) of the motif, the residue Asp is highly conserved in yeast FDHs (for example, Asp195 for FDHs from *Candida methylica* and *Candida bodinii*, and Asp196 for a FDH from *Saccharomyces cerevisiae*) [11,12,15]. In bacterial and plant FDHs the conserved Asp is the 17th residue downstream from the end of the motif (for example, Asp 221 for a formate dehydrogenases from bacterium *Pseudomonas* sp. 101) [9]. The known X-ray structures of formate dehydrogenases show that the conserved Asp residue interacts with

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the 2′- and 3′-OH groups of adenosine ribose, which has been proposed to be major determinant of the strict cofactor specificity for NAD^+ [16–19]. This is confirmed by the finding that single mutation of Asp195 residue of the yeast *Candida methylica* FDH (CmeFDH) to serine (Asp195Ser) removed the absolute requirement for NAD^+ over $NADP^+$ [12]. Based on affinity labeling and molecular modeling studies, Labrou et al. predicted that the best approach to alter the cofactor specificity of formate dehydrogenase from *Candida boidinii* (CboFDH) to $NADP^+$ was to mutate residues Asp195 and Gln197 [15,20]. They argued that these mutations might create a new cavity necessary for the accommodation of the bulky phosphate group and to eliminate the repulsion of Asp195 by the new phosphate group of $NADP^+$, since the binding groove for NAD^+, especially around the adenine moiety and adjacent ribose unit, was narrow for $NADP^+$. The crystal structures of CboFDH mutants (K47E and K328V) show that the adenine ring is likely to interact with His232 and Tyr196, and a hydrophobic cluster is formed by Tyr194 and Tyr196 [17]. This analysis suggests that residues Asp195, Tyr196 and Gln197 may be very important in controlling the enzyme's cofactor specificity. Fig. 1 shows the NAD^+ binding site of CboFDH obtained by docking NAD^+ in the crystal structures of CboFDH mutants (K47E and K328V). Very recently, Andreadeli et al. have reported that mutation of residue Asp195 of CboFDH and subsequent mutation at residue Tyr196 generated a few mutant FDH enzymes with $NADP^+$ specificity [21]. In present study, the site-saturation mutagenesis of residues Asp195 and Tyr196 of CboFDH were performed simultaneously, and screening of the double-mutant library resulted in two mutant enzymes with significant $NADP^+$ specificity. Introduction of mutation at residue Gln197 of one of the mutant enzymes further increased the enzyme's $NADP^+$ specificity, indicating the critical roles of these residues in determining the enzyme's cofactor specificity.

2. Experimental

2.1. Strains and chemicals

The host strains BL21 (DE3), Rosetta2 (DE3) and Rosetta2 (DE3) pLysS, and the vectors pET15b and pET22b were purchased from Novagen. Polymerase pfx amplifying kit was from Invitrogen. The plasmid extracting kit and QIAquick spin column 50 were from QIAgen. All endorestriction enzymes including NdeI, EcoRV, DpnI and XhoI were from New England BioLabs Inc. Cofactor NAD^+ and $NADP^+$ were from Biocatalytics. Sodium formate, K_2HPO_4, KH_2PO_4, and NaCl were analytical grade and used as received. Primers used in the mutant library construction were synthesized by Integrated DNA Technologies.

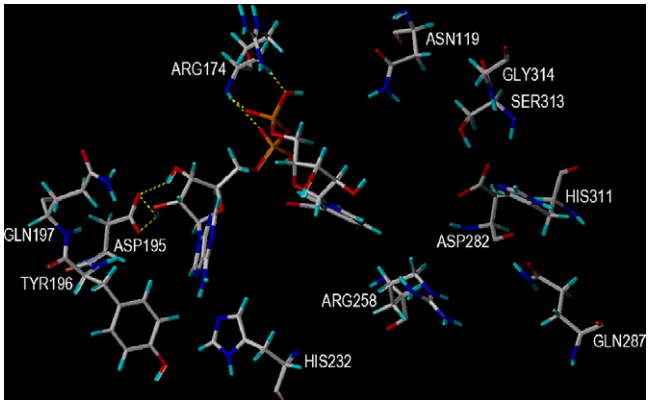

Fig. 1. The binding comformation of NAD^+ in CboFDH (PDB ID: 2FSS) obtained by docking with Sybyl7.3 of Tripos.

