Engineering of coenzyme specificity of formate dehydrogenase from *Saccharomyces cerevisiae*

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A eukaryotic formate dehydrogenase (EC 1.2.1.2, FDH) with its substrate specificity changed from NAD^+ to $NADP^+$ has been constructed by introducing two single-point mutations, $Asp^{196} \rightarrow Ala$ (D196A) and $Tyr^{197} \rightarrow Arg$ (Y197R). The mutagenesis was based on the results of homology modelling of a NAD^+-specific FDH from *Saccharomyces cerevisiae* (SceFDH) using the *Pseudomonas* sp.101 FDH (PseFDH) crystal structure as a template. The resulting model structure suggested that Asp^{196} and Tyr^{197} mediate the absolute coenzyme specificity of SceFDH for NAD^+.

Key words: cofactor preference, mutagenesis, *Pseudomonas* sp.101, substrate binding, three-dimensional structure modelling.

INTRODUCTION

The molecular basis of coenzyme specificity remains an issue of fundamental interest in molecular biology. Despite the structural similarity of NAD^+ and $NADP^+$, dehydrogenases exhibit a pronounced specificity for either one of these coenzymes, suggesting strict steric and electrostatic requirements for the binding of the 2′-phosphate group of $NADP^+$ within the active sites of these enzymes. The directed change in coenzyme specificity of a selected dehydrogenase is an intellectually challenging task. The problem is also of considerable commercial importance, since NAD(P)H is widely used as a coenzyme in the synthesis and biotransformation of valuable chiral compounds and needs to be recovered by a cheap and efficient regeneration system.

Although more than 200 attempts to change the coenzyme specificity of $NAD(P)^+$-specific enzymes have been published over the last decade, only a few of them can be considered successful, despite the introduction of multiple mutations. Significantly improved binding of $NADP^+$ to NAD^+-specific enzymes has been reported for dihydrolipoamide dehydrogenase [1] and 3-isopropylmalate dehydrogenase [2]. The only example of a single amino acid replacement resulting in a pronounced change in substrate specificity, i.e. from NAD^+ to $NADP^+$, is for a *Drosophila* alcohol dehydrogenase [3].

NAD^+-specific formate dehydrogenase (EC 1.2.1.2, FDH) catalyses the NAD^+-dependent oxidation of formate anion to carbon dioxide and is an ideal model system for studying the molecular basis of coenzyme specificity. FDH is of considerable commercial interest as a catalyst for the regeneration of reduced coenzymes in the synthesis of biologically active compounds [4,5], because of the low cost of the substrate and the volatility of the reaction product that greatly facilitates its removal.

FDH belongs to the superfamily of D-specific 2-hydroxy acid dehydrogenases [6]. FDH genes are present in diverse organisms, such as bacteria, yeasts, fungi and plants. FDHs from evolutionarily distant sources show 40–50 % amino acid sequence similarity and, thus, are predicted to be structurally highly conserved. All known FDHs exhibit a high preference for NAD^+ over $NADP^+$. However, the wild-type bacterial FDHs can oxidize the formate anion at high concentrations of $NADP^+$. Therefore the construction of a $NADP^+$-binding site in bacterial FDH should be feasible. A mutant *Pseudomonas* sp.101 FDH (PseFDH) with its substrate specificity changed from NAD^+ to $NADP^+$ has been reported [7], but the amino acid substitutions were not described. This $NADP^+$-specific mutant of PseFDH is commercially available from Juelich Fine Chemicals (Juelich, Germany, http://www.juelich-chemicals.de). The same is not true for the yeast FDH orthologue, which exhibits an absolute specificity for NAD^+. An attempt to make a single-point mutant of yeast *Candida methylica* FDH (CmeFDH) lacking an absolute requirement for NAD^+ over $NADP^+$ has been reported by Gul-Karaguler et al. [8], but disappointingly the mutant enzyme still demonstrated 40 times higher efficiency (k_{cat}/K_m) with NAD^+ than with $NADP^+$ [8].

In the present study, we describe the construction of a yeast *Saccharomyces cerevisiae* FDH (SceFDH) mutant with its coenzyme preference shifted from NAD^+ to $NADP^+$. The site-directed mutagenesis of SceFDH was only possible because the SceFDH gene has been recently cloned and overexpressed in *Escherichia coli* as a soluble, catalytically active protein in our laboratory.

EXPERIMENTAL

Materials

All chemicals used for genetic engineering manipulations were of Molecular Biology Grade (Sigma). T4 DNA ligase, and *Eco*RI, *Sph*I and *Bss*HII restriction endonucleases were from New England Biolabs. *Pwo* DNA polymerase was from Boehringer Mannheim. Recombinant wild-type NAD^+-specific and mutant $NADP^+$-specific PseFDHs were from Juelich Fine Chemicals. Oligonucleotides were prepared with an Applied Biosystems DNA Synthesizer 380B. Amplification reactions were performed employing a Techne PHC-2 thermal cycler. NAD^+, $NADP^+$ ($\geqslant 99$ % purity; Sigma) and sodium formate (analytical grade; Reachim, Russia) were used in kinetic experiments. Absence of NAD(H) in $NADP^+$ was confirmed using isocratic HPLC

Abbreviations used: D196A, $Asp^{196} \rightarrow Ala$; D195S, $Asp^{195} \rightarrow Ser$; FDH, formate dehydrogenase; CmeFDH, *Candida methylica* FDH; PseFDH, *Pseudomonas* sp.101 FDH; SceFDH, *Saccharomyces cerevisiae* FDH; Y197R, $Tyr^{197} \rightarrow Arg$.

