Effect of pH on kinetic parameters of NAD$^+$-dependent formate dehydrogenase

Alexander V. MESENTSEV*, Victor S. LAMZIN†, Vladimir I. TISHKOV‡, Tatyana B. USTINNIKOVA* and Vladimir O. POPOV*§

*A. N. Bakh Institute of Biochemistry, Russian Academy of Sciences, Leninsky pr. 33, Moscow 117071, Russia, †European Molecular Biology Laboratory (EMBL), c/o DESY, Notkestrasse 85, 22603, Hamburg, Federal Republic of Germany, and ‡Department of Chemical Enzymology, M.V. Lomonosov State University, Leninskie Gori, Moscow 119899, Russia

To define in detail the molecular mechanism of NAD$^+$-dependent formate dehydrogenase, the pH dependences of various kinetic and spectroscopic parameters have been studied: V_{max}, K_m (NAD$^+$), K_m (formate), inhibition constants for structural analogues of substrate (NO_3^-) and product (CNS^-, CNO^-, N_3^-), CD and fluorescence properties. The value of V_{max}, rate-limiting hydride transfer, is nearly constant throughout the entire pH range of enzyme stability (6.0–11.2) but decreases below 6. The K_m values for both substrates remain constant within the pH range 6–10. At pH values below 6 (for the coenzyme) and above 10 (for both substrate and coenzyme) the K_m values increase. In the acidic range this change is attributed to the ionization of two carboxy groups (pK approx. 5.5–6.0) located at the NAD$^+$-binding site of the enzyme active centre. The pH transition in the basic region (pK 10.5 ± 0.2) has a conformational origin and affects the enzyme's affinity for substrates and anion inhibitors. A similar transition has been observed for formate dehydrogenases from yeast *Candida boidinii* and *Hansenula polymorpha*. The results complement the conclusions about the catalytic mechanism deduced from the crystal structure of the enzyme.

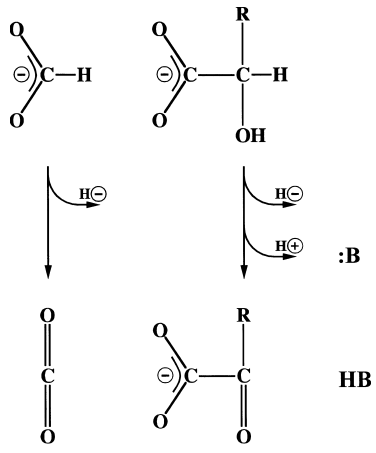
INTRODUCTION

Acid–base catalysis is an important feature of NAD$^+$-dependent dehydrogenases acting on 2-hydroxyacids [1–6]. An invariant pair of residues, a histidine and a carboxylic acid occupying conserved spatial positions, is found in the active centres of a number of these enzymes. A conserved histidine, hydrogen-bonded to the carboxylic residue (aspartic in L-specific dehydrogenases, glutamic in D-specific ones) comprises a composite acid–base catalyst providing a proton shuttle to and from the 2-hydroxy group of the substrate [7].

NAD$^+$-dependent formate dehydrogenase (FDH; formate: NAD$^+$ oxidoreductase, EC 1.2.1.2) from the methylotrophic bacterium *Pseudomonas* sp. 101 (pFDH) is one of the most extensively characterized NAD$^+$-dependent dehydrogenases. Three high-resolution crystal structures of this protein are available [8,9]. Structural information combined with kinetic data and physicochemical studies in solution provided the basis for a tentative molecular mechanism of FDH [8,10].

FDH is a member of the family of D-specific 2-hydroxyacid dehydrogenases acting on D-stereoisomers of the respective substrates. There is extensive similarity between FDH and these dehydrogenases both at the level of amino acid sequence [10–12] and three-dimensional structures [13–15]. However, FDH occupies a special place within the family. The FDH substrate, the formate anion, contains a single carbon atom. During the reaction a hydride anion is transferred to NAD$^+$ C4N. No abstraction or proton uptake is expected, thus making a proton relay chain, characteristic of dehydrogenases acting on 2-hydroxyacids, unnecessary [1–3] (Scheme 1). The substrate-binding site (Figure 1), as deduced from the crystal structure of the ternary complex pFDH-NAD-azide (regarded as a transition state analogue) is formed by Arg-284 and Asn-146. These two residues are supposed to form hydrogen bonds to the oxygen atoms of formate [8,10].