2.2. Construction of first generation mutant library

The mutant D195/Y196 DNA library was constructed by PCR using pfx DNA polymerase. The plasmid of recombinant wild-type CboFDH, which was constructed by following similar procedure as previously reported [20], was used as template. Primers which introduced two mutations at positions D195 and Y196 were as follows: forward primer FDH_D195Y196F1: 5′-G AAA GAA CTG CTG TAC TAC NNK NNK CAG GCT CTG CCG AAA GAAG-3′; backward primer FDH_D195Y196R1: 5′-C TTC TTT CGG CAG AGC CTG MNN MNN GTA GTA CAG CAG TTC TTT C-3′. The total PCR volume was 50 μl including 0.3 mM dNTP, 1 mM $MgSO_4$, 0.3 pmol primers, 5.0 μl 10× pfx polymerase amplifying buffer, 150 ng template, 34 μl of fresh sterile DI water, and 2.5 U pfx DNA polymerase. The template was denatured at 94 °C for 2 min followed by 30 cycles as follows: 94 °C for 15 s, 55 °C for 45 s, and 68 °C for 6.8 min. Then, it was extended for another 10 min at 68 °C. The remained templates in the PCR products were digested by 30 U of endorestriction enzyme DpnI at 37 °C for 2 h.

The DpnI digested PCR product was transformed into host Rosetta2 (DE3) using electro-transformer (Bio-Rad). Two microliters of PCR products and 50 μl of electro-competent cell Rosetta2 (DE3) with the efficiency of 109 were used in the transformation. The voltage used in transformation was 1.8 kV under bacterial mode. All materials used in the process, such as DNA, SOC media, pipette tips, and cuvettes were ice-cooled for 10 min before use. The transformant was immediately transferred to 500 μl of SOC media and cultured at 37 °C for 1 h. The culture was centrifuged at 5000 rpm for 5 min and supernatant was decanted. The pellets were resuspended in 200 μl of fresh LB media containing antibiotics Amp and Cam, and the resulting mixture was spread onto fresh LB agar plate containing 100 μg ml^{-1} of Amp and 34 μg ml^{-1} of Cam. The plates were cultured at 37 °C for at least 12 h until visible colonies grew. To obtain 99% coverage of all possibilities of two codons encoding double mutations in position D195/Y196, according to the calculation, the size of mutant library should be at least as big as 3072 colonies. 3300 colonies were picked and each was cultured in 130 μl fresh LB media contain Amp and Cam in 96-well plates. Meanwhile, eight colonies of wild-type CboFDH were picked and cultured at the middle column in each plate as positive control. The 96-well plates were incubated at 37 °C for 12 h with vigorous shaking at speed of 250 rpm as master library, which was duplicated into 160 μl of fresh LB media containing Amp and Cam to make screening library. The screening library plates were cultured at 37 °C for 16 h, and then centrifuged at 4000 rpm for 30 min and supernatant was decanted. The pellets were used for screening.

2.3. Construction of second generation mutant library

A second generation library was constructed based on mutant B7 obtained from the screening of first generation library. Single mutation at position Q197 was introduced using the following primers: FDHMUT_B7_Q197X_Q195R196_F1: 5-CTG CTG TAC TAC CAG CGT NNK GCT CTG CCG AAA GAA GCA-3; FDHMUT_B7_Q197X)Q195R196_R1: 5-TGC TTC TTT CGG CAG AGC MNN ACG CTG GTA GTA CAG CAG-3. The plasmid of FDH mutant B7 was used as template. The procedures were same as described in the construction of first generation library.

2.4. Library screening

The pellets in 96 well plates of screening library were resuspended with 20 μl of TES buffer. Then, 10 μl of TES-lysozyme buffer including 100 U lysozyme was added into each well and incubated at room temperature for 40 min to break cell wall. For each well, the lysate was diluted with 90 μl of Potassium phosphate buffer

(pH 7.5) followed by centrifuging at 4000 rpm at 4 °C for 30 min. The cell debris precipitate was removed and cell free extract was screened in the presence of cofactor $NADP^+$ and substrate sodium formate. The activities were measured at room temperature in a 96-well plate at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) using a Spectra Max M2 microplate reader. The total screening volume was 200 μl containing 0.5 mM of $NADP^+$ (40 μl of 2.5 mM stock) and 100 mM of sodium formate (40 μl of 0.5 M stock). The reading interval was 50 s as well as the time span was 5 min.

