REVIEW ARTICLE
NAD$^+$-dependent formate dehydrogenase

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INTRODUCTION

NAD(P)$^+$-dependent dehydrogenases comprise a substantial and diverse group of proteins differing in structure and function. They catalyse a number of key metabolic steps and belong to one of the most extensively studied protein families. Nevertheless, the majority of mechanistic studies on NAD$^+$-dependent dehydrogenases are still performed using only a few selected enzymes acting on carbonyl compounds: alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and glyceraldehyde-3-phosphate dehydrogenase (GPDH). The biochemistry and structure of these NAD$^+$-linked dehydrogenases have been extensively studied and reviewed over the past decades [1–8], leading to a number of important structural and mechanistic generalizations.

One of the simplest examples of NAD$^+$-dependent dehydrogenation of carbonyl compounds is the oxidation of formate anion to CO_2, catalysed by NAD$^+$-dependent formate dehydrogenase (FDH; EC 1.2.1.2). This reaction is devoid of proton release or abstraction steps, and entails cleavage of a single carbon–hydrogen bond in the substrate and formation of a single new one in the product. Thus FDH is perhaps the most suitable model for investigating the general mechanism of catalysis involving hydride ion transfer. In addition, FDH is one of the most promising candidates for the development of so-called coenzyme regeneration systems [9–11], and is at present applied on the laboratory and pilot scale in fine organic and asymmetrical synthesis for production of high-value-added products [12,13].

PHYSICO-CHEMICAL PROPERTIES

Enzymes capable of oxidizing formic acid are found in microorganisms from different taxons [14–37] and higher plants [38–42]. They may be classified into two major families. The first includes a diverse group of conjugated iron–sulphur metal-containing proteins of microbial origin differing in physiological role, cellular location, substrate specificity, nature of the physiological electron acceptor, content and type of prosthetic groups [29–37]. These enzymes are distinguished by their high molecular mass, complex quaternary structure, the presence of various prosthetic groups and their lability towards oxygen. The second family comprises NAD$^+$-dependent FDHs (EC 1.2.1.2), and with few exceptions is represented by proteins devoid of any prosthetic groups (Table 1).

The present review will be limited to discussion of NAD$^+$-dependent FDHs. Its scope is to summarize the molecular and kinetic properties of these enzymes, compare the FDH structure with those of other enzymes, outline the tentative molecular mechanism and highlight possible areas of practical application. Throughout this review, the numbering of amino acid residues in FDH from the methanol-utilizing bacterium *Pseudomonas* sp. 101 (FDH-Ps) will be used.

Occurrence and physiological role

In methylotrophs, NAD$^+$-dependent FDHs play a key role, catalysing the terminal step of catabolism of C_1 compounds and supplying these organisms with energy and reducing equivalents [43,44]. Prokaryotes and eukaryotes employ different biochemical systems for methanol utilization. Methylotrophic bacteria oxidize methanol to CO_2 either through cyclic mechanisms operating at the level of formaldehyde or through linear chains of dehydrogenases, while all known methylotrophic yeasts oxidize methanol to CO_2 via formaldehyde [43,44].

The general scheme of the dissimilative methanol pathway in methylotrophic yeasts is presented in Figure 1. In these organisms FDH plays an important regulatory role, being under the control of the adenine nucleotide pool [20,43,44]. When the cell is saturated with reducing equivalents, these enzymes are inhibited and the majority of formaldehyde is channelled to assimilation purposes. When the energy status of the cell is low the inhibition is removed and formaldehyde is oxidized to CO_2, providing reducing equivalents.

FDHs as a rule are inducible enzymes synthesized in large quantities (up to 10–15 % of the net protein content) when organisms are grown on C_1 compounds [23,45]. Some organisms, e.g. the methylotrophic bacteria *Mycobacterium vaccae* 10 [45] and *Pseudomonas* sp. 101 [46], contain more than one FDH, which differ in cellular location, molecular mass and specificity towards the electron acceptor. In the presence of molybdenum the dominant form is the high-molecular-mass (280–440 kDa), presumably Mo-containing, NAD$^+$-dependent FDH, with broad acceptor specificity and high affinity (0.3–0.6 mM) for formate [46]. In the absence of added molybdenum or in the presence of tungsten in the growth medium, the prevailing form in both organisms is the low-molecular-mass (80–93 kDa) non-metal containing enzyme with lower affinity towards the substrate (8-15 mM).

Isolation procedures

The majority of highly purified FDH preparations have been isolated from methylotrophic yeasts (Table 1), with only a few homogeneous enzymes being obtained from other sources. In most cases the isolation procedures used are rather straightforward and employ various chromatographic procedures (hydrophobic or ion-exchange chromatography being the principal purification step). Purification methods based on affinity chromatography have also been described [40,41,47]. Industrial techno-

Abbreviations used: ADH, alcohol dehydrogenase; DHFR, dihydrofolate reductase; FDH, formate dehydrogenase; FDH-Ps, formate dehydrogenase from *Pseudomonas* sp. 101; GPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; ADPR, adenosine diphosphoribose; DTNB, 5,5′-dithiobis(2-nitrobenzoate); GSF, *S*-formylglutathione; PCMB, *p*-chloromercuribenzoate.

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Table 1 Physico-chemical properties of NAD$^+$-dependent FDHs