The pH profiles of the catalytic parameters of 2-hydroxyacid dehydrogenases reveal a profound pH transition at approx. pH 7 (which may be shifted to the basic region owing to pairing with the acidic residue) attributed to the ionization of a catalytic histidine residue [3,6,7,12]. In pFDH, His-332 corresponds to the conserved histidine residue, whereas Gln-313 substitutes for the conserved glutamic residue. His-332 forms a tight hydrogen bond (2.8 Å) with the amide nitrogen of Gln-313 and is thus



Scheme 1 Comparison of the proton relay systems in FDH and 2-hydroxyacid dehydrogenases

Abbreviations: B, a composite acid–base catalyst, imidazole of histidine coupled with carboxylate of an aspartate (L-specific dehydrogenases) or glutamate (D-specific dehydrogenases).

Abbreviations used: FDH, NAD$^+$-dependent formate dehydrogenase; pFDH, FDH from the methylotrophic bacterium *Pseudomonas* sp. 101; cFDH, FDH from the methylotrophic yeast *Candida boidinii*; hFDH, FDH from the methylotrophic yeast *Hansenula polymorpha*.

§ To whom correspondence should be addressed.

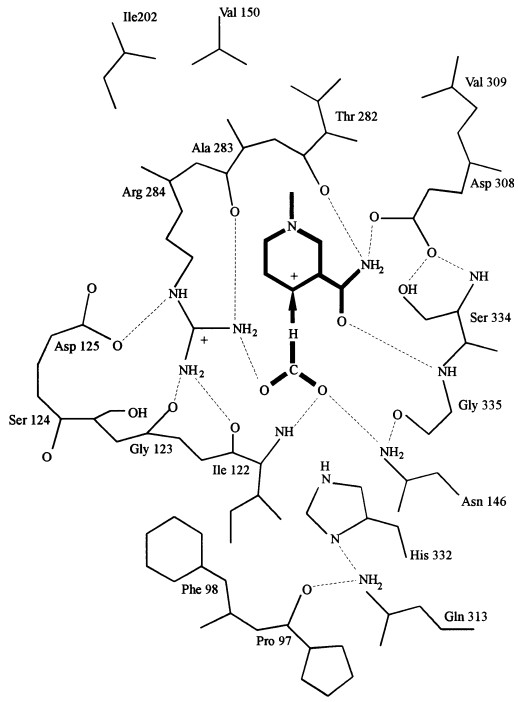


Figure 1 Diagram of the pFDH active centre

trapped in a non-protonated state [8]. The replacement of a glutamic residue by glutamine is highly specific and is conserved throughout all known FDH sequences [8].

Studies of the pH dependences of the kinetic, equilibrium and physicochemical parameters of an enzymic reaction is an informative approach towards elucidation of the molecular mechanism and can provide valuable insight into details of the active centre organization and functioning. All non-metal-containing FDHs have similar kinetic properties and are characterized by a wide pH optimum of catalytic activity in the neutral pH range [10]. The pH dependences of the kinetic parameters have been previously described for the enzyme from the methylotrophic yeast *Candida boidinii* (cFDH) [16] and for pFDH [17]. Here we undertake a systematic investigation of the pH dependences of the kinetic parameters of the reaction catalysed by pFDH to allow a combination of the results with the information available on the structure of this enzyme and its tentative molecular mechanism of action.

EXPERIMENTAL

Materials

The following chemicals were used: NAD^+ (Reanal, Budapest, Hungary), EDTA, TRIZMA-base (Sigma, St. Louis, MO, U.S.A.). Reagents used for mutagenesis were of 'molecular biology' or 'DNA purification' grade. All other chemicals were from Reachim (Moscow, Russia) of the highest purity available.

Methods

pFDH was purified as described [18]. FDH from the methylotrophic yeast *Hansenula polymorpha* (hFDH) was obtained by a similar procedure, modified by adding a thermoinactivation step (10 min at 55 °C) before $(NH_4)_2SO_4$ fractionation. Enzyme samples were homogeneous as judged by SDS/PAGE [19].

Partly purified samples of cFDH were obtained as follows. A cell suspension in 10 mM potassium phosphate buffer, pH 7.5, was disrupted in a mill with glass beads at 5 °C for 15 min. The cell homogenate was centrifuged and the supernatant heated to 59 °C in the presence of 4 % (w/v) sodium formate and incubated for 10 min. After heat denaturation the suspension was cooled to 20 °C on ice and centrifuged; the resulting supernatant was applied to a Q-Sepharose (Pharmacia, Uppsala, Sweden) column (3 cm × 10 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.5. cFDH was eluted with the same buffer, pH 7.5, containing 0.15 M NaCl. Active fractions were pooled and subjected to hydrophobic chromatography on an octyl-Sepharose (Pharmacia) column (6 cm × 10 cm), equilibrated with 20 mM potassium phosphate buffer, pH 7.5, containing 60 % saturated $(NH_4)_2SO_4$. cFDH was eluted from the column with a linear descending gradient of $(NH_4)_2SO_4$ (60 % to 0 % saturation) in the same buffer. The purity of the protein thus obtained was not less than 80 % as judged by SDS/PAGE.

