Active-site characterization of *Candida boidinii* formate dehydrogenase

Nikolaos E. LABROU*¹ and Daniel J. RIGDEN†

*Laboratory of Enzyme Technology, Department of Agricultural Biotechnology, Agricultural University of Athens, 75 Iera Odos Street, GR-11855 Athens, Greece, and

†Bioinformatics Laboratory, Universidade Católica de Brasília, Brasília, Brazil

NAD⁺-dependent formate dehydrogenase (FDH) from *Candida boidinii* was cloned and expressed to a high level in *Escherichia coli* (20% of soluble *E. coli* protein). Molecular modelling studies were used to create a three-dimensional model of *C. boidinii* FDH, based on a known structure of the *Pseudomonas* sp. 101 enzyme. This model was used for investigating the catalytic mechanism by site-directed mutagenesis. Eleven forms of *C. boidinii* FDH were characterized by steady-state kinetic analysis: the wild type as well as 10 mutants involving single (Phe-69–Ala, Asn-119–His, Ile-175–Ala, Gln-197–Leu, Arg-258–Ala, Gln-287–Glu and His-311–Gln) and double amino acid substitutions (Asn-119–His/His-311–Gln, Gln-287–Glu/His-311–Gln and Gln-287–Glu/Pro-288–Thr). The kinetic results of the mutant enzymes provide the first experimental support that hydrophobic patches, formed by Phe-69 and Ile-175, destabilize

substrates and stabilize products. Also, the key role of Arg-258 in stabilization of the negative charge on the migrating hydride was established. Asn-119, besides being an anchor group for formate, also may comprise one of the hinge regions around which the two domains shift on binding of NAD⁺. The more unexpected results, obtained for the His-311–Gln and Gln-287–Glu/His-311–Gln mutants, combined with molecular modelling, suggest that steric as well as electrostatic properties of His-311 are important for enzyme function. An important structural role has also been attributed to *cis*-Pro-288. This residue may provide the key residues Gln-287 and His-311 with the proper orientation for productive binding of formate.

Key words: kinetic mechanism, protein engineering, site-directed mutagenesis.

INTRODUCTION

Yeast formate dehydrogenase (FDH; formate:NAD⁺ oxidoreductase, EC 1.2.1.2) catalyses the oxidation of formate to CO₂ with concomitant reduction of NAD⁺ to NADH. The enzyme exists as a homodimer of 42 kDa subunits each possessing an independent active centre, and has no apparent metal-ion requirement (for a review see [1]). Initial velocity studies have established an ordered kinetic mechanism in which NAD⁺ binds first to the enzyme prior to formate binding [2]. The hydride transfer takes place in the central ternary complex and is fully rate limiting [1–3].

Crystal structures of holo- (ternary complex, enzyme–NAD⁺–azide) and apo-FDH from the methylotrophic bacterium *Pseudomonas* sp. 101 at 2.05 and 1.8 Å resolution, respectively, have been reported [4,5]. FDH differs significantly from other NAD⁺-dependent dehydrogenases in primary, quaternary and domain structure but it shows strong sequence and three-dimensional fold similarities to D-specific 2-hydroxyacid dehydrogenases [5].

The crystal structures have enabled a catalytic mechanism for FDHs to be proposed [4,5]. A combination of stabilizing and destabilizing factors is suggested to be important. Among these is a hydrophobic wall consisting of Val-123 and Ile-175 (note that *Candida boidinii* numbering will be used throughout), which destabilize the positively charged NAD⁺ and stabilize the neutral product NADH. Arg-258 destabilizes the positively charged NAD⁺ and stabilizes the negatively charged hydride ion as it migrates from formate to NAD⁺. A further hydrophobic wall comprising Pro-68 and Phe-97 is suggested to destabilize the formate ion. Whereas the crystal structure with bound azide did not allow the unambiguous assignment of a formate binding site, it was clear that two residues are directly involved in formate binding, Arg-258 and Asn-119. Another notable feature of the

catalytic site is the presence of an invariant His–Gln pair in FDHs (His-311 and Gln-287 in the *C. boidinii* enzyme) replacing the His–Glu proton relay system conserved in all other NAD⁺-dependent dehydrogenases. Despite the locking of the His-311 in its unprotonated state by the proximity of Gln-287, His-311 has been suggested to be a further formate ligand based on the results of its mutation to Phe in another FDH [6]. That mutant was unable to bind formate and showed no catalytic activity. Unfortunately it is not clear if the mutant was able to accommodate the replacement of His with Phe, a highly non-conservative change [7], without gross changes in overall structure. Thus the definite function of His-311 has yet to be found in FDH. While limited investigation by mutagenesis of the His–Gln pair has been carried out [6], other aspects of the proposed mechanism (based on crystal structure) have not been tested experimentally. This work was undertaken in order to evaluate and investigate this proposed mechanism of catalysis by site-directed mutagenesis and molecular modelling studies. For this purpose, first, the FDH from *C. boidinii* was cloned, expressed, purified and characterized.

FDH has the potential for use in organic acid synthesis as a catalyst in a coenzyme regeneration system [8,9]. Thus a detailed knowledge of the catalytic mechanism of hydride transfer between NAD⁺ and formate anion under a range of conditions will improve its synthetic application, and will facilitate the design of mutants with improved properties that will circumvent the limitations of this biocatalyst (e.g. its low specific activity).