Source*	Subunits (kDa) [pI]	Activity (units/mg)†	Temp. stability (°C)	pH optimum	K_m^{NAD} (μM)	K_m^{form} (mM)	Inhibitors	Comments	Refs.
Bacteria									
Pseudomonas oxalaticus	2 × 100, 2 × 59			7.5	105	0.14	N_3^-, CN^-, NO_3^-, Hg^{2+}, PCMB	Light- and oxygen-labile; contains Fe, S^{2-} and FMN	27
Methylomonas methylica (× 32)		3.1 (37 °C		7.7–8.0	160	0.40			28
Methylomonas extorquens AM1 (× 3)		0.42 (22 °C)		8.4	90	0.25	CN^-, Cu^{2+}, Fe^{3+}, iodoacetamide	Temperature-labile	25
Pseudomonas sp. 101‡§	2 × 44 [4.6–5.2]	16.0 (37 °C)	55	6.0–9.0	110	15	N_3^-, CN^-, NO_3^-, Hg^{2+}, PCMB, DTNB	Active with NADP$^+$; uses GSF; random Bi-Bi kinetic scheme	21, 50, 67
Moraxella sp. C-1	2 × 48 [3.9]	6.0 (25 °C)	55	6.0–9.0	68	13	N_3^-, CN^-, Ag^+, Hg^{2+}, DTNB, hydroxylamine		22
Paracoccus sp. 12-A	2 × 49 [5.4]	11.6	50–55	6.5–7.5	36	5	N_3^-, CN^-, Ag^+, Hg^{2+}, PCMB		23
Mycobacterium vaccae 10	2 × 44 [4.6]	6.0 (37 °C)	57	6.0–9.0	200	20	N_3^-, SCN^-, Cu^{2+}, Hg^{2+}, DTNB		24
Yeasts									
Candida boidinii	2 × 36 [5.4]	2.4 (30 °C)	55	6.5–8.5	90	13	N_3^-, CN^-, SCN^-, NO_3^-, Ag^+, Hg^{2+}, PCMB	Ordered Bi-Bi kinetic scheme	14, 64
Candida methylica	2 × 46 [4.6–4.8]	10.0 (37 °C)	50	6.0–9.0	100	13	N_3^-, CN^-, Hg^{2+} DTNB	Ordered Bi-Bi kinetic scheme	18, 65
Candida methanolica	2 × 43 [5.5]	7.5 (30 °C)	50	6.5–9.5	110	3	N_3^-, CN^-, Hg^{2+}, Ni^{2+}, PCMB	Ordered Bi-Bi kinetic scheme	20
Kloeckera sp. 2201		0.14 (30 °C)	50	7.0–8.0	100	22	N_3^-, CN^-, Hg^{2+}, Cu^{2+}, PCMB		15
Pichia pastoris NRRL-Y-7556	2 × 47	8.2	20–25	6.5–7.5	140	16	CN^-, Hg^{2+}, Cu^{2+}, PCMB, DTNB		16
Pichia pastoris IFP 206	2 × 34	2.8 (37 °C)	47	7.5	270	15	N_3^-, CN^-, Hg^{2+}, Cu^{2+}, PCMB		17
Hansenula polymorpha‡ (× 16)	2 × 40	2.8 (37 °C)	60	7.0	70	40		Uses GSF	19, 85
Plants									
Phasoleus aureus	2 × 46‖				7.2	1.6		Ordered Bi-Bi kinetic scheme	38
Pisum sativum	2 × 42	3.7 (25 °C)		6.0–8.0	43	1.7	DTNB, PCMB		39
Pisum sativum sp. Onwards	2 × 42	4.1 (25 °C)			23	2.1		Uses GSF	40
Glycine soja var. Beeson	2 × 47	1.4 (25 °C)		6.0	5.7	0.6		Uses GSF; ordered Bi–Bi kinetic scheme	41
Solanum tuberosum¶‖	2 × 42 [6.8]								42

* For partially purified preparations, purification (fold) is shown in parentheses.
† 'Units/mg' are μmol/min per mg of protein; the temperature at which the activity was measured is given in parentheses.
‡ Gene sequenced and expressed.
§ Three-dimensional structure available.
‖ Date on subunit composition not available; the molecular mass of the whole protein was divided by two.
¶ Gene sequenced.

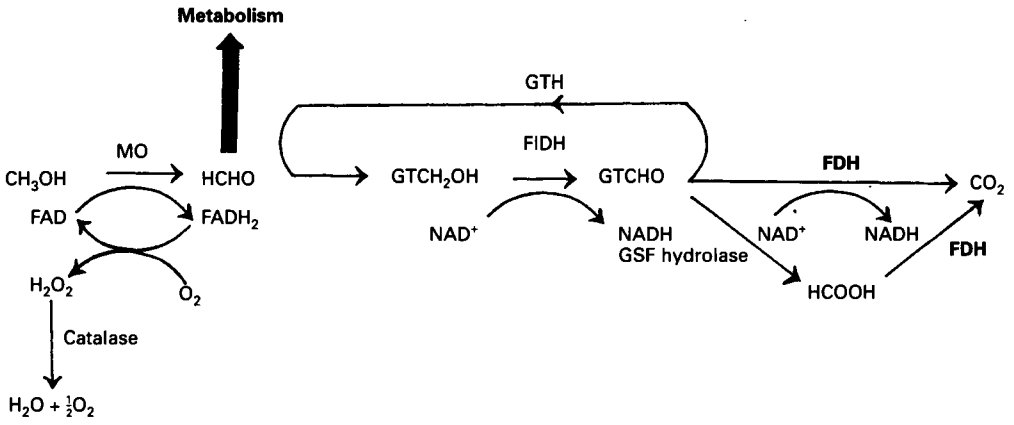


Figure 1 General scheme of methanol metabolism in yeasts

MO, methanol oxidase; FIDH, formaldehyde dehydrogenase; $GTCH_2HO$, *S*-hydroxymethylglutathione; GTCHO, *S*-formylglutathione (GSF). See the text for further details.

logies for obtaining large quantities of FDH from the yeast *Candida boidinii* have been elaborated [48,49]. These procedures, based on separation in two-phase systems, enable the isolation of hundreds of grams of technical-grade enzyme starting from up to 200 kg of cells.

Basic molecular properties

Molecular mass, subunit composition, prosthetic groups and specific activity

The majority of characterized NAD⁺-dependent FDHs do not contain any prosthetic groups or metal ions. The enzymes from eukaryotic organisms [14–20,38–42], as well as from some methylotrophic bacteria [21–24], have molecular masses ranging from 70 to 100 kDa and are composed of two chemically identical subunits. They display relatively low specific activity, a low affinity for formate ion and a broad pH optimum for catalytic activity at neutral pH (Table 1).

The enzyme from *Pseudomonas oxalaticus* is the only example among highly purified NAD⁺-dependent FDHs of a quite different molecular organization. It has a high molecular mass (315 kDa), a complex quaternary structure of the $2\alpha2\beta$ type and contains a number of prosthetic groups including iron (18–25 per molecule), acid-labile sulphur (15–20) and two molecules of flavin mononucleotide [27]. This enzyme displays diaphorase activity and uses oxygen, ferricyanide and various dyes as electron acceptors. According to its structure and composition, as well as other properties such as light and oxygen lability, higher specific activity and affinity for formate, *Ps. oxalaticus* FDH resembles NAD⁺-independent enzymes and constitutes a special subclass of FDHs.

Studies on NAD⁺-dependent FDHs from prokaryotes are not numerous. Homogeneous preparations have been obtained from *Pseudomonas* sp. 101 (former names *Bacterium* sp. 1 and *Achromobacter parvulus* T1) [21], *Moraxella* sp. C-1 [22], *Paracoccus* sp. 12-A [23] and *Mycobacterium vaccae* N10 [24]. According to their molecular properties, FDHs from these bacteria belong to the same family as the NAD⁺-dependent FDHs from yeasts and higher plants (Table 1). On the other hand, FDHs partially purified from other bacterial strains, e.g. *Methylomonas extorquens* AM1 (former name *Pseudomonas* AM1) [25] and *Methylomonas methylica* [28], resemble the enzyme from *Ps. oxalaticus* in their molecular mass, affinity for formate and substrate specificity.

Fine details of interrelationships among FDHs are revealed by immunological studies. While antiserum obtained against homogeneous enzyme preparations from *Pichia pastoris* or *Candida methanolica* reacted with FDHs from other methylotrophic yeasts (detailed studies reveal at least three subtypes among yeast FDHs), it showed no cross-reactivity with enzymes of bacterial origin [16,20]. Both polyclonal and monoclonal antibodies obtained against FDH-Ps showed cross-reactivity with the enzyme from *Mycobacterium vaccae* N10, but did not bind FDH preparations from the yeasts *Candida methylica* and *C. boidinii* [24] or from potato (C. Colas des Francs, personal communication). Thus classification of NAD⁺-dependent FDHs into two major families according to their molecular properties is further corroborated by immunological studies. 'Simple' non-metal-containing bacterial, yeast and probably plant FDHs seem to comprise separate subgroups within the same family.

