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Site-directed mutagenesis of the formate dehydrogenase active centre: role of the His^{332}-Gln^{313} pair in enzyme catalysis

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Abstract **Gln^{313} and His^{332} residues in the active centre of NAD^+-dependent formate dehydrogenase (EC 1.2.1.2, FDH) from the bacterium *Pseudomonas* sp. 101 are conserved in all FDHs and are equivalent to the glutamate-histidine pair in active sites of D-specific 2-hydroxyacid dehydrogenases. Two mutants of formate dehydrogenase from *Pseudomonas* sp. 101, Gln^{313}Glu and His^{332}Phe, have been obtained and characterised. The Gln^{313}Glu mutation shifts the pK of the group controlling formate binding from less than 5.5 in wild-type enzyme to 7.6 thus indicating that Gln^{313} is essential for the broad pH affinity profile towards substrate. His^{332}Phe mutation leads to a complete loss of enzyme activity. The His^{332}Phe mutant is still able to bind coenzyme but not substrate or analogues. The role of histidine in the active centre of FDH is discussed. The protonation state of His^{332} is not critical for catalysis but vital for substrate binding. A partial positive charge on the histidine imidazole, required for substrate binding, is provided via tight H-bond to the Gln^{313} carboxamide.**

Key words: NAD^+-dependent formate dehydrogenase; Active center; Site-directed mutagenesis; Molecular mechanism

1. Introduction

NAD^+-dependent formate dehydrogenase (EC 1.2.1.2, FDH) from the methylotrophic bacterium *Pseudomonas* sp. 101 is a member of the family of D-specific 2-hydroxyacid dehydrogenases acting on D-stereoisomers of the respective substrates. There is strong sequence similarity between FDH and these dehydrogenases [1]. Crystal structures of FDH [2] and two D-specific dehydrogenases, D-phosphoglycerate dehydrogenase [3] and D-glycerate dehydrogenase [4], revealed significant similarities in the three-dimensional fold of these proteins.

The imidazole moiety of histidine is a ubiquitous acid-base catalyst found in a number of enzymes. One of the classic examples is the proton relay system comprising the serine-histidine-aspartate triad in serine proteases [5]. Acid-base catalysis is an important feature of a number of NAD^+-dependent dehydrogenases acting on 2-hydroxyacids [6–8]. An invariant pair of residues, histidine and a carboxylic acid, occupying conserved positions is found in the active centres of a number of D- and L-specific dehydrogenases [9]. They function as a composite acid-base catalyst facilitating proton transfer to and from the 2-hydroxylic group of the substrate [1,3,4,6–9].

Comparison of the three-dimensional structures of NAD^+-dependent dehydrogenases with that of FDH [10] showed that His^{332} (FDH numbering) is the 'essential' histidine residue, while Gln^{313} substitutes for the conserved carboxylate. Replacement of glutamate for glutamine is highly specific and conserved throughout all known FDH sequences [11]. The glutamine residue is located within the Pro-**Gln**-Pro-Ala-Pro fingerprint. In FDH the two prolines flanking Gln^{313} are in the *cis*-conformation and force the amide group of the glutamine to face His^{332} NE2. As a result of this interaction His^{332} forms a tight H-bond (2.8 Å) with Gln^{313} NE2 and is trapped in a non-protonated state [2,10,11].

A schematic view of the FDH active centre is presented in Fig. 1. Three amino acid residues in the vicinity of the catalytic C4 position of the nicotinamide moiety of NAD^+, Asn^{146}, Arg^{284} and His^{332}, can be involved in substrate binding. In the FDH-NAD^+-azide crystal structure Arg^{284} and Asn^{146} are directly involved in binding azide, the closest structural and electronic analogue of the CO_2 molecule, the product of the reaction [12]. On this basis it was proposed that the substrate binding site overlaps with that for azide and that formate anion binds to FDH via two hydrogen bonds to Arg^{284} and Asn^{146} [2,11]. This was further corroborated by Arg^{284} mutagenesis studies in FDH (Galkin, A., unpublished data) and comparison with the known three-dimensional structures of a number of D- and L-specific dehydrogenases of 2-hydroxyacids which contain a conserved arginine residue responsible for binding the carboxylate group of the substrate [2–4]. The role of His^{332} in FDH remains somewhat unclear. Computer modeling suggests that His^{332} may bind substrate giving rise to equally good geometry of the putative transition state complex [11] although this is not evident from the FDH-NAD^+-azide crystal structure [2].