EXPERIMENTAL

Materials

A poly(A)-mRNA purification kit was obtained from Ingenius (R&D Systems Europe). A first-strand cDNA synthesis kit,

Abbreviations used: FDH, formate dehydrogenase; IPTG, isopropyl β-D-thiogalactoside.

¹ To whom correspondence should be addressed (e-mail lambrou@aia.gr).

The FDH nucleotide sequence has been submitted to the EMBL Nucleotide Sequence Database under the accession no. AJ011046.

dNTPs and restriction enzymes were obtained from Boehringer Mannheim, Mannheim, Germany. The unique-site-elimination mutagenesis kit and the expression vector pKK223-3 were purchased from Pharmacia (Uppsala, Sweden). XL1-Blue *Escherichia coli* cells, *Pfu* DNA polymerase and all other molecular biology reagents were from Stratagene (La Jolla, CA, U.S.A.). NAD⁺ and sodium formate were obtained from Sigma (St. Louis, MO, U.S.A.).

C. boidinii cultures and native FDH purification

C. boidinii (N.C.Y.C. 1513) was grown at 30 °C in a medium containing 1% peptone, 1% yeast extract and 2% glucose. After 24 h, cells were pelleted by centrifugation and resuspended in minimal medium supplemented with methanol (4%), to induce FDH expression. The culture was grown for another 96 h. The cells were harvested by centrifugation and disintegrated according to Labrou et al. [10]. Native FDH was purified to homogeneity according to Labrou [11].

CNBr cleavage of protein and N-terminal amino acid sequence analysis

CNBr cleavage of protein was performed according to Aitken et al. [12]. Protein fragments were separated by SDS/PAGE (15% separating gel [13]); and were transferred electrophoretically on to PVDF membranes. Bands of interest were excised from the membranes and gas-phase sequenced on an Applied Biosystems protein sequencer, model 477A, equipped with an online phenylthiohydantoin analyser, model 120 A.

Molecular cloning

A cDNA library in λZAP II was constructed using poly(A)⁺ mRNA from *C. boidinii* cells. Poly(A)⁺ mRNA was purified using the RNA isolation kit as described by the manufacturer (Ingenius, R&D Systems Europe). The library was screened with an approx. 430 bp PCR product generated from *C. boidinii* cDNA as follows: first-strand cDNA synthesis was done with oligo-p(dT)₁₅ primer and avian myeloblastosis virus (AMV) reverse transcriptase according to the manufacturer's recommendations (Boehringer Mannheim). The PCR primers were designed from the N-terminal amino acid sequence of the native enzyme and from the N-terminal amino acid sequence of a CNBr-cleaved fragment of the enzyme. The primers were designed with codon usage information from *Candida* sp. The resulting upstream 5' primer was 5'-AAG ATC GTT TTA GTC TTA TAT GAT GCT GGT-3' and the 3' primer was 5'-AAC AAA ATT TCT AAC CAA GAC AAG-3'. The PCR reaction was carried out in a total volume of 50 µl containing 4 pmol of each primer, 1 µg of template cDNA, 0.2 mM of each dNTP, 5 µl of 10 × *Pfu* buffer and 1 unit of *Pfu* DNA polymerase (Stratagene). The PCR procedure comprised 30 cycles of 45 s at 95 °C, 1 min at 53 °C and 2 min at 72 °C. A final extension time at 72 °C for 10 min was performed after the 30 cycles. The PCR product (430 bp) was gel-purified and was labelled by the random-priming method (Pharmacia).

Heterologous expression and purification of *C. boidinii* FDH

The cDNAs to be expressed in *E. coli* were generated by PCR using the oligonucleotide primers synthesized for the 5' region of the gene from the ATG start codon (5'-ATG AAG ATC GTT TTA GTC TTA TAT GAT GCT GGT-3') and for the 3' end of the gene finishing at the TAA stop codon (5'-TTA TTT CTT ATC GTG TTT ACC-3'). These primers were used to amplify the open reading frame of the cDNA. The PCR reaction, carried