Isoforms

The presence of multiple isoforms is a characteristic feature of FDHs. Isoforms were detected for the enzymes from *Pseudomonas* sp. 101 [21], the yeast *Candida methylica* [18] and higher plants [40,41]. The microheterogenity of FDH from soya beans was attributed to protein glycosylation [41]. In some cases, e.g. for the enzyme from the pea *Pisum sativum* [40], heterogeneity might be an artefact of the isolation procedure, as the cell-free extract of this organism contains only a single form of FDH.

Isoelectric focusing of both highly purified preparations and crude extracts from *Pseudomonas* sp. 101 reveals up to between four and seven enzymically active components which differ in their stability and affinity for formate [21,24]. The number of components depends on the isolation protocol, and is reduced to three in the presence of SH-containing compounds. Similar behaviour was observed for the enzyme from *Candida methylica* [18].

The reasons for the microheterogeneity of FDH preparations from these sources remain unknown. One proposal attributes different forms of FDH to the variable oxidation state of its SH groups [21], while the another [24,50] implies post-translational modification associated with digestion of C-terminal amino acids.

Coenzyme and substrate specificity, and stereospecificity

FDH-Ps transfers hydrogen to the *pro-R* position of the nicotinamide moiety of NAD^+ [51] and thus belongs to the family of A-specific dehydrogenases. The majority of NAD^+-dependent FDHs are highly specific towards NAD^+ and do not utilize $NADP^+$ as a coenzyme. However, at least one of them, FDH-Ps [24], displays dual coenzyme specificity. Under optimal reaction conditions the activity of FDH-Ps towards $NADP^+$ reaches nearly 30% of that with NAD^+.

Data on the substrate specificity of FDHs are more controversial. A number of enzymes have been shown to oxidize esters and thioesters of formic acid [19,40,41,52,53]. The activity towards one of the thioesters, *S*-formylglutathione (GSF), might be of physiological significance. This compound constitutes a physiological product of the reaction catalysed by glutathione-dependent formaldehyde dehydrogenase, the enzyme preceding FDH in the dissimilatory pathway of methanol metabolism in plants, certain methylotrophic yeasts and some bacteria (Figure 1) [43,44].

Partially purified FDH from *Hansenula polymorpha* can utilize both formate and GSF as substrates [19]. The K_m for GSF was 40 times lower than that for formate ion. Moreover, the enzyme was unable to hydrolyse GSF in the absence of NAD^+. All these observations enabled van Dijken and co-workers to suggest GSF as a physiological substrate of FDH in this organism [19].

GSF-dependent activity was also demonstrated for homogeneous FDH preparations from pea [40] and *Pseudomonas* sp. 101 [52,53]. These enzymes were active with a number of esters and thioesters of formic acid [40,53]. Formation of a binary complex between FDH-Ps and GSF was monitored directly by fluorescence measurements. The value of the dissociation constant obtained (2.5 mM) agreed with estimates derived from kinetic experiments (2.3 mM) [53].

However, the GSF-dependent activity of FDHs from methylotrophic yeasts has been seriously questioned by several research groups [17,54,55]. A specific and highly active enzyme hydrolysing GSF was isolated in a homogeneous form from two species of methylotrophic yeast, *Candida boidinii* [54] and *Kloeckera* sp. 2201 [55]. The hydrolase activity was shown to be closely associated with the dehydrogenase activity in the course of purification. A discrete GSF hydrolase activity has been demonstrated in *Hansenula polymorpha* [55].

Thus, at present, the prevailing view is that FDHs from methylotrophic yeasts are strictly specific towards formate, while enzymes from other sources might use GSF as a substrate as well. The latter conclusion is in agreement with all the information, both structural and kinetic, available for FDH-Ps (see below).

Stability

Thermostability, chemical stability and oxygen sensitivity

All NAD^+-dependent FDHs, except the enzyme from *Ps. oxalaticus*, are stable in air (Table 1). The oxygen sensitivity of the latter FDH is expected, taking into account its complex structure and composition as well as the extreme oxygen lability of a vast number of conjugated iron–sulphur proteins [56].

The majority of NAD^+-dependent FDHs have closely similar thermostabilities and are rapidly inactivated at 55–60 °C (Table 1). Heat treatment at 50 °C is widely used as a purification step in the course of FDH isolation [14,19].

Many FDHs are labile on storage in the absence of activity stabilizers. Detailed studies of the FDH inactivation mechanism have been undertaken for the enzymes from *Pseudomonas* sp. 101 and *Candida methylica* [18,21,57–61]. Inactivation of FDH-Ps at temperatures below 50 °C was attributed to the oxidation of an essential SH-group by atmospheric oxygen, catalysed by the traces of transition metal ions present in the solution [59,60]. The redox state of SH-groups seems to be of vital importance for the stability of a number of other FDHs from eukaryotes.

Stabilization strategy

SH-containing compounds, EDTA, poly(ethylene glycol) and glycerol effectively preserve FDH catalytic activity [16,18,21,40,41,48,59], increasing the 'shelf life' of the enzyme from days to months or even years [58]. Based on established mechanisms of inactivation, several rationales for the stabilization of enzyme catalytic activity have been suggested. Effective stabilization of FDH-Ps under real operational conditions was achieved by providing protection of its essential thiols by adding SH-compounds or by scavenging the traces of transition metal ions with chelating agents [21,59]. Protection of the essential SH-groups of the enzyme from solution by the formation of water-soluble enzyme–polymer complexes also resulted in a 300–500-fold increase in stability [60,61]. Long-term storage life times of FDHs from *Pseudomonas* sp. 101, *Candida methylica* and *C. boidinii* increased remarkably in the presence of glycerol [18,58] and poly(ethylene glycol) [48].

KINETIC PROPERTIES

Formal kinetic schemes

All recently kinetically characterized FDHs follow an ordered Bi-Bi two-substrate kinetic scheme (or its variants), with NAD^+ being the first substrate [20,38,41,62–65]. The only exception is FDH-Ps, which binds substrates and releases products independently [66,67]. The mechanism of this enzyme was probed by transient-state kinetic experiments [68]. These studies confirmed the random order of substrate binding and showed its near-equilibrium character, a feature suggested formerly on the basis of steady-state kinetics [66]. The kinetic studies of FDH-Ps suggest that both substrate and coenzyme binding sites pre-exist and are not formed *in situ* during transition of the apo form of the dehydrogenase to the holo state on coenzyme binding. However, binding of one of the substrates increases the affinity for the other 3.5-fold.

The majority of FDHs display Michaelis-type kinetics and independent functioning of the active centres. However, some enzymes from higher plants show deviations from simple Michaelis dependencies [40] or non-equivalence of the active sites with respect to formate or inhibitor binding [69].

CO_2 was shown to be the true product of the reaction catalysed by FDH-Ps [70] and undergoes a rather slow transformation to bicarbonate (the delay between NADH formation and proton release is about 24 s). Thus no water molecules seem to participate in the enzymic reaction.