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chromatography on a Silasorb C18 column with 20 mM phosphate buffer (pH 7.0).

Mutant construction

The *E. coli* TG-1 cell line was used for genetic engineering manipulations with the pFY4d plasmid. The $Asp^{196} \rightarrow Ala$ (D196A)/$Tyr^{197} \rightarrow Arg$ (Y197R) double replacement in the SceFDH gene was performed by a two-step PCR. Two synthetic oligonucleotides were designed to obtain the mutant P-2 (5′-GTAGTTCCTGG**CGCG**CGTAGTACAGTAACTTC-3′) and P-3 (5′-CTGTACTACG**CGCG**CCAGGAACTACCTGCGG-3′). The introduction of the CGCG sequence into the SceFDH gene provided both the D196A/Y197R double mutation and the *Bss*HII restriction site. The P-1 (5′-GGTCCATGGCATCGA-AGGGAAAGGTTTTGCT-3′) and P-4 (5′-CAAGAATTCAA-ACAAGAACAAAAGGAGACC-3′) synthetic oligonucleotides, previously designed to clone the SceFDH gene, were also used to perform mutagenesis reactions. Two gene fragments, containing the desired mutations, were obtained by PCR with the P-1/P-2 and P-3/P-4 oligonucleotide pairs (1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C; in total 15 cycles followed by 5 min at 72 °C). The SceFDH gene with the appropriate mutations was prepared by amplification (same conditions as above, but with 35 cycles) from the mixture of two fragments purified from a 1 % agarose gel and the P-1 and P-4 primers. The end product was precipitated from the reaction mixture by 70 % ethanol in the presence of 0.7 M ammonium acetate and dissolved in deionized water. To clone the mutant gene, the PCR fragment was cut with the *Eco*RI and *Sph*I restriction endonucleases, purified from 1 % agarose gel and ligated with a DNA fragment prepared by digestion of the pFY4d plasmid with the same enzymes. The initial screening of mutants was performed using *Bss*HII restriction endonuclease followed by sequencing the entire gene on an Applied Biosystems Automated DNA Sequencer (model 373A) and an ABI PRISM Dye-Labelled Terminators DNA Sequencing Kit.

The mutant and wild-type SceFDHs were expressed in *E. coli* cells and purified as described for the wild-type recombinant PseFDH [9]. The purity of the recombinant enzymes was 95–98 % as judged by SDS/PAGE. The D196A/Y197R mutant was expressed at a level similar to that of the wild-type SceFDH.

Characterization of the mutant

Steady-state kinetic measurements were performed in 0.1 M potassium phosphate buffer (pH 7.0) at 340 nm with a Schimadzu UV 1601PC spectrophotometer equipped with a thermostatic cell holder at 30 °C. The concentrations of NAD^+, $NADP^+$ and sodium formate were in the ranges 0.01–40 mM, 0.5–40 mM and 0.1–1.0 M respectively. The kinetic parameters V_{max} and K_m for the wild-type and mutant SceFDHs and PseFDHs were measured by varying the concentration of one substrate at a fixed concentration of the second substrate as described earlier for PseFDH [9]. The values of V_{max} and K_m for the wild-type SceFDHs, and wild-type and mutant $NADP^+$-specific PseFDH were determined at saturating concentrations of the second 'fixed' substrate and, therefore, they are true kinetic parameters. V_{max} and K_m for the mutatnt SceFDH are apparent and not true values because the fixed concentrations of the second substrate were not saturating (for details see section 'Kinetic properties of double mutant').

The thermal stability of the enzyme was studied at 42.5 °C in 0.1 M potassium phosphate buffer (pH 7.0). Aliquots (200 μl) of the enzyme solution (0.2 mg/ml) were added to 1.5 ml Eppendorf tubes and incubated in a water bath (42.5 $\pm$ 0.1 °C). At intervals, the tubes were removed from the thermostat and cooled on ice for 3 min. The residual enzymic activity was then measured under the standard conditions given above. Kinetic data were analysed using a SigmaPlot 2000 software package (SPSS Inc., San Rafael, CA, U.S.A.).

The dependence of the Michaelis constant for NAD^+ on the ionic strength was studied in 0.01 M potassium phosphate buffer (pH 7.0) at 30 °C. The concentration of ammonium formate was varied over the range 0.2–2.5 M.