out in a total volume of 100 µl, contained 200 ng of each primer, 1 µg of template cDNA, 0.2 mM of each dNTP, 10 µl of 10 × *Pfu* buffer and 2 units of *Pfu* DNA polymerase. The PCR procedure comprised 30 cycles of 45 s at 95 °C, 1 min at 55 °C and 3 min at 72 °C. A final extension time at 72 °C for 10 min was performed after the 30 cycles. The PCR products were run on a 1.2% (w/v) agarose gel and a fragment of about 1100 bp was excised, purified by adsorption to silica beads, cloned into the pCR®-Blunt cloning vector, sequenced, digested with *Eco*R1 and subsequently ligated into the isopropyl β-D-thiogalactoside (IPTG)-inducible expression vector pKK223-3. The resulting expression construct pFDH2 was used to transform competent *E. coli* JM 105 cells. *E. coli* cells, harbouring plasmid pFDH2, were grown at 37 °C in 1 litre of Luria-Bertani medium containing 100 µg/ml ampicillin. The synthesis of FDH was induced by the addition of 1 mM IPTG when the absorbance at 600 nm was 0.6. After induction (4 h), cells (approx. 3.5 g) were harvested by centrifugation at 4000 g for 15 min, resuspended in potassium phosphate buffer (50 mM, pH 7.5, 9 ml), sonicated and centrifuged at 10000 g for 20 min. The supernatant was collected and dialysed overnight against Mes/NaOH buffer (20 mM, pH 6.0, 2 litres). The dialysate was loaded on to a column of mercapto-pyruvic-VBAR-Sepharose (where VBAR is Vilmafix® Blue A-R; 1 ml) [10], which was previously equilibrated with Mes/NaOH buffer (20 mM, pH 6.0). Non-adsorbed protein was washed off with 10 ml of equilibration buffer, followed by 10 ml of Mes/NaOH buffer (20 mM, pH 6.0) containing 10 mM KCl. Bound FDH was eluted with equilibration buffer containing 0.15 mM NAD⁺ and 2 mM Na₂SO₃. Collected fractions (1 ml) were assayed for FDH activity and protein. All mutants were purified according to the procedure described above for the wild-type enzyme, with the single exception of the Asn-119-His mutant, which was purified according to the anion-exchange method [14] because of its reduced ability to bind to the dye-ligand affinity adsorbent. Nucleotide sequencing was performed along both strands using an Applied Biosystems sequencer model 373 A.

Site-directed mutagenesis

Site-directed mutagenesis was performed according to the unique-site-elimination method described by Deng and Nickoloff [15]. The oligonucleotide primer sequences used were as follows (with altered nucleotides given in *italics*): for the Phe-69-Ala mutation, 5'-ACT CCT *GCC* CAT CCT GCT-3'; Asn-119-His mutation, 5'-ACC GGT TCT CAT GTT GTC TCT GTT-3'; Ile-175-Ala mutation, 5'-GCC GGT AGA *GCA* GGT TAC-3'; Arg-258-Ala mutation, 5'-C AAT ACT GCA *GCA* GGT GCC ATT TGT G-3'; Gln-287-Glu mutation, 5'-TGG TTC CCA GAA CCA GCG CCA AAA-3'; Gln-287-Glu/Pro-288-Thr mutation, 5'-TGG TTC CCA GAA *ACC* GCG CCA AAA-3'; His-311-Gln mutation, 5'-ATG ACT CCT CAA TAC TCT GGT ACT -3'. The selection primer's sequence was as follows: 5'-GAA TTC TCG TGG ATC CGT CGA CCT-3'. This primer contains a mutation in a unique *Sma*I restriction site of the pFDH2 vector. The primers were phosphorylated before use by polynucleotide kinase and were then used in pairs with one mutant primer and the selection primer in each mutagenesis reaction. The expression construct pFDH2 encoding *C. boidinii* FDH was used as template DNA in all mutagenesis reactions. The double mutant Asn-119-His/His-311-Gln was constructed by subcloning the *Hind*III restriction fragment of the His-311-Gln mutated DNA to the *Hind*III-digested Asn-119-His mutated DNA. The double mutant Gln-287-Glu/His-311-Gln was constructed by subcloning the *Nco*I/*Sph*I restriction fragment of the His-311-Gln mutated DNA to the *Nco*I/*Sph*I-

digested Gln-287–Glu mutated DNA. All mutations were verified by DNA sequencing on Applied Biosystems sequencer model 373 A with the DyeDeoxy Terminator Cycle sequencing kit.

Molecular modelling

A molecular model of *C. boidinii* FDH was constructed as described elsewhere [16], using the known crystal structures of *Pseudomonas* sp. 101 FDH [5], with which it shares 46 % sequence identity. An alignment of the *C. boidinii* and *Pseudomonas* sequences was generated with CLUSTAL [17]. An iterated protocol involving multiple model construction (10 for each tested alignment) and rigorous protein structure quality assessment, using PROSA II [18], PROFILER_3D [19] and PROCHECK [20], was used [21].

Comparison of the final model with the known structures confirmed that the side-chain conformations of the *C. boidinii* FDH residues considered in this study are likely to be the same as those observed for corresponding residues in the *Pseudomonas* enzyme. This consideration, combined with the good resolution of the known structures, allowed our results for mutant enzymes to be placed in a detailed structural context and to be compared directly with results from other FDHs.

Predictions of side-chain conformations at mutated positions were made using the side-chain rotamer libraries contained in the program O [22]. A search was made for the new side-chain rotamer giving the fewest clashes with the rest of the protein structure.

Assay of enzyme activity and protein

FDH assays were performed at 25 ± 0.1 °C according to a published method [14] using a Hitachi U-2000 double-beam UV-visible spectrophotometer equipped with a thermostatically controlled cell holder (10 mm path length) connected to a circulating water bath. One unit of enzyme activity is defined as the amount that catalyses the conversion of 1 μ mol of NAD⁺ into NADH/min. Protein concentration was determined by the method of Bradford [23], using BSA (fraction V) as standard.

SDS/PAGE

SDS/PAGE was performed according to Laemmli [13] on a 1.5 mm-thick vertical slab gel (14 cm $\times$ 16 cm), containing 12.5 % (w/v) polyacrylamide (running gel) and 2.5 % (w/v) stacking gel. Protein bands were stained with Coomassie Brilliant Blue R-250.