2.5. Cloning of His-tagged wild-type CboFDH and mutants

A six-histidine tag was introduced using pET22b vector. Five micrograms of pET22b vector was sequentially digested by 40 U of endo-restriction enzyme NdeI/XhoI, and then purified using 1% agarose gel and concentrated by QIAquick spin columns 50. The gene fragment of His-tagged CboFDH wild-type and mutants were amplified by PCR using plasmids of CboFDH wild-type and mutants as template. The primer pair used in reactions was as follows. SMUHUA005FDH_NdeI F1: 5′-CAC ATA TGG GCA AAA TCG TTC TGG TTC-′3; FDHSMU005_XhoI R1: 5′-GGA TCT CGA GTT TTT TGT CCT GTT TGC-3′. The PCR reaction mixture (100 μl) contained 0.3 mM of dNTP, 1 mM of $MgSO_4$, 30 pmol of forward and backward primers, 105 ng of DNA template, 10 μl of pfx amplifying buffer, and 2.5 U of pfx DNA polymerase. The template was denatured at 94 °C for 2 min followed by 30 cycles: 94 °C for 15 s, at 55 °C for 50 s, and 68 °C for 70 s. Then, the PCR was extended for another 10 min at 68 °C. After being desalted using QIAquick spin columns 50, the PCR products were sequentially digested with 40 U of endo-restriction enzyme NdeI/XhoI, and then purified using 1% agarose gel and concentrated by QIAquick spin columns 50.

The concentration of purified vector pET22b, FDH wild-type, and FDH mutants were quantitated by agarose gel electrophoresis using 10 μl of 1.0 kB DNA ladder as DNA standard. The concentrations of samples (2 μl of vector pET22b, FDH wild-type and each mutant digested with NdeI/XhoI) were calculated based on the density of bands compared to DNA standard. The ligation was carried out using Fast-link DNA ligation kit (Epicentre Biotechnologies). The ligation reaction mixture contained 100 ng of vector, 81 ng of insert DNA fragment (FDH or its mutant gene), 1.5 μl of 10× Fast link ligation buffer, 1 μl of Fast link DNA ligase (2 U) and 1 mM of ATP, and fresh autoclaved DI water was added to make up total reaction volume to 15 μl. The resulting mixture was incubated at room temperature for 30 min and then transferred to 70 °C to deactivate DNA ligase for 15 min.

Transformation of ligation product was performed by electrotransformer. Before transformation, all materials including ligation products, cuvette, SOC media, and tips were pre-cooled for 10 min. Two microliters of ligation products were transformed into 50 μl of electro-competent cell EC100. The voltage in the transformation was 1.8 kV under bacterial mode. The transformants were immediately transferred to 500 μl of SOC media and cultured at 37 °C with vigorous shaking at the speed 250 rpm for 1 h. The cultures were centrifuged at 5000 rpm for 5 min. Supernatant was discarded and pellets were resuspended with 200 μl fresh LB media containing antibiotics 100 $\mu g\ ml^{-1}$ Amp. All transformants were spread onto fresh LB agar plate containing 100 $ng\ ml^{-1}$ of Amp. The plates were cultured at 37 °C for at least 12 h until visible colonies grew.

Twelve positive colonies of His-tagged wild-type FDH or each mutant were picked from LB agar plate and cultured into 2 ml of fresh LB media containing 100 $\mu g\ ml^{-1}$ Amp in 12 wells plate. The culture was incubated at 37 °C for 10 h, and 1.5 ml of the resulting culture was transferred into enpendorf tubes. The tubes were centrifuged at 8000 rpm for 10 min and the supernatants were discarded. The plasmids in pellets were extracted by methods described in the mini-preparation protocol. The obtained plasmids were conformed by double-digestion with NdeI/XhoI and single digestion with EcoRV, respectively. Total reaction volume was 10 μl containing 1 μl enzyme digestion buffer (where NEB2 + BSA for double digestion NdeI/XhoI, and NEB3 + BSA for EcoRV), 0.3 μl of enzyme (6 U of each enzyme), and 2 μl of DNA solution. The digestion results were checked by 1% agarose gel.

2.6. Expression of His-tagged wild-type CboFDH and mutants

The plasmids of His-tagged wild-type and mutant FDHs were transformed into host Rosetta2 (DE3) pLysS, respectively. The expression of His-tagged FDHs was optimized. Under the optimal condition, one colony from each His-tagged FDH was picked and cultured in 50 ml fresh LB media at 37 °C for 10 h. Then 20 ml of the culture was transferred into 1 l fresh LB media containing 100 $\mu g\ ml^{-1}$ Amp and 34 $\mu g\ ml^{-1}$ Cam, which was incubated at 37 °C until OD at 595 nm reached 0.6. Thereafter, the culture was induced with 0.5 mM IPTG at 18 °C for 10 h. Subsequently, the cells were harvested by centrifuging at 8000 rpm for 20 min.