The goal of the present work was to investigate the role of His^{332} and Gln^{313} in FDH catalysis and the implication of 'locking' of His^{332} in only one protonation state.

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Abbreviations: FDH, NAD^+-dependent formate dehydrogenase (EC 1.2.1.2); K_d^{NAD}, dissociation constant of NAD^+ from the binary FDH-NAD^+ complex

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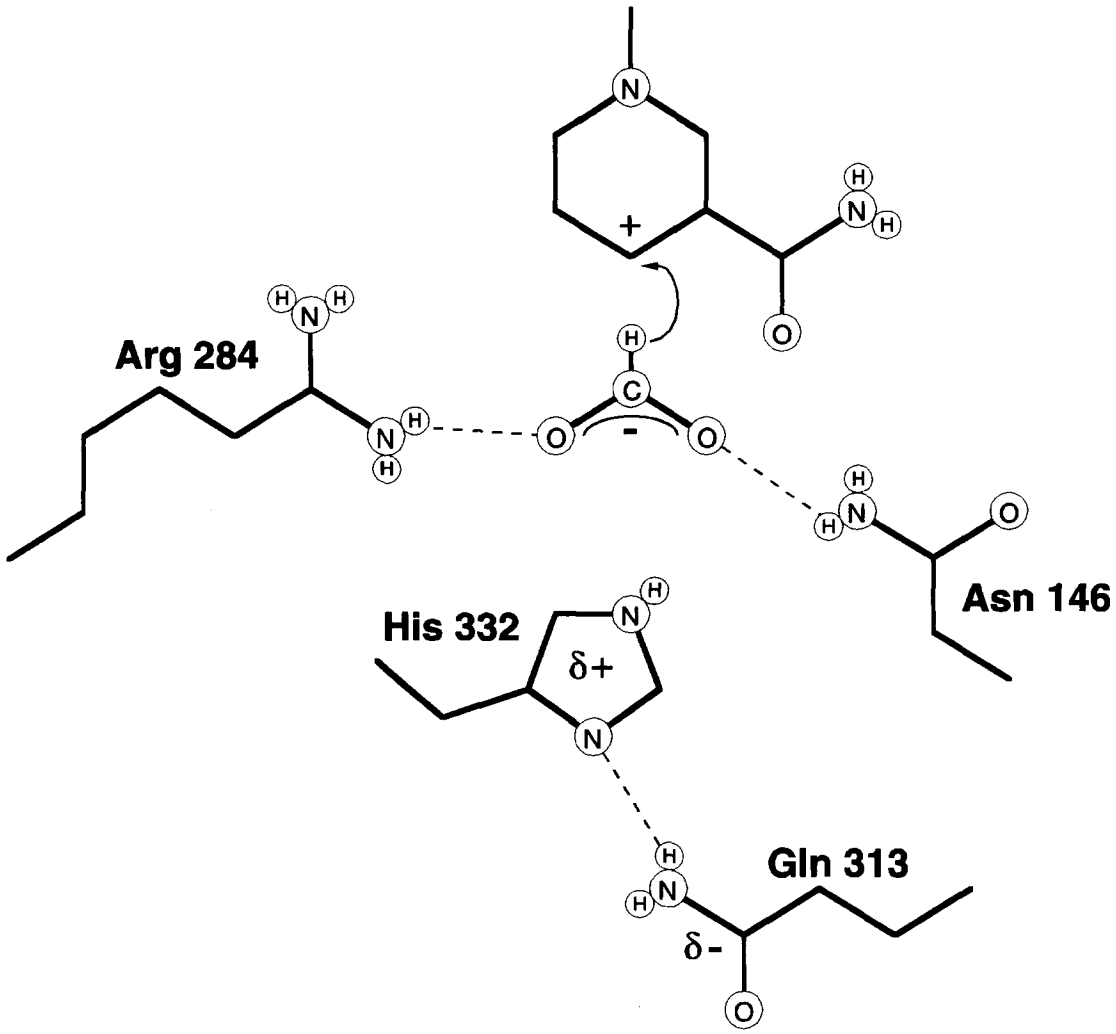