Mutants of pFDH were obtained as described [20].

The enzymic activity was measured spectrophotometrically with a Hitachi 557 spectrophotometer by monitoring the increase in NADH concentration (ϵ_{340} 6.22 $mM^{-1} \cdot cm^{-1}$) at 37 °C. Fresh solutions of both the substrate and coenzyme in a buffer solution of specified pH were prepared immediately before the experiment. The reaction was initiated with an aliquot of the enzyme (pH 7.0). The total volume of the reaction mixture was 2 ml.

The kinetic parameters (K_m and V_{max}) were obtained as follows. Preliminary estimates of V_{max} and saturating substrate concentrations were obtained at each pH value by successively doubling the concentration of each of the reactants from initial values of 80 μM for NAD^+ and 20 mM for formate. If a change in the initial rate of the reaction after the last increase of the reactant concentration did not exceed 1 % this value was considered to be close to V_{max} and the concentration of the reactant last varied was assumed to be saturating.

The following concentrations (M) of formate obtained as described were used as saturating at the following pH values: 0.2 (pH 5.2–9.4), 0.35 (9.4–10), 0.6 (10–10.35), 0.85 (10.35–11.0). The following concentrations (mM) of NAD^+ obtained as described were used as saturating at the following pH values: 13 (pH 5.2), 5 (5.4), 4 (5.55), 2 (6), 1 (6.5–9.6), 2 (9.6–10.4), 5 (10.4–11).

When determining kinetic parameters the concentration of one of the substrates was kept constant at saturation, while the concentration of the other substrate, [S], was varied from saturating to close to K_m. At least eight data points for each data set were obtained. K_m and V_{max} values were obtained by nonlinear regression to the Michaelis–Menten equation:

$$v = V_{max}[S]/(K_m + [S]) \quad (1)$$

The inhibition constants at each pH value were obtained from a data set of at least 36 points. The formate concentration was varied from saturation to K_m (at least six data points) at a fixed saturating NAD^+ concentration (see above) at each value of inhibitor concentration (from zero to about $10K_i$, at least six data sets). Reciprocal initial rates were plotted against the reciprocal of formate concentration (primary plot) at each inhibitor concentration and the slopes were obtained by the least-squares method, assuming equal variances for v. The slopes then were plotted against inhibitor concentrations, [I] (secondary plot), and fitted to eqn. (2) with the statistical weight for each data point proportional to $1/\sigma^2$ obtained from the primary plot:

$$slope = (K_m/V_{max})(1 + [I]/K_i) \quad (2)$$

The following buffer systems were used throughout the pH ranges studied: 0.1 M potassium phosphate (pH 5.4–8.5) (buffer A); 0.5 M potassium phosphate (pH 7–9.8 and 10.4–11.2) (buffer B); 0.5 M sodium EDTA titrated with sodium phosphate (pH 9.3–11.2) (buffer C). Buffers A and B contained 5 mM EDTA as a stabilizer of the enzyme activity. In the overlapping regions (pH 7–8.5 for buffers A and B and 10.4–11.2 for buffers B and C) no effect of buffer concentration (buffer A compared with buffer B) or buffer type (buffer B compared with buffer C) on enzyme activity was observed. The buffer solutions were made with doubly distilled water. The pH was monitored with a pH meter OP211/1 (Radelcis, Budapest, Hungary) with a precision of 0.05 pH units.

The lifetime of FDH at extreme pH values has been determined. The half-inactivation period of FDHs used in the present study at pH 5.2 and 11.2 were 2 and 2.5 min respectively, long enough to obtain the unbiased initial rate of the reaction. Outside these pH values the half-inactivation period was less than 0.5 min, too short for reliable measurements of kinetic parameters, precluding extension of the pH range studied. The rapid spectrophotometric assay of enzyme activity used in the present work enabled a reliable determination of the initial rate of enzyme reaction within 10–15 s. The initial rate of the reaction at the extreme pH values (5.2 and 11) where irreversible inactivation could contribute to the enzyme assay was evaluated as the tangent to the experimental curve at zero time.

To evaluate ΔH of ionization the respective pK values were obtained at 40, 37 and 25 °C. At least two independent sets of experiments (five to nine data points) for each particular temperature were performed and an average value of pK was used for further estimates. The following values were obtained: pK_H 5.85 ± 0.20 (25 °C), 6.00 ± 0.10 (37 °C) and 5.95 ± 0.05 (40 °C); pK'_H 5.30 ± 0.20 (25 °C), 5.40 ± 0.10 (37 °C) and 5.20 ± 0.10 (40 °C). A linear relation between pK and the reciprocal of temperature ($1/T$) was used for the evaluation of ΔH.