Kinetic analysis of wild-type and mutant enzymes

Steady-state kinetic measurements were performed in 0.1 M potassium phosphate buffer, pH 7.5, by varying the concentration of one of the corresponding substrates, at the saturating concentration of the second substrate, as described by Blanchard and Cleland [2]. All initial velocities were determined in triplicate. The kinetic parameters k_{cat} and K_{m} were calculated by non-linear regression analysis of experimental steady-state data using the GraFit (Erithacus Software) program. Turnover numbers were calculated on the basis of one active site/40 kDa subunit.

pH dependence of k_{cat} and $k_{\text{cat}}/K_{\text{m}}^{\text{HCOOH}}$

The steady-state kinetic parameters for wild-type and the mutants were determined by non-linear regression analysis of initial velocity data in the presence of a saturating concentration of NAD⁺ and varying concentrations of sodium formate in 0.25 M potassium phosphate buffer ranging in pH from 5.3 to 10.5 at 25 °C. The ionic strength of the buffer varies in the pH range

used. However, FDH activity did not show any appreciable dependence on ionic strength. pK_{a} values were estimated by fitting the experimental data to the equation reported by Blanchard and Cleland [2], using the computer program GraFit.

Quenching of the tryptophan fluorescence of the wild-type and Asn-119–His mutant enzymes

Quenching of the tryptophan fluorescence of wild-type and Asn-119–His mutant enzymes was carried out in 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C with an enzyme concentration of 2.3 μ M. The excitation wavelength used was 300 nm and the emission wavelength was 342 nm.

UV difference spectroscopic studies

Far-UV difference spectra were measured with a Perkin–Elmer Lambda 16 recording spectrophotometer at 25 °C. The enzyme (0.5 mg/ml) was dialysed against the appropriate buffer (0.25 M potassium phosphate buffer, pH 5.3–10.5) and its UV difference spectra were recorded after additions of NAD⁺ (0.2 μ mol) and azide (0.1 μ mol).

RESULTS

Cloning, expression and purification of *C. boidinii* FDH

N-terminal amino acid sequencing of purified FDH gave the following sequence: Met-Lys-Ile-Val-Leu-Val-Leu-Tyr-Asp-Ala-Gly-Lys-His-Ala-Asp-Glu-Glu-Lys-Leu-Tyr-Gly-Xaa-Glu-Asn-Lys-Leu-Gly-Ile. CNBr cleavage of the protein gave three different fragments of approx. 20, 15 and 6.5 kDa. Separation of these fragments by SDS/PAGE, and N-terminal amino acid sequencing of the middle fragment gave the following N-terminal sequence: Leu-Val-Leu-Val-Arg-Asn-Phe-Val-Pro. Two synthetic oligonucleotides were designed based on the N-

Figure 1 SDS/PAGE of FDH

SDS/PAGE was performed on a 1.5 mm-thick vertical slab gel (14 cm $\times$ 16 cm) containing 12.5 % (w/v) polyacrylamide (running gel) and 2.5 % (w/v) stacking gel. The protein bands were stained with Coomassie Brilliant Blue R-250. Left lane, uninduced *E. coli* crude extract; middle lane, *E. coli* crude extract after induction with 1 mM IPTG; right lane, FDH purified according to our method.

Figure 2 Stereo ribbon diagram of FDH

Stereo ribbon diagram of a subunit (**A**) and a dimer (**B**) of the *C. boidinii* FDH model with NAD⁺, shown in space-filling mode, bound to the active site.

terminal amino acid sequence and the internal CNBr-cleaved protein fragment sequence, respectively. PCR using *C. boidinii* cDNA amplified a 430 bp fragment. This PCR product was used to screen a cDNA library in λ ZAP II that was constructed using poly(A)⁺ mRNA isolated from *C. boidinii* cells. The positive cDNA clone contained an open reading frame of 1095 bp which encoded a 364-amino-acid polypeptide with a predicted molecular mass of 40 142 Da. The deduced amino acid sequence exhibits 97 % identity (the nucleotide sequence exhibits 93 % identity) with the *fdh1* gene isolated from *C. boidinii* strain S2 [24] and has a predicted isoelectric point of 6.28 (that of FDH1 is 5.86).

FDH was overproduced as a soluble active protein to about 20 % of *E. coli* soluble cell protein (Figure 1), and was purified to homogeneity in a single-step procedure based on affinity chromatography on immobilized biomimetic dye as described previously [10], except that the bound FDH was eluted with a mixture of NAD⁺/sulphite [11]. This purification procedure resulted in 85 % recovery of the enzyme with 5-fold purification. The eluted enzyme was homogeneous, as judged by SDS/PAGE with Coomassie Brilliant Blue staining (Figure 1). All mutants were expressed at approximately the same level as the wild-type enzyme, and were purified according to the same procedure. The only exception was the Asn-119–His mutant, which was purified according to the method of Schütte et al. [14], due to its reduced ability to bind to the biomimetic dye-affinity adsorbent.