Fluorescent measurements were performed with a PerkinElmer LS50B luminescence spectrometer using 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C. The fluorescence was measured at 345 nm with the excitation at 295 nm.

Three-dimensional structure modelling

The model structure of SceFDH was constructed using an Automated Protein Modelling Server SWISS-MODEL (http://www.expasy.ch/swissmod/) with the crystal structure of holo-PseFDH (PDB2NAD.ENT) as a template. The structures were analysed using RasMol version 2.7 software.

RESULTS

Coenzyme preference

NAD^+-specific FDHs from bacterial and yeast species show different activities with respect to $NADP^+$ as the substrate. PseFDH is capable of catalysing the reaction with $NADP^+$. The kinetic curves recorded at 340 nm are linear for at least 10 min and display no initial burst due to product accumulation. In the presence of 50 μM recombinant wt-PseFDH and 0.3 M formate, the reaction rate was a linear function of the $NADP^+$ concentration in the 1–40 mM coenzyme concentration range. The coenzyme preference for $NADP^+$ expressed as the ratio $(k_{cat}/K_m)^{NADP+}/(k_{cat}/K_m)^{NAD+}$ was approx. 4.2×10^{-4} (Table 1). This value demonstrates that the wt-PseFDH is 2400 times more effective with NAD^+ than with $NADP^+$ as a substrate.

The results presented in [8] show that the wild-type recombinant yeast CmeFDH is essentially specific for NAD^+. The coenzyme preference of CmeFDH for NAD^+ expressed as $(k_{cat}/K_m)^{NAD+}/(k_{cat}/K_m)^{NADP+}$ was estimated as 2.5×10^5 [8], which is 100 times higher when compared with PseFDH. The recombinant wild-type SceFDH also exhibits essentially absolute specificity for NAD^+. In the presence of 25 μM SceFDH, 10 mM $NADP^+$ and 0.25 M sodium formate, no NADPH accumulation was detected for at least 30 min. Under the same conditions, with NAD^+ instead of $NADP^+$ and an enzyme concentration of 2.5 μM, the reaction rate was 2×10^{-5} M/s. Assuming the accuracy of the spectrophotometric measurements (approx. $\pm$0.001 absorbance units), the molar absorption coefficient for NADH/NADPH at 340 nm (6220 $M^{-1} \cdot cm^{-1}$) and $K_m^{NAD^+}$ for the wild-type SceFDH (36 μM), a lower limit for the preference for NAD^+ versus $NADP^+$ of the baker's yeast FDH can be estimated to be 3×10^9. Thus, the coenzyme preference of SceFDH for NAD^+ exceeds the value reported for CmeFDH [8] by four orders of magnitude.

Computer modelling and structural analysis

The amino acid sequence alignment for FDHs from different sources reveals key differences in the N-terminal regions and coenzyme-binding domains (Figure 1). N-termini of bacterial enzymes are 29 amino acids longer than those of other FDHs.

Table 1 Kinetic properties of recombinant wild-type and mutant FDHs from yeasts *S. cerevisiae* and *C. methylica* and bacterium *Pseudomonas* sp.101

Formate dehyrogenase	$K_m^{NAD^+}$ (μM)	k_{cat} with NAD^+ (s^{-1})	$K_m^{NADP^+}$ (μM)	k_{cat} with $NADP^+$ (s^{-1})	$K_m^{formate}$ (mM)	$\frac{k_{cat}^{NADP^+}}{K_m^{NADP^+}} / \frac{k_{cat}^{NAD^+}}{K_m^{NAD^+}}$
Wild-type SceFDH	36 ± 5 (0.25 M formate)	6.5 ± 0.4	ND*	ND	5.5 ± 0.3	$< 3.3 \times 10^{-10}$
SceFDH D196A/Y197R	7600 ± 800 (0.25 M formate)	0.095 ± 0.01 (0.25 M formate)	4500 ± 500 (0.25 M formate)	0.13 ± 0.01 (0.25 M formate)	1000 ± 200 (40 mM $NADP^+$)	2.3 (0.25 M formate)
	8400 ± 900 (0.5 M formate)	0.12 ± 0.02 (0.5 M formate)	7600 ± 900 (0.5 M formate)	0.16 ± 0.02 (0.5 M formate)		1.5 (0.5 M formate)
Wild-type CmeFDH [8]	55 ± 4 (0.2 M formate)	1.4 ± 0.1	ND	ND	NR†	$< 4 \times 10^{-6}$
CmeFDH D195S [8]	4700 ± 300 (0.2 M formate)	1.6 ± 0.1	ND (> 0.4 M‡)	ND	NR	2.4×10^{-2} (0.2 M formate)
Wild-type PseFDH	60 ± 5 (0.3 M formate)	10.0 ± 0.6	> 0.4 M	ND	7.0 ± 0.8	4.2×10^{-4} (0.3 M formate)
Mutant $NADP^+$-specific PseFDH	1000 ± 150 (0.3 M formate)	5.0 ± 0.4	150 ± 25 (0.3 M formate)	2.5 ± 0.15	9.0 ± 3.0	3.5 (0.3 M formate)

* ND, not detectable.
† NR, not reported.
‡ Estimated from data in Figure 2 [8].