CD spectra were measured at 18 °C with a JASCO J-40AS instrument equipped with a 0.1 mm cell. The FDH concentration was 2 μM.

The fluorescence measurements were performed with a Hitachi 3MF spectrofluorimeter at 18 °C and a protein concentration of 2 μM (emission wavelength 300 nm). Before spectroscopic measurements the protein samples were filtered through a membrane filter with a pore size of 0.22 μm.

RESULTS AND DISCUSSION

Effect of pH on V_{max}

The pH dependences of V_{max} and K_m for the coenzyme and the substrate are presented in Figure 2. V_{max} was nearly constant throughout the pH range 6.0–11.2 but decreased by 70 % at the acidic edge of the enzyme's pH stability. The pH transition is controlled by a pK of 5.35 ± 0.05 and implicates the uptake of one proton (Figure 2). The best fit to the experimental data was obtained by assuming that the protonated enzyme form is catalytically incompetent.

Earlier studies revealed that hydride ion transfer in the central ternary complex is the rate-limiting step in the reaction [16,21]. The independence of the rate of hydride transfer over a wide pH range, spanning more than 5 pH units, suggests that such acid–base catalysts as the imidazole of histidine, the amino group of lysine, the hydroxy group of tyrosine and the thiol of cysteine with pK values within this interval are probably not involved in the catalysis. Similar conclusions have been derived from the analysis of the crystal structure of pFDH [8,10].

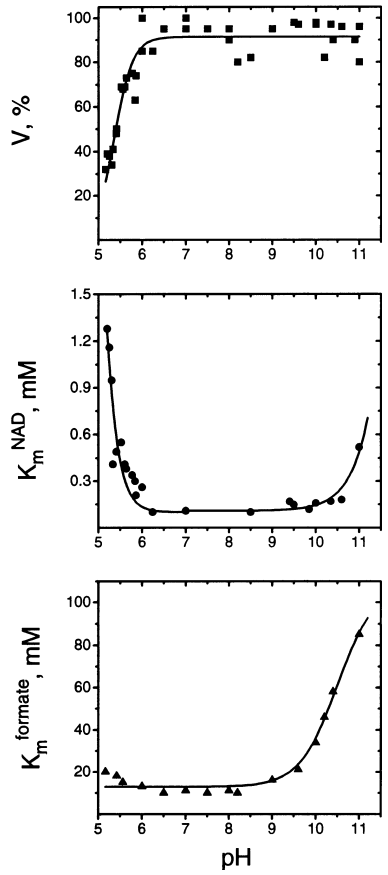


Figure 2 pH dependences of the pFDH reaction kinetic parameters at 37 °C

Curves drawn are theoretical, obtained by computer simulation. Top panel, V_{max} against pH [pK 5.35, n (number of protons) = 1]; middle panel, K_m (NAD^+) against pH [pK_H 6.0, pK'_H 5.4, $n = 2$ (acidic region) (see Scheme 2), pK 10.6, $n = 1$ (basic region)]; bottom panel, K_m (formate) against pH (pK 10.4, $n = 1$).

Effect of pH on substrate and coenzyme binding in the acidic range

K_m values for both formate and NAD^+ remained constant within a wide pH range. At pH values above 10 (for formate) and below 6 and above 10 (for NAD^+) K_m values increased. These changes reflect the ionization of the groups responsible for substrate binding because the catalytic constant remained essentially unchanged over this pH range. For pFDH, which follows a random equilibrium kinetic mechanism, the K_m for the respective substrate is equal to the dissociation constant of the reactant from the central ternary complex [22]. A statistical analysis (Figure 3) revealed that the best fit to the experimental data in the acidic pH range was achieved with a synchronous protonation of two ionogenic groups responsible for NAD^+ binding and assuming that the protonated enzyme form, EH_2^{2+}-NAD, is not catalytically competent (Scheme 2).