Fig. 1. Schematic diagram of the active centre of formate dehydrogenase from *Pseudomonas* sp. 101. Side chains shown are those of the residues involved in formate binding. The nicotinamide moiety of NAD^+ is shown. H-bonds are indicated by dashed lines.

2. Materials and methods

2.1. Materials

All chemicals used for genetic engineering manipulations were of molecular biology grade (Sigma). T4 polynucleotide kinase, T4 DNA polymerase and ligase were from New England Biolabs. Oligonucleotides were prepared with an Applied Biosystems DNA Synthesizer 380B. NAD^+ (grade V), NADH (grade IV), sodium azide (Sigma) and sodium formate (analytical grade, Reachim, Russia) were used in kinetic and spectrofluorimetric experiments.

2.2. Methods

2.2.1. Mutant preparation Uracil single stranded plasmid pFDH6 was prepared according to [13]. The plasmid pFDH6 was obtained by cloning the *Bam*HI-*Eco*RI fragment containing the FDH gene and *tac* promoter, from the plasmid pFDH4 [14] to the phagemid pUC119. Site-directed mutagenesis reactions were performed using phosphorylated 25-mer 5′-CGGCGCCGGCTCCGGAAACCACACG-3′ and 23-mer 5′-GCCGGAGATGAACGGGGTCATGC-3′ primers to produce $Gln^{313}Glu$ and $His^{332}Phe$ mutations, respectively. The *E. coli* TG1 cell line was used for the transformation by the reaction products. Screening of mutants was performed by DNA sequencing with an Applied Biosystems Automated DNA Sequencer 370A and a PCR Taq dye-labeled terminators sequencing kit. The mutants were expressed in *E. coli* TG1 cells and purified as for wild-type recombinant enzyme [14]. The concentration of inactive $His^{332}Phe$ mutant enzyme was measured by ELISA as described in [15].

2.2.2. Characterisation of mutants V_{max} and K_m for wild-type and $Gln^{313}Glu$ FDH were measured in 0.1 M sodium phosphate, Tris-HCl and sodium pyrophosphate buffers by varying the concentration of one of the corresponding substrates at the saturating concentration of the second as described earlier [16]. Spectrofluorimetric determination of binding of NAD^+ and azide to wild-type and $His^{332}Phe$ FDH were carried out according to [17].

3. Results and discussion

Crystallographic studies do not exclude the possibility of His^{332} being involved in substrate binding [2]. To bind formate anion efficiently His^{332} imidazole should carry a positive charge while high resolution crystal structures unambiguously reveal that His^{332} protonation is hindered because of the tight H-bond to the conserved Gln^{313}. Moreover the involvement of histidine is not supported by the pH profiles of kinetic parameters. Maximal reaction rate, defined by the rate of hydride ion transfer [16,18], is constant within the whole range of enzyme pH stability (5.5–11.2), while the affinity for substrate, substrate analogues and coenzyme simultaneously decreases at pHs higher than 10 with an apparent pK of 10.5 ± 0.2. This has been attributed to a protein conformational change (Mesentsev, A., unpublished data).