The structural data [10] and sequence analysis (Figure 1) show that the coenzyme-binding domain of FDHs has a classical Rossmann fold. All FDHs and other NAD^+-specific oxidoreductases from the superfamily of D-specific 2-hydroxy acid dehydrogenases contain a conserved 'fingerprint' sequence G(A)XGXXG and a conserved aspartic acid residue (Asp^{221}, Asp^{195} and Asp^{196} in PseFDH, CmeFDH and SceFDH respectively) (Figure 1). This conserved Asp residue interacts with the 2′- and 3′-OH groups of adenosine ribose and is a major determinant of the specificity for NAD^+, according to the X-ray data for PseFDH (PDB2NAD.ENT), D-lactate (PDB2DLD.ENT), 3-phosphoglycerate (PDB1PSD.ENT) and D-glycerate (PDB1GDH.ENT) dehydrogenases and model structures for the yeast FDHs from *C. methylica* [8] and *C. boidinii* [11]. This Asp residue is located 18 residues downstream from the Gly residue at the end of the 'fingerprint' sequence in yeast FDHs. The bacterial and plant sequences (Figure 1) have the conserved Asp as the 17th residue downstream from the end of the 'fingerprint'. Gul-Karaguler et al. [8] described a mutant $Asp^{195} \rightarrow$ Ser (D195S) CmeFDH reacting with both coenzymes but still showing a preference for NAD^+ over $NADP^+$ (Table 1) [8]. The dependence of enzymic activity of this mutant on $NADP^+$ concentration was linear up to 40 mM of the coenzyme. According to the Michaelis–Menten equation, a linear dependence of velocity on substrate concentration [S] is observed when $[S] < (0.05–0.07)K_m$. This allows an estimate of the $K_m^{NADP^+} > 0.4$ M for the mutant D195S CmeFDH. Hence, in addition to the conserved Asp residue, the absolute requirement for NAD^+ in yeast FDHs must be under the control of another residue(s).

To elucidate the molecular determinants of the SceFDH coenzyme specificity, the structure of the enzyme was modelled and compared with the structure of the PseFDH holoform. In addition to the conserved Asp^{196} residue, the structural analysis (Figure 2) suggests a contribution of Tyr^{197} residue to the high specificity of SceFDH towards NAD^+. This tyrosine residue is present in all yeast and fungal FDHs (Figure 1). According to the SceFDH model, the Tyr^{197} side chain is located near the 2′-OH group of NAD^+ in the enzyme's active site (Figure 2). It should prevent $NADP^+$ binding both by creating a hydrophobic environment unfavourable for the negatively charged 2′-phosphate and by sterically blocking the cavity, which could otherwise accommodate this group. The D-specific 2-hydroxy acid dehydrogenases, which prefer NAD^+ as a coenzyme, also contain hydrophobic amino acids with bulky side chains at the +1 position with respect to the conserved Asp residue (Figure 1, D-lactate dehydrogenases, glycerate dehydrogenase and 3-phosphoglycerate dehydrogenase).

In contrast, an Arg residue at the above-mentioned position was found in the bacterial FDHs (Arg^{222}, Figure 1). The presence of either an Arg or Lys residue in this position is a common feature of the coenzyme-binding domains of $NADP^+$-dependent dehydrogenases [12,13]. In particular, vancomycin-resistant protein from *Enterococcus faecium* (EfaVanH), which is a $NADP^+$-specific dehydrogenase from the same superfamily as FDH [14], also contains Arg in this position (Figure 1). The positively charged guanidinium group of this Arg residue directly interacts with the negatively charged 2′-phosphate of $NADP^+$ in glutathione reductase [13]. The side chain of Arg^{222} in the active site of PseFDH is orientated away from the coenzyme (Figure 2). In apo-PseFDH, it does not interact with other residues, whereas in holo-PseFDH, Arg^{222} forms three hydrogen bonds – one with Glu^{260} from the coenzyme-binding domain and two bonds with His^{379} and Tyr^{381} residues from the catalytic domain [10]. These data suggest that the main function of this Arg^{222} residue in the PseFDH is to maintain the optimal conformation of the active site but not to participate in coenzyme binding. The orientation of the Arg^{222} side chain in the holo-PseFDH suggests a low probability of ionic interaction between the 2′-phosphate group of $NADP^+$ and guanidinium group of the Arg^{222} residue (Figure 2). At the same time, this orientation of the Arg^{222} residue does not create steric hindrance for the binding of the 2′-phosphate of $NADP^+$ in the enzyme's active site. Thus a comparison of the structure of the holo-PseFDH and the model structure of SceFDH shows that mutation Y197R in SceFDH should result in enhanced $NADP^+$ binding to the yeast FDH active site, relative to that of the wild-type enzyme.