2.7. Purification of His-tagged wild-type CboFDH and mutants

The pellets of His-tagged FDH were resuspended into lysis buffer (50 mM NaH_2PO_4, 1 M NaCl, 10 mM imidazole, pH 8.0, 5 $ml\ mg^{-1}$ pellets), and were broken using Homogenizer (Avestin). The cell lysate was centrifuged at 19,000 rpm at 4 °C for 2 h and the supernatant was transferred to another new tube, which was centrifuged for additional 1 h to completely remove aggregated protein. Initially, 12 ml of cell free extract was mixed with 3 ml of Ni-NTA resin and incubated with slightly shaking at 4 °C for 1 h. The resulting mixture was transferred into a column and 8 ml of washing buffer (50 mM NaH_2PO_4, 1 M NaCl, 20 mM imidazole, pH 8.0) was then added into the column and flown through to wash away junk proteins, DNA, and lipid in the cell free extract. The protein was eluted by adding 8 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) to flow through the column and collected with vials (0.75 ml in each vial). The fractions containing the target protein were collected and exchanged with 15 ml of 10 mM potassium phosphate buffer using Vivaspin tube containing ultra filtration membrane (Sartorius group). The concentration of purified protein was determined by Bradford methods.

2.8. Kinetic assay of His-tagged wild-type CboFDH and mutants

The activities were measured at room temperature in a 96-well plate at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) using a Spectra Max M2 microplate reader. The reaction (200 μl) was performed in potassium phosphate buffer (10 mM, pH 7.5) containing sodium formate (500 mM), cofactor (0–20 mM) and FDH. The concentration of enzyme was chosen at which a reasonable reaction rate was retained. For each His-tagged FDHs, the enzyme concentration was as follows. FDH wild-type: 0.1 μM for NAD^+; FDH mutant B7: 1.0 μM for both NAD^+ and $NADP^+$; FDH mutant F2: 1.0 μM for $NADP^+$ and 0.1 μM for NAD^+; FDH mutant B7Q197-1-E3: 0.5 μM for $NADP^+$ and 0.5 μM for NAD^+.

3. Results and discussion

The mutations at D195/Y196 were introduced simultaneously and the corresponding mutant library was constructed as described in Section 2. The library was screened for the activity toward $NADP^+$ in the presence of sodium formate. The wild-type of recombinant CboFDH was used as control in each screening plate. In the initial screening, 50 colonies showed activity toward $NADP^+$. These 50 hits were further screened and 10 were chosen to be cul-

Table 1
Purification of His-tagged wild-type and mutant CboFDH proteins.

Protein	Cell free extract		After purification		Protein yield (%)	Purification factor
	Total protein (mg)	Specific activity ($NADP^+$, mU mg^{-1})[a]	Total protein (mg)	Specific activity ($NADP^+$, mU mg^{-1})[a]		
Wild-type CboFDH	113.7	0	94.4	0	83	–
B7 (D195Q/Y196R)	122.1	1.32	64.7	1.81	53	1.4
F2 (D195S/Y196P)	138.0	1.91	103.5	3.99	75	2.1
B7Q197-1-E3 (D195Q/Y196R/Q197N)	60.5	3.48	46.0	5.89	76	1.7

[a] The activity unit (U) was defined as 1 μmol of NADPH formed in 1 min (μmol min^{-1}) using formate as substrate.

tured at 5 ml scale, and their activity toward $NADP^+$ was measured. Among them, F2 exhibited the highest activity toward $NADP^+$ and NAD^+, which were 1.9×10^{-3} and 7.7×10^{-3} U mg^{-1}, respectively. Another clone B7 showed higher activity toward $NADP^+$ than that for NAD^+. The specific activities of cell free extract toward $NADP^+$ and NAD^+ were 1.3×10^{-3} and 8.0×10^{-5} U mg^{-1}, respectively. F2 and B7 were sequenced and possessed mutations D195S/Y196P and D195Q/Y196R, respectively, which are different from the three mutants reported by Andreadeli et al. [21].