Inhibition studies

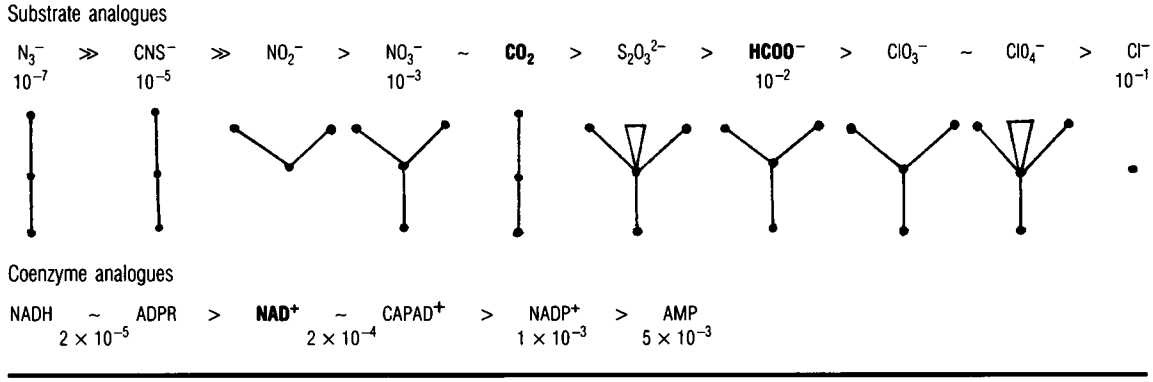
Much attention has been given to inhibition studies of FDHs using coenzyme and substrate analogues. These studies have on the one hand confirmed the proposed kinetic schemes for particular enzymes [38,62–67], and on the other revealed some important mechanistic features.

Coenzyme analogues

Various coenzyme analogues modified at the C-3 position of the nicotinamide moiety (thio-, deamino-, pyridinealdehyde- and

Table 2 Affinity of FDH from *Pseudomonas* sp. 101 towards substrate- and coenzyme analogues

CAPAD, 3-tricloroacetylpyridine adenine dinucleotide. The dissociation constant (K_d; in M) is given below the relevant analogue (pH 7.0, 37°C). The structures of inorganic anions are presented in relative scale.



acetylpyridine-NAD^+) can substitute for NAD^+ as substrate for FDHs from the yeast *Candida boidinii* [62,63] or *Pseudomonas* sp. 101 (I. A. Shumilin, personal communication). However, the affinities of such coenzyme analogues were 15–40-fold lower compared with normal NAD^+.

Inhibition studies of FDH-Ps revealed that electrostatic effects play an important role in coenzyme binding (Table 2). The positive charge of the nicotinamide ring of NAD^+ hindered coenzyme binding compared with the neutral reduced form of the coenzyme (NADH) or with a compound completely devoid of the nicotinamide moiety [adenosine diphophoribose (ADPR)]. Better binding of neutral NADH compared with positively charged NAD^+ is a feature shared by several FDHs [38,62,64,65], as well as by many other NAD^+-dependent dehydrogenases [1–8].

FDH from *Candida boidinii* was effectively inhibited by adenine nucleotides (ATP, ADP, AMP) in the physiological concentration range. This enabled Kato and co-workers [64] to propose that FDH activity in the cell is under the control of the pool of these nucleotides and NADH.

Substrate analogues

The nearest structural homologues of formic acid (acetate, propionate, oxalate, pyruvate, methanol and hydrazine) show only weak affinity ($K_i > 0.5$ M) for FDH. However, many simple inorganic anions compete with formate for the FDH binding site [62,66]. The inhibition constants span seven orders of magnitude (Table 2). Tetrahedral anions such as perchlorate, phosphate and sulphate (with the exception of thiosulphate) show very weak binding. Linear and planar anions with delocalized negative charge are the best inhibitors of FDHs. Linear triatomic anions, e.g. azide or SCN^-, may be considered as structural analogues of the product of the reaction, CO_2, while planar anions such as NO_2^- might be regarded as analogues of the formate anion. Azide, which is isoelectronic to CO_2, is the most powerful inhibitor. Tight binding of this anion resulting in formation of the abortive ternary complex, FDH–NAD^+–azide, was exploited in a fluorimetric procedure for titration of FDH active sites [71].

Several features seem to be important for tight binding of inhibitors: (1) overall geometry, i.e. linear compounds bind better then planar ones, e.g. $N_3^- \gg NO_2^-$ or $CO_2 > HCOO^-$; (2) nature of the central atom, i.e. $SO_3^{2-} \gg NO_3^- \gg ClO_3^-$; (3) nature of the distal atoms, i.e. $S_2O_3^{2-} > ClO_4^-$; thio-formate is a potent inhibitor of FDH from *Candida boidinii* with $K_i \sim 80$ μM [62].

Because of the high electrophilicity of the C-4 position of nicotinamide, NAD^+ is known to form adducts with various nucleophiles, such as CN^-, HSO_3^- and hydroxylamine [72]. Comparison of the binding constants of the inhibitor with free NAD^+ and with FDH–NAD^+ binary complex enables evaluation of the stabilization energy for formation of the ternary complex FDH–NAD^+–inhibitor, which in the case of HSO_3^- is estimated to be about 29 kJ/mol (7 kcal/mol) A strong stabilizing effect is obtained when formate ($K_d \sim 15$ mM) is replaced in the ternary complex by azide ($K_d \sim 0.5$ μM). Extremely tight binding of linear anions by the active site allows us to regard such abortive complexes as stable analogues of the transition state.

One group of compounds occupies a special place among FDH inhibitors. Pyridoxal ($K_i \sim 0.9$ mM) competes with formate both in the free enzyme and in the binary FDH–NAD^+ complex for FDH-Ps [73], while pyridoxal phosphate and some aromatic aldehydes partially inhibit *Candida methanolica* FDH [20]. The substantial molecular dimensions of pyridoxal and related compounds suggest that they may bind not to the formate binding subsite of the active centre, but to some other site, and act as allosteric inhibitors.

pH-dependencies

All NAD^+-dependent FDHs have a broad pH optimum of catalytic activity in the neutral pH range (Table 1). pH-dependencies of the catalytic parameters were studied in detail for the enzymes from *Candida boidinii* [62] and *Pseudomonas* sp. 101 [53,66]. Blanchard and Cleland [62] investigated the pH-dependence of the kinetic parameters of the enzymic reaction ($V_{max.}$, K_m^{form}, K_m^{NAD}) as well as the pH-dependence of the inhibition constant for azide. It was shown that a group in the FDH–NAD^+ binary complex with a pK of 8.3–8.5 must be protonated for formate or azide binding. A cationic acid (probably lysine) was implicated in substrate binding, based on its abnormally high ΔH_{ion} [84.5 ± 8.4 kJ/mol (20 ± 2 kcal/mol)] and on the fact that the pK of this group was displaced to 9.8 on binding of formate. A group of p$K \sim 6.4$ and no temperature-dependence (carboxylic acid) must be ionized for binding of azide and formate, and another group of p$K \sim 5.9$ must be ionized for catalysis. Apparently there might be two or more unresolved pK values at low pH, since the pH-dependence had a slope of about 2.