Kinetic properties of the double mutant

To confirm the role of Tyr^{197} residue in the absolute specificity of SceFDH towards NAD^+, the double mutant D196A/Y197R was

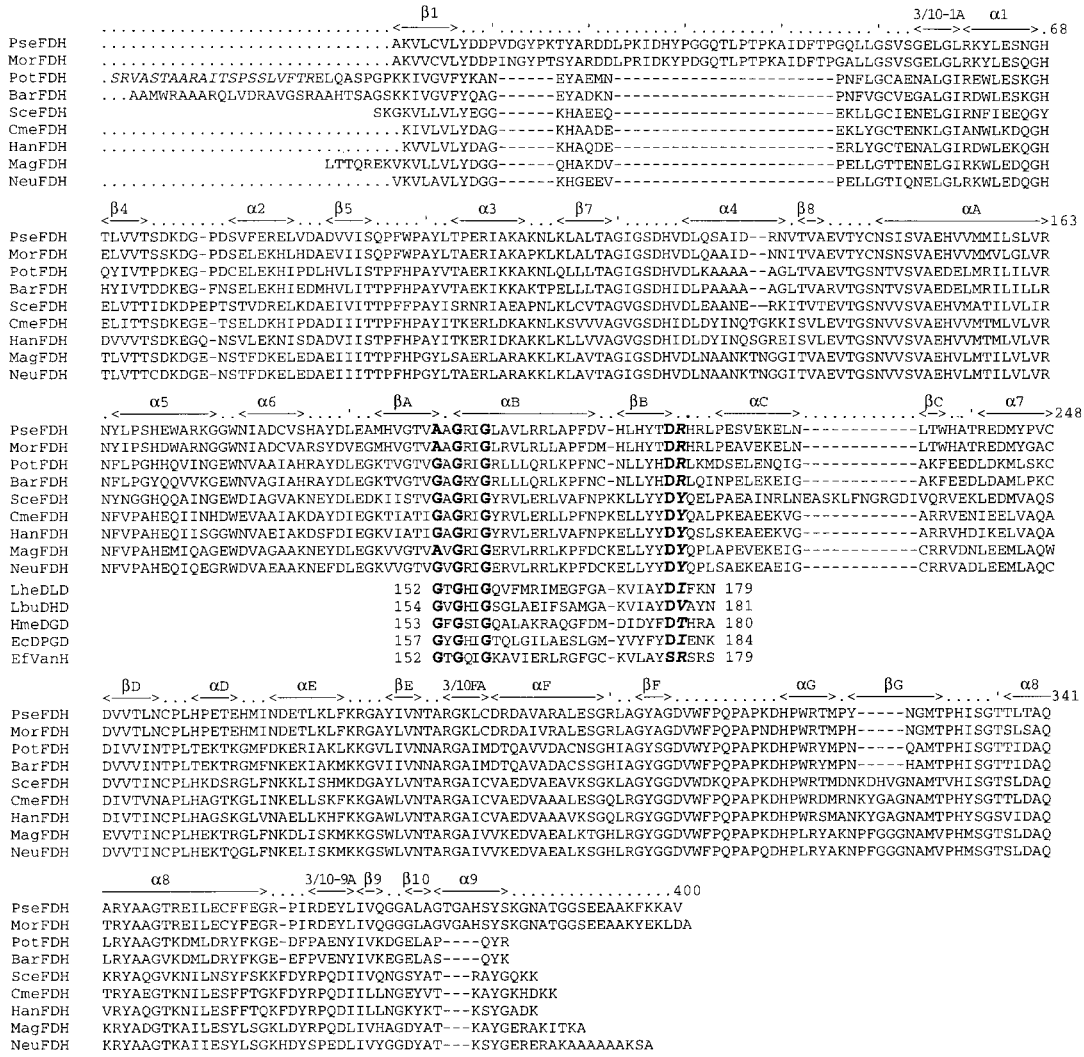

Figure 1 Alignment of amino acid sequences of FDHs from different sources

Bacteria: *Pseudomonas* sp. 101 (PseFDH, SWISS-PROT:FDH_PSESR) and *Moraxella* sp. C-1 (MorFDH, EMBL accession Y13245); plants: potato (PotFDH, EMBL Z21493) and barley (BarFDH, EMBL D88272); yeasts: *S. cerevisiae* (SceFDH, EMBL Z75296), *Candida methylica* (CmeFDH, EMBL X81129) and *Pichia angusta* (HanFDH, formerly *Hansenula polymorpha*, EMBL P33677); fungi: *Neurospora crassa* (NeuFDH, EMBL L13964) and *Magnaporthe grisea* (MagFDH, EMBL AA415108); amino acid sequences of some other D-specific 2-hydroxy acid dehydrogenases in the regions of coenzyme-binding domains: D-lactate dehydrogenases from *Lactobacillus helveticus* (LheDLD, EMBL U07604) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LbuDLD, EMBL X60220), glycerate dehydrogenase from *Hyphomicrobium methylovorum* (HmeDGD, SWISS-PROT:DHGY_HYPME), 3-phosphoglycerate dehydrogenase from *Escherichia coli* (EcDPGD, EMBL L29397) and vancomycin-resistance protein VanH from *Enterococcus faecium* (EfVanH, GenBank® M64304) are also shown. Numeration of residues and structural elements corresponds to FDH from *Pseudomonas* sp. 101.