Characteristic pK values obtained for the acidic part of the pH profile, a pK_H of 6.0 ± 0.1 and a pK'_H of 5.4 ± 0.1, can be attributed to either the carboxy groups of aspartate or glutamate (typical pK 4.0–5.5) or an acidically shifted imidazole of histidine (typical pK 6.6–7.2) [23]. However, investigation of the temperature dependence of the observed pH transition showed that within the range 25–40 °C the ΔH of ionization was close to

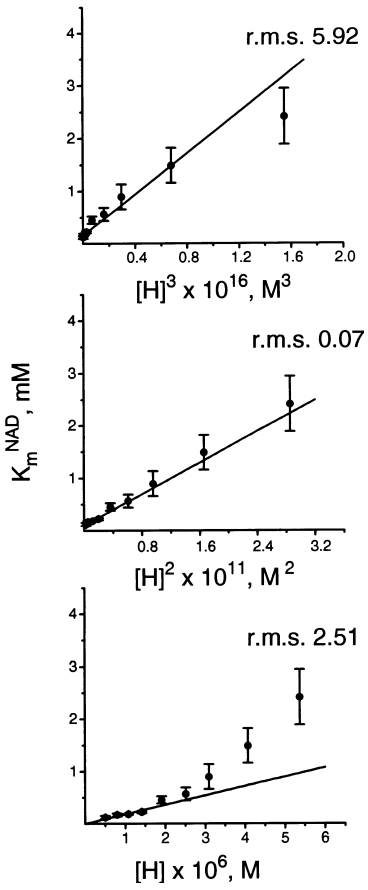
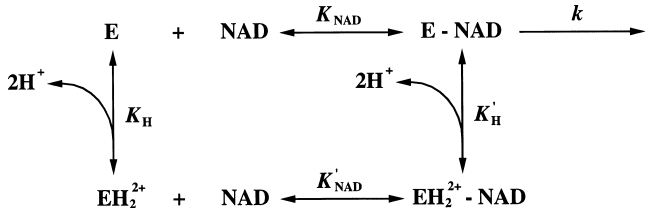


Figure 3 Linear regression of K_m (NAD^+) in the acidic pH range as a function of proton concentration ([H], $[H]^2$, $[H]^3$)

Error bars represent S.D. for each data point. The reciprocal value of the squared S.D. has been taken as a statistical weight. Abbreviation: r.m.s., root-mean-square deviation.



Scheme 2 Ionization of pFDH in the acidic region

Abbreviations: K_{NAD} and K'_{NAD} are the NAD^+ dissociation constants from the E–NAD^+ and EH_2^{2+}–NAD^+ complexes, K_H and K'_H are the protonation constants of the apo-enzyme and the holo-enzyme, and E stands for the FDH–formate binary complex.

zero (-7.5 ± 7.9 kJ/mol). This is consistent only with carboxy groups. The ΔH of ionization of the imidazole group of histidine is much higher, 29–34 kJ/mol [23,24].

The results suggest that the pFDH active centre comprises two ionogenic groups, aspartate or glutamate, with similar pK values. The ionization states of these groups affect coenzyme binding, whereas one of them is implicated in catalysis. The acidic shift of pK of these carboxylates on coenzyme binding suggests a stabilization of their ionization state in the binary complex with

Table 1 Characteristic pH transitions of FDHs in the basic region

Measurements were carried out at 37 °C as described under 'Methods'. Abbreviation: n.d., not determined.

Parameter	Value for pFDH (μM)	pK pFDH	pK cFDH	pK hFDH
K_m (NAD^+)	110	10.6 ± 0.2	10.5 ± 0.1	n.d.
K_m (formate)	15000	10.4 ± 0.1	10.5 ± 0.1	10.2 ± 0.1
K_i (NO_3^-)	1000	10.5 ± 0.1	n.d.	n.d.
K_i (N_3^-)	0.15	10.7 ± 0.2	n.d.	n.d.
K_i (rodanide)	300	10.5 ± 0.1	n.d.	n.d.
K_i (CNO^-)	0.20	10.5 ± 0.1	n.d.	n.d.
Fluorescence	–	10.6 ± 0.1	n.d.	n.d.
CD at 210 nm	–	10.2 ± 0.2	n.d.	n.d.

NAD^+, possibly through electrostatic interaction with the electropositive groups of coenzyme.

Chemical modification of the carboxy groups in pFDH by Woodward's reagent also revealed two concertedly ionizing carboxylic residues with a pK of 5.20 ± 0.05 [25], close to the pK of the groups controlling NAD^+ binding described above. One of these carboxylic residues has been shown to be screened from modification in the presence of NAD^+ and to be essential for coenzyme binding [25].

Somewhat different results were obtained earlier for cFDH [16]. A group with a pK of approx. 6.4 and no temperature dependence (carboxylic acid) must be ionized for binding of the azide and formate, and another group with a pK of approx. 5.9 must be ionized for catalysis. There might be two or more unresolved pK values at low pH because the pH dependence had a slope of approx. 2. However, no carboxylic residues are in the vicinity of the formate-binding site in the crystal structure of pFDH.