FDH mutant B7 obtained from the screening of the first generation mutant library was used as template to construct the secondary generation mutant library. Mutation was introduced at residue Q197. The library was screened for the activity toward $NADP^+$ at room temperature in the presence of sodium formate. Five promising hits, which showed higher activity toward $NADP^+$ than positive control FDH mutant B7, were picked and re-screened at 5 ml scale. The mutant hit B7Q197-1-E3, in which Q197 was substituted with asparagine (N), had the highest specific activity toward $NADP^+$ (1.16×10^{-1} U mg^{-1}) and the lowest toward NAD^+ (2.2×10^{-4} U mg^{-1}) among all the hits.

Since the introduction of Histidine tag into *Candida methylica* FDH facilitates the purification procedure and does not affect the enzyme's kinetic behavior [22], wild-type CboFDH and mutants F2, B7 and B7Q197-1-E3 were His-tagged and then expressed in Rosetta2 (DE3) pLysS with induction of 0.5 mM IPTG at 18 °C for 10 h. The proteins were purified with Ni-NTA resin, and the purification conditions were optimized to achieve high purity by means of changing the concentration of NaCl and imidazole in the lysis buffer and washing buffer. The best concentration of NaCl and imidazole in lysis buffer was 1 M and 10 mM, respectively, and 1 M and 20 mM in washing buffer. The elution fractions containing His-tagged FDH proteins were collected and exchanged with potassium phosphate buffer (10 mM). The purification parameters are summarized in Table 1.

The activity of His-tagged FDH enzymes were assayed by measuring the concentration of NAD(P)H accumulated in the reaction, in which the concentration of NAD^+ or $NADP^+$ was varied (0–20 mM) and that of sodium formate was kept at 0.5 M. The Michaelis constant (K_m) and catalytic constant (k_{cat}) were calculated using Lineweaver–Burk plot, which derives from the Michaelis–Menten equation. The results are summarized in Table 2.

Mutant B7 (D195Q/Y196R) exhibited Michaelis constant K_m value of 0.05 mM toward $NADP^+$, comparable to that of wild-type CboFDH for NAD^+. The K_m value of mutant B7 toward NAD^+ was 0.084 mM, which was higher than that of wild-type enzyme (0.051 mM). This mutant showed comparable catalytic constants (k_{cat}) for $NADP^+$ and NAD^+ (0.53 and 0.45 s^{-1}, respectively). Introduction of mutations D195Q/Y196R switched the cofactor specificity from NAD^+ to $NADP^+$. The overall catalytic efficiencies (k_{cat}/K_m) toward $NADP^+$ and NAD^+ were 1.14×10^4 and 5.44×10^3 $M^{-1}s^{-1}$, respectively, with the ratio of catalytic efficiencies $(k_{cat}/K_m)^{NADP+}/(k_{cat}/K_m)^{NAD+}$ being 2.1. Although mutant F2 relaxed the tight specificity of wild-type CboFDH from NAD^+ to $NADP^+$, the change in cofactor specificity was not as significant as that of mutant B7. Mutant F2 with mutations D195S/Y196P had a K_m of 0.11 mM and catalytic constant k_{cat} of 0.31 s^{-1} for $NADP^+$, leading to a catalytic efficiency k_{cat}/K_m of 2.91×10^3 M^{-1} s^{-1}(about a quarter of that of mutant B7). Compared to the wild-type CboFDH, the K_m of mutant F2 to NAD^+ increased about 4 times, but the catalytic constant almost did not change.

The Michaelis constant K_m and catalytic constant k_{cat} values of the reported mutant CboFDH enzymes by Andreadeli et al. are also listed in Table 2 [21]. It can be seen that all mutant CboFDHs with double mutation at residues D196/Y196 showed similar catalytic constant (k_{cat}) toward $NADP^+$, but the K_m value could change up to two orders of magnitude. Contrastively, both Michaelis constant K_m and catalytic constant k_{cat} values toward co-factor NAD^+ were greatly affected by the double mutation at

Table 2
The kinetic parameters of wild-type CboFDH and mutants.