constructed. The results of the steady-state kinetic measurements made for this double mutant are presented in Table 1. The SceFDH mutant exhibited Michaelis–Menten kinetics with both coenzymes and showed a lower K_m value for $NADP^+$ compared with NAD^+ at a formate concentration of 0.25 M (Table 1). However, the mutations lowered the enzyme's efficiency with formate. Saturation kinetics with respect to formate was not reached over the range up to 1 M at 20 mM of $NADP^+$ (results not shown). Increasing the coenzyme concentration to 40 mM gave a hyperbolic dependence of velocity on formate concentration (Figure 3) with $K_m = 1000 \pm 200$ mM for formate (Table 1). This value is 180-fold greater than that for wild-type SceFDH (Table 1). Therefore, $K_m^{NAD^+}$ and $K_m^{NADP^+}$ values for the double-mutant D196A/Y197R determined at 0.25 M formate (approx. 0.25 $K_m^{formate}$) must be higher than the true values of the Michaelis constants for the coenzymes.

To obtain the true K_m values for NAD^+ and $NADP^+$ either a saturating concentration of formate has to be used or the dependence of the apparent Michaelis constant on the formate concentration needs to be determined. Unfortunately, neither approach can be used because increasing the formate concentration decreases the efficiency of mutant SceFDH with NAD^+ and $NADP^+$. The $K_m^{NAD^+}$ and $K_m^{NADP^+}$ for the SceFDH D196A/Y197R mutant determined in the presence of 0.5 M formate (Table 1) were found to be even higher than those observed at 0.25 M formate, despite the fact that neither concentration was saturating. We suggest that ionic strength greatly influences coenzyme binding. To verify this hypothesis, we

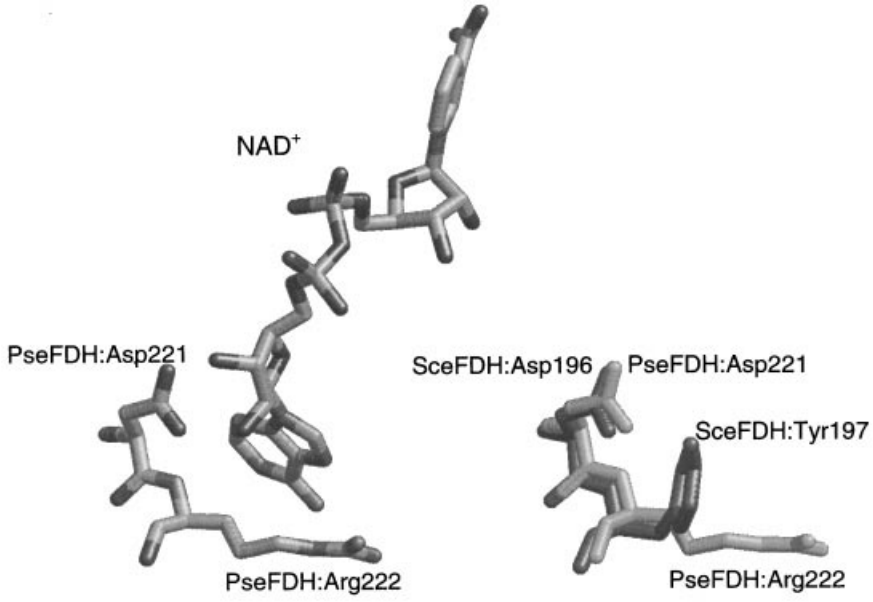

Figure 2 Interaction between NAD^+ and Asp^{221} in the active site of PseFDH (left) and superposition of PseFDH crystal structure (light grey) and SceFDH 3-D model structure (dark grey) (right)

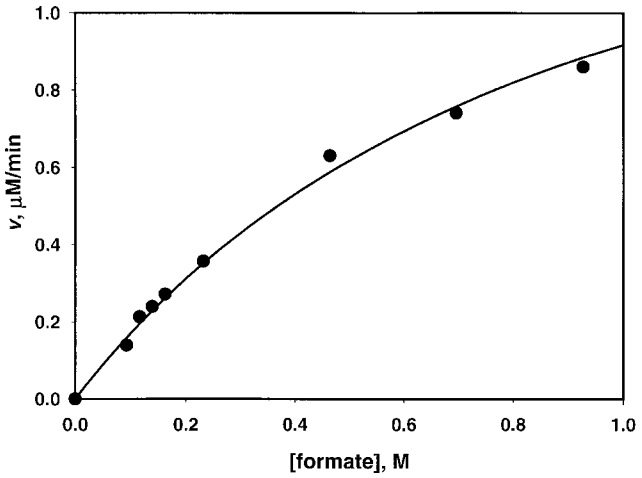

Figure 3 Dependence of enzymic activity on formate concentration for the SceFDH D196A/Y197R mutant [40 mM $NADP^+$, 0.1 mM potassium phosphate buffer (pH 7.0), 30 °C]