In the pFDH crystal structure [8] the coenzyme-binding site comprises two aspartic residues that play an important role in cofactor binding. Asp-221 governs the enzyme preference towards NAD^+ compared with $NADP^+$ by making two hydrogen bonds with the 2′- and 3′-OH groups of the NAD^+ adenine ribose. Asp-308 is located on a subsite that co-ordinates the carboxamide group of the coenzyme. The subsite ensures the proper orientation and polarization of the NAD^+ nicotinamide. A change in the catalytic constant of FDH at acidic pH suggests that one of these groups, presumably Asp-308, is also implicated in catalysis as discussed in [8,10].

Thus the acidic part of the pH dependence for pFDH is attributed to the ionization of two aspartic residues, Asp-221 and Asp-308, involved in coenzyme binding and catalysis (Asp-308). This interpretation differs from the scheme suggested for cFDH where carboxylic residues were implicated in substrate binding [16].

Effect of pH on substrate and coenzyme binding in the basic range

In the basic pH region (Figure 2) V_{max} does not change, whereas the affinity for both coenzyme and substrate is determined by similar pH transitions (Table 1). The pK values (10.4–10.7) can be attributed to the amino group of lysine, the thiol group of cysteine, the hydroxy group of tyrosine, an extremely shifted histidine imidazole or the guanidinium group of arginine. How-

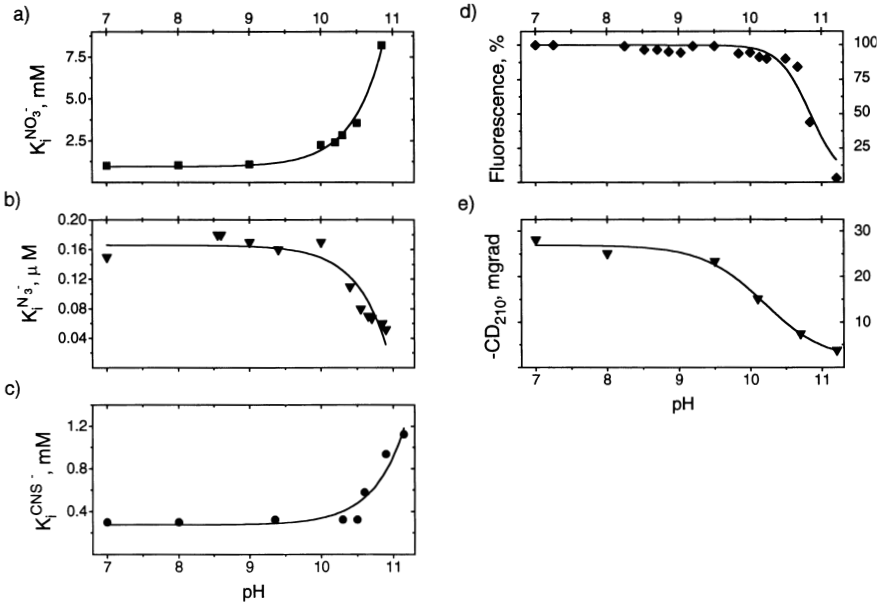


Figure 4 pH dependences of K_i for some of the pFDH substrate analogues at 37 °C [NO_3^- (a), N_3^- (b) and rodanide (c)], and pH dependences of the CD (e) and fluorescence (d) properties of the enzyme at 18 °C

Curves drawn are theoretical, obtained by computer simulation.

ever, in the vicinity of the substrate-binding site there are no lysine residues (the closest lysine is 15 Å from NAD^+ C4), tyrosine residues (9 Å) or cysteine residues (7 Å). Only three residues, Asn-146, Arg-284 and His-332, might participate in substrate binding [8,10]. The amide group of asparagine is highly basic and for histidine or arginine the observed pH transitions are very uncommon. Hydrogen-bonding of formate to the guanidinium group of Arg-284 will shift the pK of this residue to an even more basic value than the typical average (pK 11.6–12.6). As His-332 is trapped in a non-protonated state through the tight hydrogen-bonding with the amide nitrogen of Gln-313, its pK should be shifted to the acidic side. A basic shift of histidine is only possible if it is complexed with some proton-donating species, e.g. a carboxylic acid. This makes the interpretation of the basic region of the pH dependence of pFDH a highly challenging task.

The pH dependences have been studied of the K_i values for various anionic inhibitors competitive with formate. The pH profiles of the linear triatomic anions (which are structural and electronic analogues of the reaction product, the CO_2 molecule) CNS^-, CNO^-, N_3^- as well as the planar anion NO_3^- (the structural analogue of the formate anion) have all been investigated (Table 1). The binding of inhibitors follows the same pattern as the binding of substrate but with one notable exception. The pH dependence of N_3^- binding has the same pK (10.7 ± 0.2) as the other anionic inhibitors. However, the affinity of N_3^- for the active centre of FDH increases at high pH values, whereas it decreases for formate and other inhibitors (Figure 4). We have no reasonable explanation for this phenomenon.