Fig. 2 presents the expected shifts in the acid-base proper-

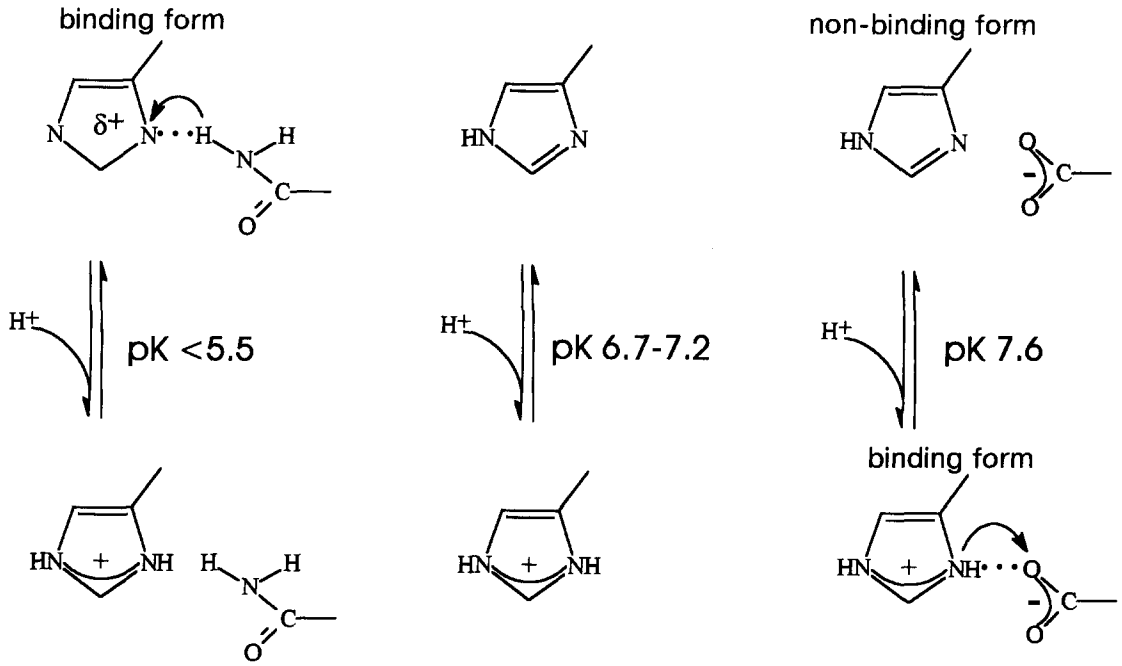

Fig. 2. Protonation of the histidine imidazole: (left) H-bonded to an amide group; (middle) isolated; (right) H-bonded to a carboxylate group.

ties of a histidine H-bonded to a carboxylate group of aspartate or glutamate (as in D- and L-specific dehydrogenases, respectively) or glutamine (as in FDH) compared to the isolated imidazole group of a non-H-bonded histidine. Usually the imidazole of histidines in proteins is characterised by a pK of 6.4–7.2 [19]. Pairing to a carboxylate group of a glutamic or aspartic acid shifts the acid-base equilibrium towards the protonated state of histidine, making it a stronger base and thus increasing its pK. In contrast, the counterpart (glutamate or aspartate) becomes a stronger acid. H-bonding to a more basic group, e.g. the amide of glutamine, stabilises the imidazole moiety in the non-protonated state and lowers its pK.

The tight H-bond between His^{332} and Gln^{313} NE2 shifts the pK of the histidine to the acidic side, probably far beyond the pH range in which the enzyme is stable (5.5). A partial proton transfer from glutamine to histidine induces a positive charge on the imidazole and facilitates negatively charged formate binding. This plays an important role in the mechanism. The stronger the partial charge, the stronger formate binding should be. Fully protonated His^{332} (a $HisH^+/Glu^-$ ion pair) would be the best ligand for formate. Absence of a (partial) positive charge would hinder formate binding. Mutation of His^{332} to any residue without the ability to form H-bonds is thus expected to destroy formate binding.

The histidine and glutamine residues in the FDH active site were replaced by phenylalanine and glutamate, respectively. The first mutation is expected to preserve the overall active site architecture but does not allow H-bonding to other groups. Mutation of Gln^{313} to glutamate should release the 'protonation lock' imposed onto His^{332}. The results are presented in Table 1 and Figs. 3 and 4.