Selection of residues for mutagenesis

Most of the residues mutated (Phe-69–Ala, Asn-119–His, Ile-175–Ala, Gln-197–Leu, Arg-258–Ala, Gln-287–Glu, Pro-288–Thr and His-311–Gln) were selected based on our model for

Table 1 Kinetic properties of wild-type and mutants of FDH

Steady-state kinetic measurements were performed at 25 °C in 0.1 M potassium phosphate buffer, pH 7.5. All initial velocities were determined in triplicate. The kinetic parameters k_{cat} and K_m were calculated by non-linear regression analysis of experimental steady-state data using the GraFit program.

Enzyme	$K_m^{\text{NAD}^+}$ (mM)	K_m^{HCOOH} (mM)	k_{cat} (% of wild type)
Wild type	0.04 ± 0.003	2.42 ± 0.2	100
Phe-69–Ala	0.05 ± 0.004	5.23 ± 0.4	8.2
Asn-119–His	50 ± 3.22	190 ± 8.25	0.1
Ile-175–Ala	0.48 ± 0.04	5.42 ± 0.3	10
Gln-197–Leu	0.05 ± 0.005	9.34 ± 0.5	70
Arg-258–Ala	Inactive	Inactive	Inactive
Gln-287–Glu	0.15 ± 0.01	2.91 ± 0.1	87
Gln-287–Glu/Pro-288–Thr	0.11 ± 0.02	920 ± 29	3.1
His-311–Gln	0.04 ± 0.003	24.92 ± 1.25	7.4
Asn-119–Ala/His-311–Ala	108 ± 9.50	225 ± 19	0.05
Gln-287–Ala/His-311–Ala	0.12 ± 0.01	4.52 ± 0.4	64

FDH (Figure 2). These residues are situated in and around the catalytic site and have proposed binding or catalytic functions (Figure 3), based on the crystal structure for the *Pseudomonas* sp. 101 enzyme [5].

Kinetic analysis of wild-type and mutant enzymes

Steady-state kinetic analysis was used to evaluate the effect of each mutation on the catalytic parameters of the reaction. The kinetic parameters k_{cat} and K_m as determined by steady-state kinetic analysis are listed in Table 1. All the mutants show significant kinetic differences when compared with the wild-type

Figure 3 Stereo ball-and-stick representation of the putative interactions of the nicotinamide portion of bound NAD⁺ and azide with the FDH model

The NAD⁺ and azide ligands are drawn with grey bonds and labelled N and A, respectively. Protein residues are drawn with white bonds. Those mutated in this work are shown larger than the others. Possible hydrogen bonds are shown as dashed lines.

Figure 4 pH dependence of kinetic parameters

pH dependence of $\log k_{\text{cat}}$ (○) and $\log k_{\text{cat}}/K_m^{\text{HCOOH}}$ (●) for the wild-type enzyme (A) and mutants Gln-287-Glu (B) and His-311-Gln (C).

enzyme. In particular, the mutants Asn-119-His, His-311-Gln and the double mutants Asn-119-His/His-311-Gln and Gln-287-Glu/Pro-288-Thr exhibited dramatic increases in K_m^{HCOOH}

Table 2 pH dependence of kinetic parameters (k_{cat} and $k_{\text{cat}}/K_m^{\text{HCOOH}}$) of wild-type and mutant FDH

The steady-state kinetic parameters for wild-type and mutant enzymes were determined by non-linear regression analysis of initial velocity data in 0.25 M potassium phosphate buffer ranging in pH from 5.3 to 10.5 at 25 °C. pK_a values were estimated using the computer program GraFit.

Enzyme	pK_a (from the pH/k_{cat} profile)	pK_a (from the $\text{pH}/k_{\text{cat}}/K_m^{\text{HCOOH}}$ profile)
Wild type	$5.95 \pm 0.2, 10.16 \pm 0.2$	$6.1 \pm 0.4, 8.24 \pm 0.3$
Gln-287-Glu	5.92 ± 0.1	7.5 ± 0.3
His-311-Gln	$4.2 \pm 0.1, 9.62 \pm 0.2$	$4.2 \pm 0.3, 8.2 \pm 0.1$
Gln-287-Ala/His-311-Ala	$4.9 \pm 0.2, 10.23 \pm 0.3$	$4.74 \pm 0.4, 8.28 \pm 0.1$

(about 80-, 10-, 94- and 380-fold, respectively, compared with the wild-type enzyme). However, the mutants Phe-69-Ala, Ile-175-Ala, Gln-197-Leu and the double mutant Gln-287-Glu/His-311-Gln exhibited only 2–4-fold increases in K_m^{HCOOH} . The Arg-258-Ala mutant did not show any catalytic activity, even at a very high formate concentration (1000 mM). With respect to $K_m^{\text{NAD}^+}$, all mutants show limited differences compared with the wild-type enzyme, with the exceptions of the Asn-119-His and Ile-175-Ala mutants and the double mutant Asn-119-His/His-311-Gln. These showed respective increases in $K_m^{\text{NAD}^+}$ of around 1250-, 12- and 2700-fold, compared with the wild-type enzyme. The effects on k_{cat} caused by the mutations are listed in Table 1. A significant reduction in the catalytic activity was observed for the mutants Phe-69-Ala, Ile-175-Ala, Asn-119-His, Gln-287-Glu, His-311-Gln and Asn-119-His/His-311-Gln.

pH dependence of k_{cat} and $k_{\text{cat}}/K_m^{\text{HCOOH}}$

The dependence of k_{cat} and $k_{\text{cat}}/K_m^{\text{HCOOH}}$ on pH was studied for the mutants Gln-287-Glu, His-311-Gln and Gln-287-Glu/His-311-Gln in order to define the acid/base properties of this His-Gln hydrogen-bonded pair (Figure 4, Table 2).