A common trend in the mode of coenzyme, substrate, and inhibitor binding in the basic pH range suggests a common mechanism underlying this phenomenon. The pH transition can have a conformational origin. To verify this assumption, we investigated the pH dependences of the tryptophan fluorescence and CD properties of FDH, which can serve as indicators of the conformational state of the protein. FDH contains seven tryptophan residues per enzyme subunit, of which two are located in the immediate vicinity of the enzyme active centre (10–15 Å). The closest, Trp-99, together with Pro-97 and Phe-98, constitutes a part of the hydrophobic wall of the enzyme's active centre. Trp-310 is located in the loop between two important active site residues, Asp-308 and Gln-313. These tryptophan residues might serve as probes for sensing the local conformation of the enzyme's active centre. The relative quantum yield of the tryptophan fluorescence of FDH is governed by the same pH transition (10.6 ± 0.1; Figure 4d) that determines substrate or inhibitor binding. The CD spectrum (Figure 4e) also revealed a pH transition in the basic range, pK 10.2 ± 0.2.

From the structural data, FDH has a high degree of conformational mobility. Substantial conformational changes occur on NAD^+ binding [8], which are manifested in the active centre (the essential residues are shifted by up to 2 Å in acquiring their catalytic conformation) and the whole protein globule (movement of the catalytic domain around the hinge regions by an angle of 7.5° and formation of new stretches of a secondary structure, the C-terminal helix α9). Thus the pH transitions in the basic region with an apparent pK of 10.4–10.7 can be assigned to a co-operative event leading to the reorganization of the protein conformation and alteration of the enzyme affinity towards the substrate or inhibitors. Active site residues, Asn-146, Arg-284 and His-332, are not involved in this pH transition. Indirect evidence in support of the conformational origin of the transition with an apparent pK of approx. 10.5 comes from an observation that at least some of the observed pH transitions are co-operative events and implicate a transfer of more than one proton.

Comparison with yeast FDHs

The data on pH dependences in the basic region obtained for pFDH are substantially different from those reported earlier for

cFDH [16]. For the yeast enzyme it was claimed that the functional group with a pK of approx. 8.3 and a ΔH of ionization of 20 ± 2 kJ/mol in a protonated form is involved in formate binding. On these grounds it was attributed to a cationic acid, most probably histidine or lysine residues.

All the FDHs so far sequenced display approx. 50 % similarity to pFDH and strict conservation of the essential active site residues [10,26]. It is probable that the sequence of cFDH, which is not yet available, will follow the same general pattern. Thus discrepancies between the results obtained for the enzymes with presumably similar organization of the active centres require further explanation. To validate our experimental results with pFDH we have re-examined the pH behaviour of yeast FDHs in the basic pH range. cFDH and hFDH have been investigated. Both showed pH transitions similar to those of the bacterial enzyme (Table 1). These transitions were of a clearly co-operative nature, implying the simultaneous abstraction of several (three or more) protons. pH dependences for cFDH reported about two decades ago [16] differ considerably from those obtained in this study. We are not in a position to give a reasonable explanation for this.

Conclusions

A flat pH profile of the enzyme's kinetic parameters over a wide pH range, spanning 5 pH units, is in accord with the known three-dimensional structure of pFDH, indicating only three amino acid residues that can participate in substrate binding: Asn-146, Arg-284 and His-332. None of these, either because of their intrinsic properties (Asn-146 and Arg-284) or specific structural organization of the FDH active centre (His-332), can undergo ionization in the specified pH range.

The pH transition in the acidic range is attributed to synchronous ionization of two aspartic residues involved in pFDH in coenzyme binding, Asp-221 and Asp-308. One of these residues, Asp-308, is also implicated in catalysis.

The pH transitions around pH 10.5 observed in FDHs from various sources have a conformational origin and are caused by a substantial reorganization of the enzyme's active centre. These structural changes can be regarded as preceding the rapid protein inactivation that occurs at pH values above 11.2. The common nature of the pH transitions, as well as the similar pK values for the FDHs from different organisms, suggests that these proteins have a rather similar structure, as would be expected from their close physicochemical properties and high sequence similarity.