The His^{332}Phe mutant has no catalytic activity, attributed to loss of ability to bind substrate. The mutant does not bind azide, the strongest inhibitor competitive to formate. It is revealed by a lack of quenching of the protein tryptophan fluorescence while formation of the FDH-NAD^+-azide complex [17] (Fig. 3). In contrast azide strongly binds to wild-type FDH-NAD^+. According to experiments on quenching of protein fluorescence by coenzyme (Table 1), the His^{332}Phe mutant has nearly the same affinity for NAD^+ as wild-type FDH. This indicates that any possible changes in the structure of the active centre are local and do not extend to the coenzyme binding subsite.

The mutation Gln^{313}Glu results in the predicted effects on FDH kinetic parameters (Table 1). The mutant has the same catalytic activity as the wild type and the same affinity for coenzyme. The mutant showed stronger formate binding than the wild type in the pH range 6.0–7.0 where His^{332} is expected to be protonated (Table 1, Fig. 4). There is a new pH transition for K_m for formate with a pK of 7.6 ± 0.2 (Fig. 4). The value of the pK is very close to that expected for a histidine H-bonded to a carboxylate (Fig. 2). The pH transi-

Table 1
Kinetic properties of wild-type and mutant formate dehydrogenases (pH 7.0, 37°C)

Enzyme	k_{cat} (% to wild type)	$K_m^{formate}$ (mM)	K_i^{azide} (μM)	K_m^{NAD} (mM)	K_b^{NAD} (mM)[a]
wild-type FDH	100	7	0.15	0.07	0.5
His^{332}Phe	0	–	no binding	–	1.2
Gln^{313}Glu	100	4	n.d.[b]	0.07	n.d.

[a] K_d^{NAD} was determined by quenching of the protein tryptophan fluorescence as described in [17].
[b] n.d., not determined.

tion is likely to be a result of the 'unlocking' of His^{332} and its H-bonding to the carboxylate group of Glu^{313}. Thus, the $Gln^{313}Glu$ mutation affects the binding of formate to the active site due to change of the pK of His^{332}. Since formate anion is bound, the protonation state of His^{332} seems not to be essential for catalysis. Both the wild type and the $Gln^{313}Glu$ mutant show the same catalytic activity within a wide range of pHs (Fig. 4).

The high degree of conservation of Gln^{313} and the 'fingerprint' sequence pattern in FDHs from various organisms suggest an important role for this residue. It provides an extremely low pK and a partial positive charge on its counterpart, the His^{332} imidazole. The affinity of the $Gln^{313}Glu$ mutant towards substrates is slightly superior to the wild type at neutral pH but rapidly decreases at basic pH. The optimum pH range for substrate binding for wild-type FDH is much broader (5.5–10.5) thus increasing the viability of the cell under unfavourable environmental conditions.

4. Conclusions

Previously it was suggested that one of the formate ligands is the guanidinium group of Arg^{284} [2,11]. Several lines of evidence confirm this assumption, including (a) the X-ray structures [2], (b) comparison with known three-dimensional structures of a number of D- and L-specific dehydrogenases of 2-hydroxyacids where a conservative arginine residue binds the carboxylate group of the substrate [10] and (c) Arg^{284}