The k_{cat} value of the wild-type enzyme decreased below pH 6.0 and above pH 9.0. These transitions were controlled by pK_a values of 5.95 and 10.16, indicating that the protonated enzyme form in the acidic pH range and the deprotonated enzyme form in the basic pH range are catalytically ineffective, in accordance to previous published work [2]. The pH dependence of k_{cat} in the mutants was different from that of the wild-type enzyme. The pH profile for the His-311-Gln mutant showed two clear ionizations with inflection points corresponding to pK_a values of 4.2 and 9.6. In the case of the Gln-287-Glu mutant, the pH/k_{cat} profile shows only a single inflection point in the acidic pH range, with a pK_a of 5.92. The double mutant Gln-287-Glu/His-311-Gln exhibits two inflection points with pK_a values of 4.2 and 10.2.

The $k_{\text{cat}}/K_m^{\text{HCOOH}}$ profiles for the wild type and mutants are shown in Figure 4. The pH profile of the wild type shows clear inflection points corresponding to pK_a values of 6.1 and 8.24. These reflect ionizations of groups relevant to the process $\text{FDH-NAD}^+ + \text{formate} \rightarrow \text{FDH-NAD}^+ - \text{formate}$. The curve as shown in Figure 4 appears to drop with an eventual slope of at least 2, indicating two or more pK_a values at low pH, but there are not enough data to support the calculation of these two pK_a values. The low pK_a is shifted outward by about 2 and 1.5 pH units in the pH profile of the mutants Gln-287-Glu and Gln-287-Glu/His-311-Gln respectively, compared with that of the wild-type enzyme. In contrast, the high pK_a remains the same in mutant and wild-type enzymes. Formate binding is prevented by a single ionization of a group with a pK_a of 7.5 in the mutant Gln-287-Glu (Figure 4).

Figure 5 Fluorescence quenching analysis

Fluorescence quenching analysis of the wild-type enzyme (■) and the Asn-119-His (◆) mutant.

Quenching of the tryptophan fluorescence of the Asn-119-His mutant

Fluorimetric titrations were used to determine the effect of the Asn-119-His mutation on NAD⁺ binding (Figure 5). From the data it is evident that the mutated protein exhibits reduced quenching of the tryptophan fluorescence, indicating that it has essentially lost its ability to bind NAD⁺. In addition, this mutant lost its ability to bind to the Cibacron Blue 3GA-Sepharose affinity column (results not shown), indicating that the mutation not only destroyed the specific ability of the enzyme to bind NAD⁺, but also abolished binding to nucleotide pseudo-analogues. This led us to believe that Asn-119 has an important structural role, as will be discussed below.

DISCUSSION

Cloning, expression and purification of *C. boidinii* FDH

In this report we present the cloning, expression and purification of a new FDH (termed FDH2) isolated from the methylotrophic yeast *C. boidinii*. The deduced amino acid sequence of FDH2 shares 97, 97 and 82% sequence identity, respectively, with the FDH-coding genes of the other methylotrophic yeasts [24,25] *C. boidinii* strain S2, *C. methylica* and *Hansenula polymorpha* (results not shown). In addition, sequence alignment of *C. boidinii* FDH2 with the known FDHs from *Pseudomonas* sp. 101, *Neurospora crassa*, *Solanum tuberosum* and *Aspergillus nidulans* revealed sequence identities of 46, 62, 49 and 63%, respectively.

pH dependence of k_{cat} and $k_{\text{cat}}/K_{\text{m}}^{\text{HCOOH}}$

The effect of pH on the kinetic parameters of the Gln-287-Glu, His-311-Gln and Gln-287-Glu/His-311-Gln mutants was investigated in order to define the acid/base properties of this His-Gln hydrogen-bonded pair. The results from the present and previous investigations [2] suggest that the active site comprises two carboxylate ionizable groups, which play important roles in formate binding and catalysis as observed in the pH/ k_{cat} and pH/ $k_{\text{cat}}/K_{\text{m}}^{\text{HCOOH}}$ profiles. Asp-308 in the *Pseudo-*

Figure 6 Far-UV difference spectroscopy

Top panel: far-UV difference spectra (25 °C) of FDH (curve a) and the FDH-NAD⁺ (curve b) and FDH-NAD⁺-azide (curve c) complexes in 0.25 M potassium phosphate buffer, pH 6.51. Bottom panel: pH dependence of the ΔA at λ_{max} on NAD⁺ (○) and azide (●) binding.