Received 8 May 1996/15 August 1996; accepted 29 August 1996

We thank Professor M.-R. Kula (Heinrich Heine University, Düsseldorf, Germany) for providing the biomass of the methanol-utilizing yeast *Candida boidinii*, Dr. V. V. Shubin (A. N. Bakh Institute of Biochemistry, Moscow, Russia) for technical support in obtaining the CD spectra, and Professor K. S. Wilson (University of York, York, U.K.) for fruitful discussions. The work was supported by grants from the International Science Foundation (MJS300) and INTAS – International Association for the Promotion of Cooperation with Scientists from the New Independent States of the Former Soviet Union (94-1309).

REFERENCES

1 Banaszak, L. J. and Brandshaw, R. A. (1975) in Enzymes, 3rd edn. (Boyer, P. D., ed.), vol. 11, pp. 369–396, Academic Press, New York
2 Harris, J. I. and Waters, M. (1975) in Enzymes, 3rd edn. (Boyer, P. D., ed.), vol. 13, pp. 1–49, Academic Press, New York
3 Holbrook, J., Lijas, A., Steindek, S. J. and Rossman, M. G. (1975) in Enzymes, 3rd edn. (Boyer, P. D., ed.), vol. 11, pp. 191–292, Academic Press, New York
4 Branden, C.-I., Jornvall, H., Eklund, H. and Furungen, B. (1975) in Enzymes, 3rd edn. (Boyer, P. D., ed.), vol. 11, pp. 103–190, Academic Press, New York
5 Rossman, M. G., Lijas, A., Branden, C.-I. and Banaszak, L. J. (1975) in Enzymes, 3rd edn. (Boyer, P. D., ed.), vol. 11, pp. 61–102, Academic Press, New York
6 Branden C.-I. and Eklund, H. (1980) in Dehydrogenases Requiring Nicotinamide Coenzymes (Jeffery J., ed.), pp. 41–84, Birkhauser, Basel
7 Birktoft, J. J. and Banaszak, L. J. (1983) J. Biol. Chem. **258**, 472–482
8 Lamzin, V. S., Dauter, Z., Popov, V. O., Harutyunyan, E. H. and Wilson, K. S. (1994) J. Mol. Biol. **236**, 759–785
9 Lamzin, V. S., Popov, V. O. and Wilson, K. S. (1993) Hamburger Synchrotronstrahlungslabor HASYLAB am Deutschen Electronen-Synchrotron DESY, Jaresbericht, pp. 819–820
10 Popov, V. O. and Lamzin, V. S. (1994) Biochem. J. **301**, 625–643
11 Vinals, K., Depiereux, E. and Feytmans, E. (1993) Biochem. Biophys. Res. Commun. **192**, 182–188
12 Kochhar, S., Hunziker, P. E., Leong-Morgenthaler, P. and Hottinger, H. (1992) Biochem. Biophys. Res. Commun. **184**, 60–66
13 Kutzenko, A. S., Korolev, S. V., Lamzin, V. S. and Popov, V. O. (1994) Mol. Biol. (Moscow) **28**, 626–632
14 Schuller, D. J., Grant, G. A. and Banaszak, L. J. (1995) Struct. Biol. **2**, 69–76
15 Goldberg, J. D., Yoshida, T. and Brick, P. (1994) J. Mol. Biol. **236**, 1123–1140
16 Blanchard, J. S. and Cleland, W. W. (1980) Biochemistry **19**, 3543–3550
17 Tishkov, V. I., Popov, V. O. and Egorov, A. M. (1983) Biokhimya **48**, 1172–1180
18 Lamzin, V. S., Aleshin, A. E., Strokopytov, B. V., Yukhnevich, M. G., Popov, V. O., Harutyunyan, E. H. and Wilson, K. S. (1992) Eur. J. Biochem. **206**, 441–452
19 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
20 Tishkov, V. I., Galkin, A. G., Marchenko, G. M., Egorova, O. A., Sheluho, D. V., Kulakova, L. B., Dementieva, L. A. and Egorov, A. M. (1993) Biochem. Biophys. Res. Commun. **192**, 976–981
21 Popov, V. O., Egorov, A. M. and Berezin, I. V. (1978) Dokl. AN SSSR **240**, 217–220
22 Egorov, A. M., Popov, V. O., Berezin, I. V. and Rodionov, Yu. V. (1980) J. Solid-Phase Biochem. **5**, 19–33
23 Jencks, W. P. (1969) Catalysis in Chemistry and Enzymology, McGraw-Hill, New York
24 Fersht, A. R. (1977) Enzyme Structure and Mechanism. W. H. Freeman, Reading
25 Tishkov, V. I., Egorov, A. M. and Popov, V. O. (1983) Bioorg. Khim. **9**, 1341–1347
26 Allen, S. J. and Holbrook, J. J. (1995) Gene **162**, 99–104