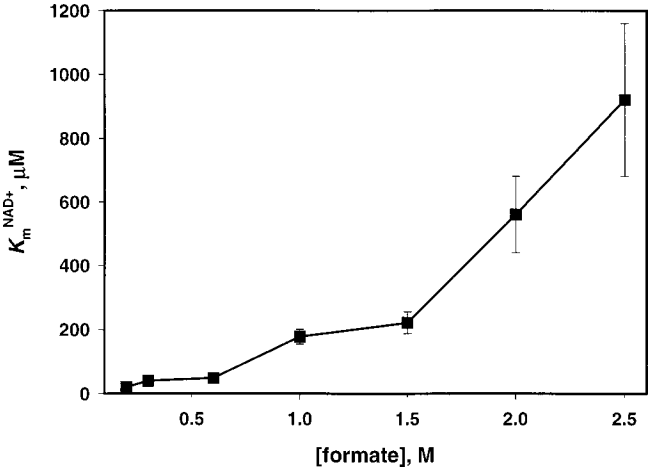

Figure 4 Dependence of the value on formate concentration for wild-type SceFDH (0.01 M potassium phosphate buffer, pH 7.0, 30 °C)

examined the dependence of the k_{cat} and $K_m^{NAD^+}$ on the formate concentration in the saturating range (35–180 $K_m^{formate}$) for the wild-type SceFDH (Figure 4). As seen in Figure 4, the K_m value increases with increasing ionic strength, whereas k_{cat} remains constant (results not shown). The efficiency of interaction of the SceFDH double mutant with the two coenzymes was also affected by ionic strength (Table 1). Electrostatic interactions are extremely important for NAD^+ or $NADP^+$ binding to the active sites of dehydrogenases [12]. The formate concentration affects the binding of these coenzymes, especially that of $NADP^+$, because the 2′-phosphate group forms additional ionic contacts with the protein.

Fluorescence of enzyme–azide–coenzyme ternary complexes

Additional evidence for the efficient interaction of $NADP^+$ with the mutant SceFDH was obtained by determining the effect of coenzymes in the presence of azide on the fluorescence of the wt-SceFDH and mutant SceFDH. Azide anion is a strong inhibitor

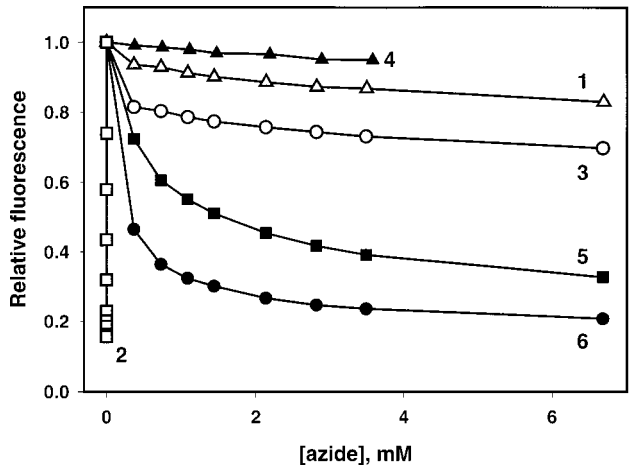

Figure 5 Quenching of tryptophan fluorescence (excitation at 295 nm, emission at 345 nm) of wild-type SceFDH and the D196A/Y196R mutant in the absence of coenzymes (1,4), in the presence of NAD^+ (2,5) and in the presence of $NADP^+$ (3,6) (100 μM coenzyme, 0.1 M potassium phosphate buffer, pH 7.0, 25 °C)

of all FDHs [18] competing with formate and showing an inhibition constant of approx. 0.1 μM for wild-type SceFDH [15]. The formation of the PseFDH–NAD^+–azide complex results in the quenching of the protein tryptophan fluorescence [9]. To estimate the enzyme affinity for cofactors independently of ionic strength, the mutant and wild-type SceFDHs were titrated with azide at fixed non-saturating NAD^+ and $NADP^+$ concentrations (0.1 mM) (Figure 5). Addition of azide to the mixture of wild-type SceFDH and NAD^+ also results in strong quenching of the protein fluorescence (Figure 5, curve 2). A decrease in enzyme fluorescence occurs at azide concentrations comparable with the inhibition constant K_i^{azide}. A constant level of fluorescence (10 % of initial intensity) is achieved at inhibitor concentrations of 3–5 μM, which are 30 times higher than the inhibition constant for azide. These data show that the quenching of protein fluorescence is a result of the production of a wild-type SceFDH–NAD^+–azide complex. The fluorescence measurements show that the SceFDH double mutant can also produce a ternary enzyme–cofactor–inhibitor complex with NAD^+ and $NADP^+$ (Figure 5, curves 5 and 6), and the extent of fluorescence quenching is similar to that observed for the wt-SceFDH–NAD^+–azide complex. By analysing curves 3 and 6 in Figure 5 the following two important conclusions can be made. First, the SceFDH D196A/Y197R mutant binds $NADP^+$ more tightly than NAD^+. Secondly, efficient fluorescence quenching of the SceFDH double mutant in the presence of NAD^+ or $NADP^+$ requires azide concentrations 300–500 times higher than inhibitor concentrations that are required for the interaction of wild-type SceFDH with NAD^+. The decrease in the affinity for azide shown by the fluorescence experiments is similar to that observed for formate affinity by the kinetic experiments.