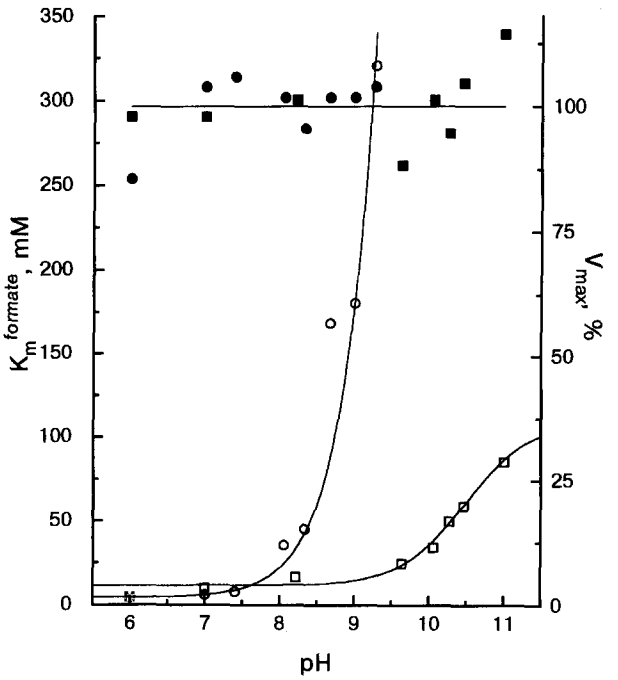

Fig. 4. pH profiles of V_{max} and $K_m^{formate}$ of wild-type FDH (■ and □) and the $His^{332}Phe$ mutant (● and ○). Measurements were carried out in 0.1 M potassium phosphate, Tris-HCl and sodium pyrophosphate buffers at 37°C in the presence of a saturating concentration of NAD^+ (2.5 mM). Enzyme concentration was 10^{-7} M.

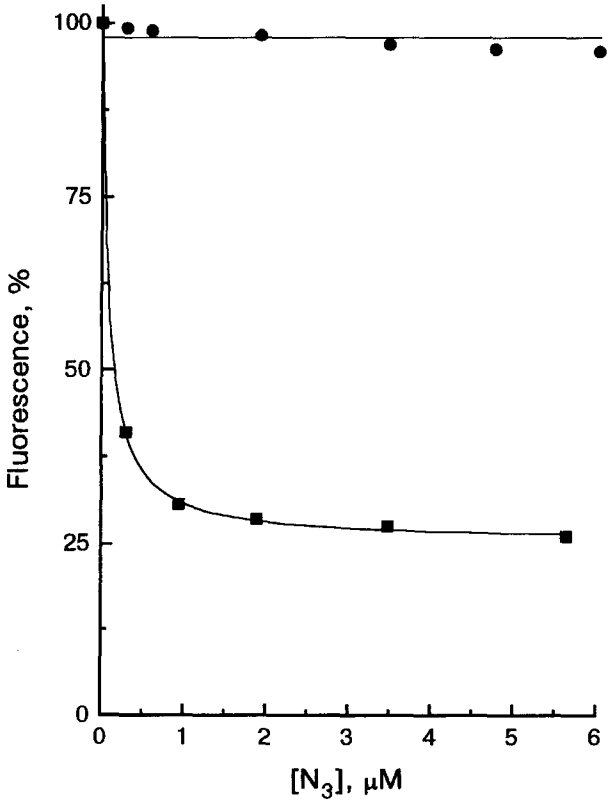

Fig. 3. Quenching of the tryptophan fluorescence of wild-type FDH (■) and the $His^{332}Phe$ mutant (●) in the presence of NAD^+ (6×10^{-4} M) as a function of azide concentration. Measurements were carried out in 0.1 M potassium phosphate, pH 7.0 at 37°C. Enzyme concentration was 4.4×10^{-6} M. Exitation wavelength 300 nm, emission wavelength 343 nm.

mutagenesis studies in FDH (Galkin, A., unpublished data). However, the position of the formate anion in the active site remains uncertain. On the basis of the FDH-NAD^+-azide crystal structure the hypothetical formate binding site can only be that of the azide, i.e. between Arg^{284} and Asn^{146} side chains (Fig. 1). There is not enough room between Arg^{284} and His^{332} for the formate anion and the large alterations in the local conformation of the active site residues are required to accommodate the substrate at this position and allow H-bonding to His^{332}.

As shown in the present study, the protonation state of His^{332} is not critical for catalysis but vital for substrate binding. Thus His^{332}, as the putative third substrate ligand, seems to provide favourable electrostatic interaction, in addition to H-bonds formed between formate and Arg^{284} and Asn^{146} side chains. Further structural studies of formate containing complexes are required to localise the substrate binding site and to draw more detailed conclusions about FDH mechanism.

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