Investigation of the pH-dependence of the kinetic parameters for FDH-Ps gave similar results which differed, however, in some

important details. In contrast to the enzyme from *Candida boidinii*, the $V_{max.}$ of the reaction was constant throughout the entire pH range (5.2–11.0) of enzyme stability. This result was expected, since hydride transfer is the rate-limiting step for FDH-Ps (see below) and no proton uptake or release is required in the course of the catalytic process.

The K_ms for both substrates remain constant in the neutral pH range and decrease below pH 6 and above pH 9. pK values obtained for K_m^{form} and K_m^{NAD} for the basic side were 10.4 and 10.6 respectively, while a value of 5.4 was obtained for K_m^{NAD} for the acidic side (K_m^{form} did not change appreciably in the acidic range). Concerted ionization of two groups with identical or close pKs was shown to be responsible for the pK value of 5.4 characterizing NAD^+ dissociation from the central ternary complex. It was displaced by 0.6 to the basic side for the apo enzyme and showed a ΔH_{ion} close to zero (V. S. Lamzin, unpublished work). On these grounds this pK value was attributed to ionization of two carboxylate groups with similar pKs. In the basic region the pH-dependence of the inhibition constant for the substrate analogue NO_3^- ($pK_i \sim 10.5$) followed the same pattern as formate binding (A. V. Mezentzev, T. B. Ustinnikova and V. O. Popov, unpublished work).

Thus studies of pH-dependencies for both yeast and bacterial FDHs indicate the presence of one or two carboxylate groups at the NAD^+ binding subsite and a group with a pK of 9.8–10.7 controlling release of formate from the central ternary complex.

Isotope effects

Primary deuterium isotope effects for the enzymes from *Pseudomonas* sp. 101 [74], *Candida boidinii* [62] and *C. methylica* [75] vary from 3.1 for the first to 2.1 for the last two. It was concluded that hydride transfer taking place in the central ternary complexes is fully rate limiting in the case of these FDHs. A relatively low value of the primary isotope effect observed with NAD^+ ($E_0' = -320$ mV) as coenzyme was attributed by Cleland and co-workers [62,63,76] to the late transition state of the FDH reaction. The transition state becomes progressively earlier with a change in the redox potential of the coenzyme and is nearly symmetrical in the case of acetylpyridine–NAD^+ ($E_0' = -258$ mV).

Determination of the secondary deuterium as well as multiple heavy atom (^{13}C, ^{18}O, ^{15}N) isotope effects resulted in the formulation of the structure of the transition state (Figure 2) of the reaction for *C. boidinii* FDH [62,63,76]. Its main features are: (1) the transition state is linear, and in the case of NAD^+ resembles the products (late transition state); (2) the bending motion of the secondary hydrogen is coupled in the transition state to hydride transfer; and (3) the contribution of tunnelling of both hydrogens in the transition state is significant. Based on the substantial observed secondary ^{15}N isotope effect, a boat conformation of the pyridine ring of NAD^+ was originally suggested as the means of raising the carbonium ion character on C-4 and facilitating hydride transfer [63]. However, the values for these gross effects for FDH and ADH were reconsidered [76], and there seems to be no pyramidalization of the N-1 atom of the nicotinamide moiety in the coenzyme in the transition state. Thus the pyridine ring of the nucleotide remains planar in the transition state in these reactions.

Chemical modification

Studies on the chemical modification of the amino acid residues with specific reagents are almost exclusively confined to FDH-Ps. The results of these experiments are summarized in Table 3.

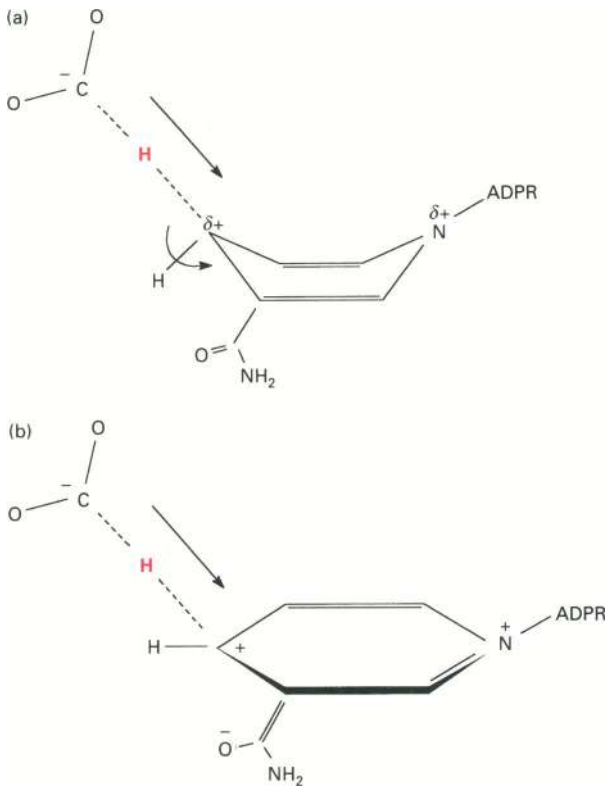


Figure 2 Two hypothetical structures of the transition state of the FDH catalysed-reaction

(**a**) Based on [63]; (**b**) based on [76]. See the text for further details.

Cysteine

The activity of various FDHs is affected by SH-modifying reagents, such as Hg^{2+}, Cu^{2+}, *p*-chloromercuribenzoate (PCMB) and 5,5′-dithiobis(2-nitrobenzoate) (DTNB) (Table 1), suggesting an important role of SH-groups in maintaining enzyme catalytic activity and stability (see above). That cysteine is essential for activity has been demonstrated for FDH-Ps and *Candida methylica* FDH [18,21]. Four SH-groups are titrated in the pea enzyme [39] by PCMB or DTNB, and this is accompanied by loss of activity.

Up to three cysteine residues per subunit, differing in their reactivities by several orders of magnitude, may be titrated in native FDH-Ps. Modification of only one SH-group per subunit by DTNB or iodoacetamide results in enzyme inactivation [21,57,77], caused by a loss of ability to bind coenzymes. Cys-255 is located at the adenine binding subsite and has been identified as the 'essential thiol', critical for activity and operational stability, on the basis of selective modifications with radioactive and fluorescent labels [78]. However, double-label (V. O. Popov, unpublished work) as well as site-directed mutagenesis [79] experiments show that at least one other cysteine is critical for activity. The probable involvement of Cys-5 is discussed in [79].

Histidine

Modification of up to four histidine residues per subunit in FDH-Ps by diethyl pyrocarbonate results in enzyme inactivation [80]. Inactivation follows pseudo-first-order kinetics, is governed by a pK of 6.7 and is retarded in the presence of coenzymes but

Table 3 Chemical modification of the functional groups of FDH from *Pseudomonas* sp. 101

n.d., not determined; pyridoxal-P, pyridoxal phosphate; Woodward's reagent is *N*-ethyl-5-phenylisoxazolium-3′-sulphonic acid).