The kinetic experiments showed that $NADP^+$ does not inhibit the wt-SceFDH and, therefore, is not bound in a wild-type SceFDH–$NADP^+$ binary complex. On the other hand, the fluorescence of wt-SceFDH in the presence of $NADP^+$ decreased on titration with azide, but never more than 30 %. This may be interpreted as the formation of a wild-type SceFDH–$NADP^+$–azide complex, provided the dissociation constant of $NADP^+$ for the ternary complex is smaller than the corresponding constant for the binary enzyme–cofactor complex. Azide has different effects on wild-type SceFDH and mutant SceFDH in the absence of the coenzymes (Figure 5, curves 1 and 4)). The wild-type enzyme lost approx. 20 % of the initial fluorescence intensity, whereas the mutant was barely affected by the same concentration of azide. Thus, in spite of an apparent ordered kinetic mechanism for SceFDH [15], the free enzyme is able to bind azide when the inhibitor concentration is much higher ($\geqslant 10^4$ times) than the inhibition constant. Since the affinity of SceFDH for formate decreases dramatically with the introduced mutations, the double mutant is unable to bind an azide ion in the millimolar range in the absence of the coenzyme.

Thermal stability of double mutant

The double replacement caused a slight destabilization effect. The mutant and wild-type enzymes lost 50 % of activity when incubated at 42.5 °C for 1.5 and 3.5 min, respectively.

DISCUSSION

In the present study, we report the construction of a novel mutant eukaryotic FDH that exhibits very high specificity for $NADP^+$. The change of coenzyme specificity was achieved by protein engineering of the wild-type enzyme with an absolute requirement for NAD^+, in contrast with a $NADP^+$-specific mutant of a bacterial FDH. The coenzyme preference of SceFDH for $NADP^+$ was enhanced by 2.5×10^6 times compared with a 200-fold increase for PseFDH (Table 1). Our hypothesis that the Tyr residue adjacent to the conserved Asp residue in the $GXGXXGX_nDY$ motif is responsible for the absolute coenzyme specificity of SceFDH for NAD^+ was successfully tested. The D196A/Y197R double-mutant SceFDH exhibits a coenzyme preference for $NADP^+$, which is two orders of magnitude higher than that reported for the CmeFDH D195S single mutant [8].

The strict regulation of coenzyme specificity in yeast FDHs can be understood in an evolutionary context. Higher organisms require enzymes with an absolute specificity for only one cofactor. We are not aware of any kinetic data for plant FDHs with a requirement for $NADP^+$ as a cofactor. The plant enzymes contain the Arg residue in the +1 position with respect to the conserved Asp residue and probably could bind 2′-phosphate in the active site (Figure 1). If this were the case, the $NADP^+$-dependent catalysis would need to be prevented, possibly by compartmentalization of FDH within the plant cell [16,17].

An unexpected result of the present work was that the D196A/Y197R double mutation in SceFDH increased the Michaelis constant for formate. The $NADP^+$-specific mutant of PseFDH exhibited an affinity for formate similar to that of the wild-type enzyme (Table 1). This can be explained by the different kinetic mechanisms for these enzymes. The kinetic mechanisms of reactions catalysed by bacterial [18] and other FDHs [15,19,20] are described as random equilibrium and ordered Bi Bi kinetic mechanisms respectively. SceFDH has an ordered kinetic mechanism, which requires the coenzyme to be the first substrate to bind, with formate being second [15]. The mutations that induce the SceFDH specificity for $NADP^+$ may also produce a non-optimal conformation of the protein–coenzyme complex, and as a consequence, may impair formate binding. For the bacterial enzyme, formate binds either first or second, i.e. it can bind in the absence of a coenzyme. Thus mutations conferring specificity for $NADP^+$ do not affect the enzyme affinity for formate. Previous studies also observed an ordered kinetic mechanism for the *C. boidinii* [19] and *C. methylica* FDHs [21]. Unfortunately, the effect of the D195S mutation in CmeFDH on the Michaelis constant for formate was not reported [8]. It is quite possible that an ordered kinetic mechanism is an evolutionary defence that

protects yeast FDHs from the adverse effects of mutations, which could result in a change of coenzyme specificity. Nevertheless, our future work will be focused on the construction of the SceFDH $NADP^+$-specific mutant with an appropriate affinity for formate.

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