monas enzyme (Asp-282 in the *C. boidinii* enzyme) has been suggested to be involved in catalysis by playing an important role in proper orientation and polarization of the NAD⁺ nicotinamide moiety. This amino acid residue has been assigned the pK_{a} in the acidic pH range observed in the *Pseudomonas* enzyme [26]. Probably the single carboxylate residue seen in the *C. boidinii* FDH pH/ k_{cat} profile and presumably one of the two carboxylates seen in the pH/ $k_{\text{cat}}/K_{\text{m}}^{\text{HCOOH}}$ profile corresponds to Asp-282. An important observation is that its pK_{a} is shifted to a more acidic pH in the His-311-Gln and Gln-287-Glu/His-311-Gln mutant, but not in the Gln-287-Glu mutant. The high pK_{a} in the pH/ k_{cat} profile (pK_{a} , 10.16), which is also found in the *Pseudomonas* enzyme, is believed to have a conformational origin and has been assigned to a co-operative event, which leads to protein reorganization [26]. This is supported, in the present study, by investigating the pH dependence of the UV difference spectra of the enzyme-NAD⁺ complex. Changes in protein conformation are evident from the difference spectra upon NAD⁺ binding, as shown in Figure 6 (top panel). The perturbations observed in the spectra at about 210 nm are due to changes in the secondary structure of the protein [27]. These conformational changes in the secondary structure of FDH exhibit pH dependence with a

pK_a of 10.4 (Figure 6, bottom panel). These data complement the crystal structure information obtained for the *Pseudomonas* enzyme, which revealed substantial conformational changes on NAD^+ binding. These changes included the movement of the catalytic domain around a hinge region by an angle of 7.5° and some secondary-structure reorganization involving formation of a C-terminal helix $\alpha 9$ [5].

The other acidic ionizable group, found in the $pH/k_{cat}/K_m^{HCOOH}$ profile, which must be ionized for formate binding, has been assigned as a carboxylic acid based on the calculated heat of its ionization, ΔH_{ion} (-2.5 kcal/mol [2]) ($1 \text{ cal} \equiv 4.184 \text{ J}$). The high pK_a found in the $pH/k_{cat}/K_m^{HCOOH}$ profile, which exhibits abnormally high ΔH_{ion} (29 kcal/mol [2]), has been attributed to a basic residue such as lysine that hydrogen-bonds to the oxygen of formate [2]. However, no lysine residues, nor other ionizable residues with a possible pK_a value of about 8.2, were observed in the vicinity of the formate-binding site either in the crystal structure of the *Pseudomonas* enzyme [5] or in our model. We can therefore speculate that these pK_a values may be the results of conformational rearrangement of the protein on formate binding. To verify this hypothesis we studied the pH dependence of the UV difference spectra of the enzyme- NAD^+ -azide complex (Figure 6, top panel). The pH dependence of the FDH- NAD^+ complex spectra on azide binding exhibits inflection points corresponding to pK_a values of 6.2 and 8.3 (Figure 6, bottom panel), which are very similar to those observed for the $pH/k_{cat}/K_m^{HCOOH}$ profile (Table 2). This indicates that these transitions relate to changes in conformation of the protein. The shift of the pK_a in the acid pH range observed in Gln-287-Glu, His-311-Gln and Gln-287-Ala/His-311-Ala mutants (Table 2) probably indicates that the conformational changes of the protein on azide binding occur near this region of the protein. Unfortunately there is no crystal structure available of the enzyme- NAD^+ complex to compare with the known enzyme- NAD^+ -azide crystal structure and thereby enable these conformation changes to be assigned to specific parts of the protein structure.

There is a new pH inflection point of 7.5 in the $pH/k_{cat}/K_m^{HCOOH}$ profile for the mutant Gln-287-Glu. This pK_a may be assigned to a His residue hydrogen-bonded to carboxylate, as has been observed for His-Asp pairs in malate, lactate and alcohol dehydrogenases [28], as well as in serine proteases [29]. It seems likely that the mutation 'unlocks' the His-311 residue from the unprotonated state resulting from its interaction with conserved Gln-287 (Figure 3). Since the mutant enzyme exhibits only a moderate decrease in k_{cat} (Table 1), we concluded that the protonation state of His-311 seems to have little effect on catalysis.

Kinetic analysis of wild-type and mutant enzymes

A molecular model of *C. boidinii* FDH was constructed to put the mutant data in a structural context. Comparison with the known crystal structures shows that the *C. boidinii* FDH active-site residues mutated are likely to share the same conformations as their *Pseudomonas* enzyme counterparts. Furthermore, the residues mutated here are conserved across all FDHs, so that our data reveal functional details that will probably be shared by all FDHs.

The effects of the two single point mutations, Arg-258-Ala and Asn-119-His, on the catalytic function of FDH are readily explained using molecular models. The Arg-258-Ala mutant was completely inactive, consistent with the postulated idea that Arg-258 stabilizes the partial negative charge on the migrating hydride ion during reaction [5]. The Asn-119-His mutant retains very low

activity with raised substrate K_m^{HCOOH} values. The model shows that the side chain ND2 atom of Asn-119 forms a $2.8 \text{ \AA}$ hydrogen bond with the azide molecule, assumed to occupy the formate-binding site, while the OD1 atom has non-bonded contacts as close as $3.1 \text{ \AA}$ with the nicotinamide ring of NAD^+ , thus explaining the significant increases in K_m values observed for these substrates when the stabilizing interactions are removed. The fluorescence results shown in Figure 5 and this mutant's reduced ability to bind to Cibacron Blue 3GA-Sepharose adsorbent confirm that the Asn-119-His mutation abolishes ability to bind NAD^+ or coenzyme pseudo-analogues. It is relevant to note that Asn-119 and its following neighbour comprise one of the hinge regions around which the two domains shift on binding of NAD^+ . Hence, an indirect effect on NAD^+ binding through interference in domain motion cannot be ruled out. The drastically reduced k_{cat} value of the Asn-119-His mutant is in line with an additional possible role for this side chain in hydrogen-bonding to the transition state.