Residues	Modifier	Number of residues modified per subunit		Protection by substrates		p*K*
		Total	Essential	NAD^+	$HCOO^-$	
Cys	DTNB	6*	1	+	−	n.d.
	Iodoacetamide	2–3	1	+	−	9.6
Arg	Butandione	6	1	+	−	n.d.
Lys	Pyridoxal	2	1	−	+	n.d.
	Pyridoxal-P	5	1	−	+	n.d.
Asp, Glu	Woodward's reagent	1	1	+	−	5.2
His	Diethyl pyrocarbonate	4	1	−	−	6.7

* In 8 M urea.

not formate. It was suggested that at least one histidine is present in the active site [80].

Arginine

Twelve arginine residues can be modified by butanedione in FDH-Ps [81,82]. Only one was shown to be essential for catalytic activity. This residue was effectively shielded from the modifying agent by coenzymes but not by formate, and its modification resulted in abolition of NAD^+ binding.

Carboxylate groups

Woodward's reagent (*N*-ethyl-5-phenylisoxasolium-3′-sulphonic acid) selectively blocks only two carboxylate groups per subunit in FDH-Ps [83]. The hyperbolic nature of the concentration-dependence suggests that this reagent may be considered as an active-site-directed modifier. One of the carboxylate groups is protected from modification by coenzymes, with preservation of the catalytic activity. The p*K* governing the enzyme inactivation process (5.2) was close to the values for the p*K*s of the carboxylate groups determining coenzyme binding (see above).

Lysine

Pyridoxal, pyridoxal phosphate and formaldehyde were used to modify lysine residues in FDH-Ps [73,84]. Two, five and 13 amino groups per subunit were modified with these reagents respectively. Blocking of one lysine residue by pyridoxal resulted in complete enzyme inactivation. Formate at saturating concentrations effectively protected FDH from inactivation, and this was accompanied by specific protection of one of the lysine residues. Moreover, initial-velocity studies indicated the competitive nature of the inhibition by pyridoxal versus formate (see above).

In contrast, *Candida methanolica* FDH is resistant to 1 mM pyridoxal and only slightly (26%) inhibited by pyridoxal phosphate [20]. Some other aldehydes, e.g. benzaldehyde (20% inhibition at 1 mM) and *o*-nitrobenzaldehyde (47% inhibition), were better inhibitors of this protein. Inhibition was prevented by formate but not by NAD^+, similar to the bacterial enzyme.

Thus, according to chemical modification studies, there are several amino acid residues that are essential for FDH catalysis. These are: (1) cysteine, which is critical for enzyme operational stability and is readily oxidizable by atmospheric oxygen (see above); (2) aspartate and/or glutamate residues, which participate in coenzyme binding; (3) arginine and histidine residues, located at the coenzyme binding site; and (4) a lysine residue in the vicinity of the formate binding subsite.

STRUCTURE

Sequence

Comparisons within FDHs

One N-terminal sequence and three complete sequences of NAD^+-dependent FDHs (Figure 3) have been determined to date [22,42,85,86]. Recently one more sequence of a protein from *Aspergillus nidulans*, with unknown function but showing high similarity with FDHs, was determined [87]. It is thought that this protein might be an FDH.

FDHs from such distant organisms as *Pseudomonas* sp. 101, *Hansenula polymorpha* and *Solanum tuberosum* have strong similarity in their primary structures (about 45% similarity), pointing to a slow evolution rate of FDH. This is estimated to be about 2–2.5 point accepted mutations per 10^8 years, comparable with the values for other NAD^+-dependent dehydrogenases such as LDH and GPDH (0.9–3.4) that are considered to be amongst the most conserved polypeptides. Some regions of the sequences display exceptional similarity that reaches nearly 70% for helix αA or the βG–α8 region, and is as high as 90% for the βF–αG loop (for notation of the secondary structure elements, see the section on Three-dimensional structure and Figure 3). All amino acid residues critical for catalysis or coenzyme and substrate binding are strictly conserved in FDHs (Figure 3).

No statistically significant sequence similarities were found between FDH-Ps and iron–sulphur–molybdenum-containing FDHs from *Escherichia coli* [88], *Methanobacterium formicicum* [89] or *Wolinella succinogenes* [90] that do not use NAD^+ as a cofactor. Thus, to date, all NAD^+-dependent FDHs not only show similar physicochemical properties but have closely related primary structures.

Protein sequencing reveals only 393 amino acid residues in the polypeptide chain of FDH-Ps [86], while the gene codes for 400 residues [50,91]. Only the first 389 residues are defined in the crystal structure [92]. The C-terminal part lying on the protein surface is easily accessible from solvent and is highly disordered in the crystal. Thus at present the exact size of mature FDH-Ps remains unclear.

The translational product of the complete FDH-Ps gene cloned and expressed in *E. coli* differs from the wild-type enzyme in temperature stability and kinetic parameters [24,91]. The C-terminal fragment of FDH-Ps is rich in lysine residues (Figure 3).