Enzyme	$NADP^+$			NAD^+			$(k_{cat}/K_m)^{NADP+}/(k_{cat}/K_m)^{NAD+}$
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})	
Wild-type CboFDH				0.051 ± 0.006	3.40 ± 0.07	66.7	0
B7 (D195Q/Y196R)	0.05 ± 0.01	0.57 ± 0.04	11.4	0.084 ± 0.009	0.45 ± 0.01	5.4	2.1
F2 (D195S/Y196P)	0.11 ± 0.03	0.31 ± 0.03	2.9	0.196 ± 0.03	3.14 ± 0.13	16.3	0.18
B7Q197-1-E3 (D195Q/Y196R/Q197N)	0.029 ± 0.005	0.79± 0.02	29.1	0.360 ± 0.03	0.62 ± 0.03	1.7	17.1
CboFDH(D195Q/Y196S)[a]	6.2 ± 0.3	0.34 ± 0.02	0.055	5.1 ± 0.06	0.40 ± 0.03	0.078	0.705
CboFDH(D195Q/Y196P)[a]	3.7 ± 0.2	0.34 ± 0.03	0.092	0.13 ± 0.01	0.87 ± 0.04	6.69	0.0138
CboFDH(D196Q/Y196H)[a]	1.7 ± 0.08	0.44 ± 0.03	0.26	1.8 ± 0.09	0.49 ± 0.03	0.27	0.96
SceFDH(D196A/Y197R)[b]	7.6 ± 0.9	0.16 ± 0.02	0.02	8.4 ± 0.9	0.12 ± 0.02	0.014	1.5
CmeFDH(D195S)[c]	ND[d]	ND[d]	0.0083	4.7 ± 0.3	1.6 ± 0.1	0.34	0.024
Mutant PseFDH[b,e]	0.15 ± 0.025	2.5 ± 0.15	16.7	1.0 ± 0.15	5.0 ± 0.4	5.0	3.4

[a] Data from Ref. [21].
[b] Data from Ref. [11].
[c] Data from Ref. [12].
[d] Not determined.
[e] Mutation information is not available, wild-type PseFDH has observable activity toward $NADP^+$.

these two residues. Relaxing of cofactor specificity of formate dehydrogenases from *Saccharomyces cerevisiae*, *Candida methylica* and bacterium *Pseudomonas* sp. 101 have also been reported [11,12,23] and their reported kinetic parameters are presented in Table 2. The CboFDH mutants B7 and F2 show much higher catalytic efficiency (k_{cat}/K_m) than mutant SceFDH, although these mutant enzymes were mutated at the same highly reserved residues [11,12]. The higher catalytic efficiency k_{cat}/K_m of CboFDH mutants B7 and F2 are mainly due to the lower Michaelis constant K_m of mutants B7 and F2 with cofactor $NADP^+$. The catalytic efficiency (k_{cat}/K_m) of mutant B7 is comparable with that of mutant formate dehydrogenase from bacterium *Pseudomonas* sp. 101 (PseFDH), but no mutation information has been reported for the latter [13,23].

When a mutation Q197N was introduced into mutant B7 (D195Q/Y196R), the Michaelis constant K_m of the mutant enzyme B7Q197-1-E3 toward cofactor $NADP^+$ decreased to 0.029 mM, nearly half of that of B7. The catalytic constant (k_{cat}) for $NADP^+$ was further increased to 0.79 s^{-1}, leading to an overall catalytic efficiency (k_{cat}/K_m) of 29.1×10^3 M^{-1} s^{-1}, much higher than the reported value for mutant formate dehydrogenase from bacterium *Pseudomonas* sp. 101 [11]. The Michaelis constant K_m of the mutant enzyme B7Q197-1-E3 with cofactor NAD^+ increased about 4 times from 0.084 to 0. 36 mM, while the catalytic constant (k_{cat}) for NAD^+ slightly increased from 0.45 to 0.62 s^{-1}, resulting in a lower overall catalytic efficiency for NAD^+ with k_{cat}/K_m being 1.7×10^3 M^{-1} s^{-1}. As shown in Table 2, the mutant enzyme B7Q197-1-E3 showed the highest overall catalytic efficiency (k_{cat}/K_m) for $NADP^+$ and ratio of catalytic efficiencies $(k_{cat}/K_m)^{NADP+}/(k_{cat}/K_m)^{NAD+}$, which have been reported so far for mutant formate dehydrogenases [11].

4. Conclusion

Site-saturation mutagenesis of residues Asp195, Tyr196 and Gln197 of the formate dehydrogenase from *Candida bodinii* (CboFDH) coupled with screening has resulted in effective $NADP^+$-dependent FDH enzymes. The present study reveals that these residues are important in controlling the cofactor specificity of formate dehydrogenases, and advances one step forward to the goal of developing highly efficient $NADP^+$-dependent formate dehydrogenases.

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