Two other single mutations give 90% reductions in k_{cat} . This loss of activity seen for the Phe-69-Ala mutant is in line with the suggested contribution of this residue to a hydrophobic area, which would help to destabilize the formate substrate [5]. Ile-175 has been suggested to have a role, along with Val-123, in forming another hydrophobic face which binds to but destabilizes the positively charged nicotinamide moiety of NAD^+ . The kinetic data for the Ile-175-Ala mutant support this idea with an elevated $K_m^{NAD^+}$ accompanying the loss of catalytic activity.

The His-Gln pair seen in FDHs, corresponding to the His-Glu charge relay system in other NAD^+ -dependent dehydrogenases, was investigated by the construction of three mutants His-311-Gln, Gln-287-Glu and the double mutant His-311-Gln/Gln-287-Glu. The Gln-287-Glu mutant, as observed previously in *Pseudomonas* FDH [6], has kinetic parameters little changed at the assay pH of 7.5. However, its pH-activity profile is radically changed with the loss of the ability to function over a broad pH range. The His-311-Gln mutant, in contrast, showed substantially raised K_m^{HCOOH} and much reduced catalytic activity. When a Gln residue is modelled at position 311, one side chain rotamer is clearly favoured, both for minimizing steric clashes with the rest of the molecule and for mimicking the conformation of the previous His. Nevertheless, clashes with Gln-287, Pro-68 and Phe-69 are inevitable. The change in the pH-activity profile observed for the Gln-287-Glu mutant (Figure 4) provides evidence that His-311 is involved directly in formate binding. However, the steric clashes of the new Gln-311 side chain predicted by modelling could also have functional consequences. A change in Gln-287 conformation might impede entry of formate, since this residue lies in the outer part of the funnel through which this substrate enters. Pro-68 and Phe-69 contribute to the postulated functionally important hydrophobic surface, so that changes in their positioning could affect catalysis. If these latter factors also play a part then the importance of the electrostatic interaction between His-311 and formate in the wild-type enzyme, necessarily limited by locking of the His in its unprotonated state, may be less than previously thought.

Most interestingly, the kinetic parameters of the double mutant His-311-Gln/Gln-287-Glu are much closer to those of the Gln-287-Glu enzyme than those of the His-311-Gln mutant; apparently a Glu residue at position 287 can ameliorate the detrimental effects, on K_m^{HCOOH} and activity, of the Gln at position 311. This finding suggests an interaction between the two new side chains, an idea that can be shown to be plausible by modelling. This interaction avoids the conformational changes in Gln-287, Pro-68 and Phe-69, and their detrimental effects on formate binding and catalysis, proposed above for the His-

311–Gln mutant. When Glu replaces Gln at position 287 it interacts with Gln-311 in a new manner, presumably between OE2 of Glu and NE2 of Gln-311.

In contrast, no compensation effects are seen for the Asn-119–His/His-311–Gln double mutant, consistent with the separation of these two side chains in the structure. The results for this double mutant may be viewed simply as the combination of two deleterious mutations.

The role of the conserved Pro-288 has been investigated in the double mutant Gln-287–Glu/Pro-288–Thr. Pro-288 and Pro-286 are the only *cis*-proline residues found in the crystal structure and lie on either side of the Gln-287 residue. Probably their only function is to provide Gln-287 with the proper orientation to make a hydrogen bond to His-311. The double mutant Gln-287–Glu/Pro-288–Thr exhibits increased K_m^{HCOOH} compared with the single mutant Gln-287–Glu, which is probably due to local conformational changes due to the inability of the new residue to accept a *cis*-peptide bond. These would lead to changes in the His-311 side-chain conformation and make less favoured the hydrogen bond that His-311 would make to formate. Hence, these conformational changes could lead to unproductive binding of formate. This is also supported by the strong reduction in k_{cat} value observed for the Gln-287–Glu/Pro-288–Thr double mutant compared with the Gln-287–Glu single mutant.

In conclusion, the results of spectroscopic and kinetic studies with selected mutants of FDH help an understanding of the catalytic function of the enzyme. The suggested roles for hydrophobic patches in destabilizing substrates and stabilizing products are fully in accord with our data. The key roles of Arg-258 and Asn-119 in formate binding and catalysis are confirmed by our data. A role also for His-311 in formate binding remains possible, but factors other than electrostatic ones may play a role in the altered properties of the His-311–Gln mutant. This is also the clear conclusion from the finding that the Gln-287–Glu/His-311–Gln double mutant has 64 % of wild-type activity.

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