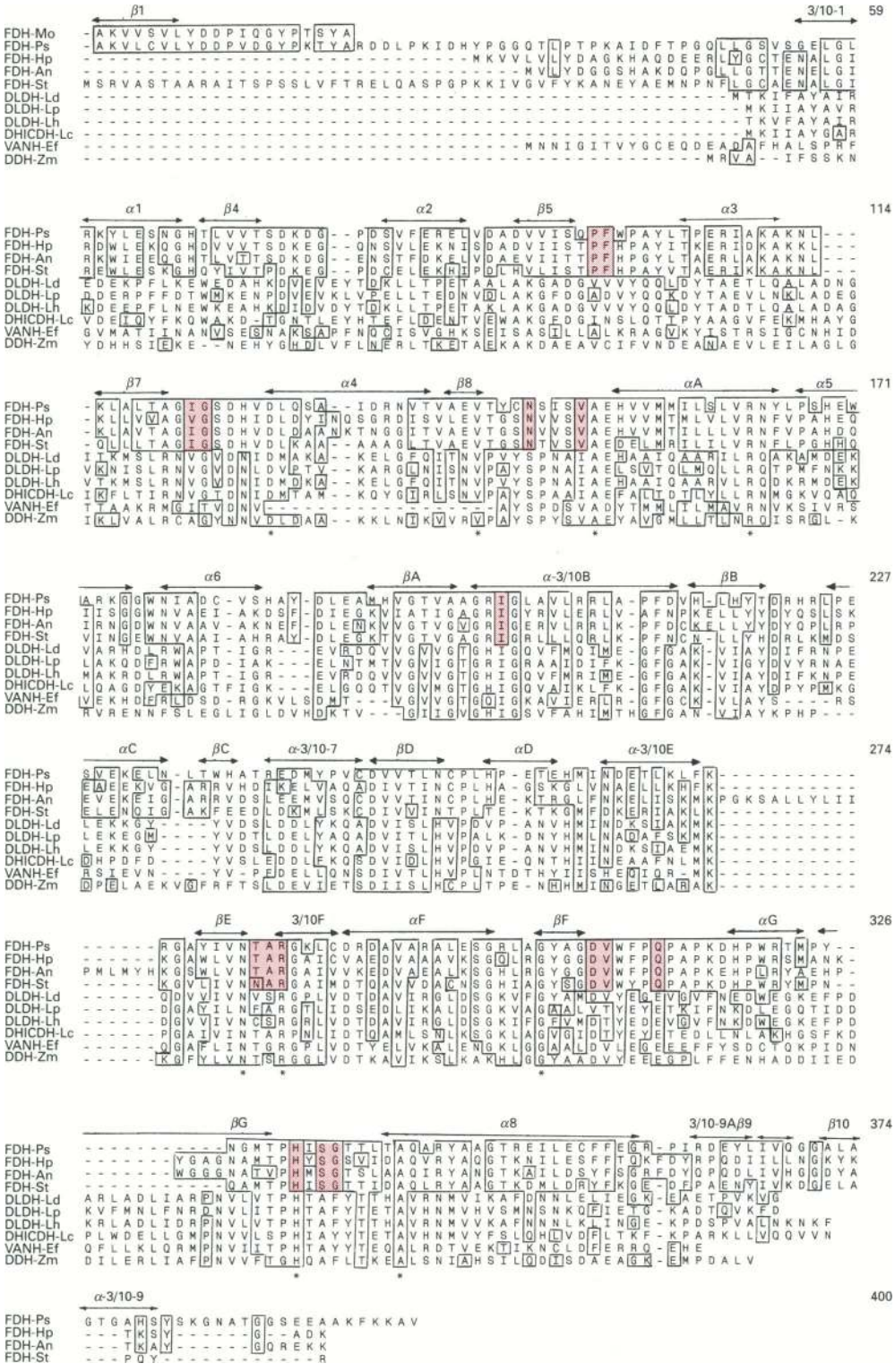


Figure 3 Alignment of FDH sequences with the sequences of D-specific 2-hydroxydehydrogenases

Sequences are FDH from *Moraxella* sp. C-1 (FDH-Mo) [22], FDH from *Pseudomonas* sp. 101 (FDH-Ps) [50,86], FDH from *Hansenula polymorpha* (FDH-Hp) [85], a polypeptide from *Aspergillus nidulans* (FDH-An) [87], FDH from potato *Solanum tuberosum* (FDH-St) [42], D-lactate dehydrogenases from *Lactobacillus delbrueckii* (DLDH-Ld) [96], from *Lactobacillus plantarum* (DLDH-Lp) [97] and from *Lactobacillus helveticus* (DLDH-Lh) [98], D-2-hydroxyisocaproate dehydrogenase from *Lactobacillus casei* (DHICDH-Lc) [99], vancomycin-resistant protein from *Enterococcus faecium* (VANH-Ef) [100] and D-dehydrogenase homologue from *Zymomonas mobilis* (DDH-Zm) [101]. The residues are numbered according to the sequence of FDH-Ps. The alignment, with minor changes, is based on that of Vinals and co-workers [94]. Asterisks mark the invariant residues. The residues essential for FDH catalysis and substrate binding are highlighted in light red. The secondary structure of FDH-Ps according to [92] is shown by the arrows. Boxes delineate the matching regions. The following residues were considered to be similar: non-aromatic non-polar (L, I, V, M); aromatic polar (F, Y, W); small with near-neutral polarity (C, S, T); small and breaking the folding pattern (G, A, P); acid and uncharged polar (D, E, N, Q); basic polar (H, R, K).

This observation led Tishkov and co-workers [50,91] to propose that the C-terminal amino acids are digested in a series of post-translational steps, producing a number of polypeptides that differ in charge and hence in electrophoretic mobility. Thus occurrence of multiple molecular forms with modified stability and kinetic properties might be associated with partial degradation of the C-terminus. In agreement with this assumption, the electrophoretic behaviour of the translational product of the complete FDH-Ps gene was identical with the mobility of the basic component observed during electrofocusing of preparations of the wild-type enzyme [50,91].

Comparison with NAD+-dependent dehydrogenases

FDHs show a relatively low degree of overall similarity (less than 20%) with polypeptides present in the sequence data banks [93]. However, the multiple alignment method indicates a significant relationship between FDHs and D-specific 2-hydroxyacid dehydrogenases acting on D-isomers of hydroxyacids [94], which have been recently shown to constitute a related family of proteins, evolutionarily distinct from L-specific 2-hydroxyacid dehydrogenases [95]. This suggests that FDH and D-specific dehydrogenases [96–101] may have similar folds.

Homologous regions (Figure 3) are found in the interdomain contact region, where some catalytically important residues are located. A high degree of conservation of the nicotinamide subsite (βD–βF) and a βA–αB structural element comprising the characteristic nucleotide binding template G(A)XGXXG$_{17}$XD [102] is also observed. The alignment does not extend to the catalytic domain. A number of residues, i.e. Gly-200, Gly-203, Gly-304, Ala-151, Ala-340, Val-142, Ile-202, Phe-213, Pro-256, Asp-128, Asp-249, Asn-281, Arg-162, Arg-284, Lys-274 and His-332, are essentially conserved in the alignment. Of these, only a small number constitute the FDH active site. The implications of the alignment will be discussed later.

Three-dimensional structure

More than 50 crystal structures of nearly 20 NAD(P)+-dependent enzymes have been determined to date at medium and high resolution, providing a vast and constantly growing database [103–109]. Recently this has been complemented by two structures of FDH-Ps, the apo form and its ternary complex with NAD+ and azide, refined at a resolution of 1.80 Å (where 1 Å = 0.1 nm) and 2.05 Å respectively [92,110,111]. The high degree of sequence similarity between various FDHs allows the extension of generalizations derived from the tertiary structure of the bacterial enzyme to other enzymes of the same family.

Domain structure

The dimeric FDH molecule has the general shape of a prolate ellipsoid, with dimensions of about 105 Å × 55 Å × 45 Å. Similar to many other related dehydrogenases, the FDH subunit is folded into a globular two-domain structure (Figure 4). A specific role is ascribed to each of the domains in NAD+-dependent dehydrogenases. The coenzyme binding domain in these enzymes is responsible for recognition and binding of the NAD+ molecule in a productive conformation and has an evolutionarily conserved structure [5–8]. The other, catalytic, domain is specific to each protein and comprises amino acid residues essential for catalysis.

Coenzyme binding domains in FDH are located near the molecular symmetry axis, comprise the central part of the polypeptide chain (amino acid residues 147–333; Figure 3) and make up the subunit interface. The catalytic domains include two polypeptide fragments (residues 1–146 and 334–400), which are

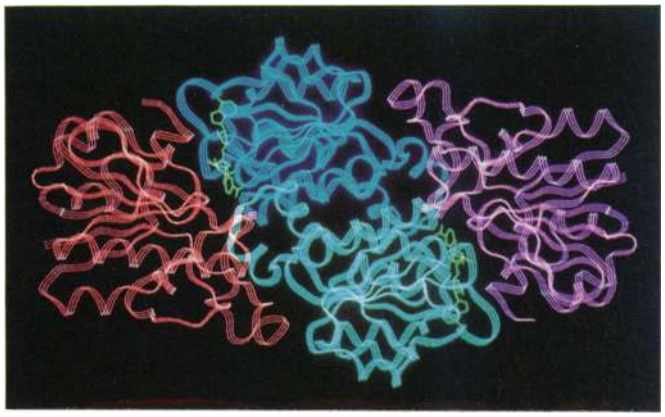


Figure 4 Ribbon plot of FDH ternary complex with NAD+ (green) and azide (not shown)

Catalytic domains are coloured in red and magenta, and coenzyme binding domains are depicted in blue and cyan.

situated on the periphery of the dimer and do not come into contact with one another. The two domains are connected via two long α-helices, αA and α8. The active centre is situated at the domain interface and is formed by residues from only one subunit. In full accordance with this observation, individual subunits of FDH-Ps stabilized in reversed micelles display catalytic activity [24].

Supersecondary structure

Each of the domains has the same type of structural organization: a left-handed twisted parallel β-sheet surrounded by α-helices. The coenzyme binding domain is based on a seven-stranded β-sheet (βC–βB–βA–βD–βE–βF–βG) and the catalytic domain on a five-stranded one (β8–β7–β5–β1–β4), with two short antiparallel strands attached (Figure 5). The same structural pattern of the coenzyme binding domain was found in a number of NAD+-dependent dehydrogenases, MDH [1], ADH [2], GPDH [3], LDH [4] and 6-phosphogluconate dehydrogenase [108], comprising the LDH subfamily; and in L-3-hydroxyacyl-CoA dehydrogenase [103], hydroxysteroid dehydrogenase [104], NADH peroxidase [107], glutathione reductase [112] and other related flavin-containing disulphide oxidoreductases [113]. Examples of other types of topologies providing the same function, i.e. binding of nucleotides, are represented by isocitrate dehydrogenase [105], 3-isopropylmalate dehydrogenase [106], medium-chain acyl-CoA dehydrogenase [114] and beef liver catalase [115].

The β-sheet in the classical coenzyme binding domains is usually composed of two supersecondary structural elements, each comprising three parallel β-strands connected by α-helices, β-strands or irregular loops (the so-called 'Rossmann fold'). However, besides two Rossmann folds, the β-sheet in the FDH coenzyme binding domain contains a seventh β-strand with corresponding α-helix (αG, βG). This distinguishes the structure of FDH from those of ADH, LDH and MDH, and makes it resemble slightly the GPDH structure (Figure 5). The fold of the catalytic domain in FDH resembles the topology of the coenzyme binding domain (Figure 5) and differs significantly from that of the catalytic domains in other NAD+-dependent dehydrogenases which comprise antiparallel β-sheets [1–4].

The similar function fulfilled by the coenzyme binding domains of the various dehydrogenases is reflected in their similar three-

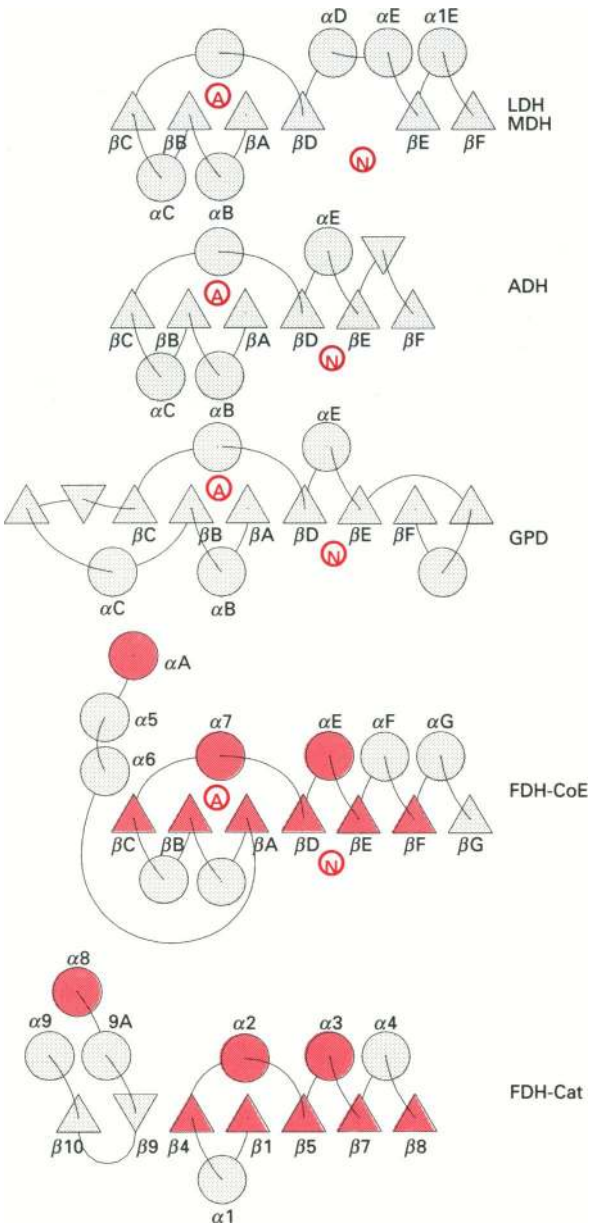


Figure 5 Diagrammatic representation of the connectivity of the coenzyme binding (FDH-CoE) and the catalytic (FDH-Cat) domains of FDH-Ps compared with the coenzyme binding domains in related NAD⁺-dependent dehydrogenases

Circles depict α-helices, and triangles indicate β-sheets. A, the adenine part of NAD⁺; N, the nicotinamide part of NAD⁺. Topologically equivalent parts of FDH-Ps (see the text) are indicated in light red.

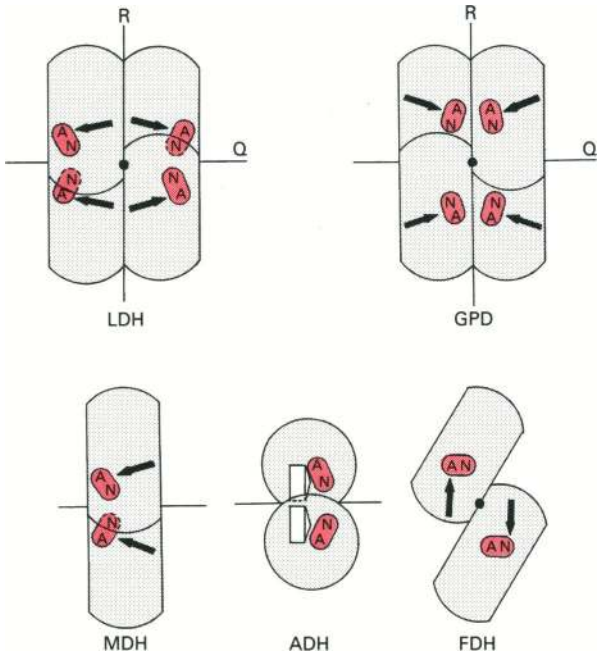


Figure 6 Quaternary structure organization of FDH-Ps compared with related NAD⁺-dependent dehydrogenases

The orthogonal PRQ set of co-ordinates [5] is employed. The pointers show directions of β-strands in sheets. A, the adenine part of NAD⁺; N, the nicotinamide part of NAD⁺. See the text for further details.

dimensional organization [5–8]. Spatial alignment reveals conservation of the β-sheet structure (root mean square deviation between C_α atoms of about 1.0 Å) in the coenzyme binding domains among several NAD⁺-dependent dehydrogenases, including FDH [111]. This is especially striking in view of the complete lack of statistically significant similarities (below 20%) in the respective primary structures.

The coenzyme binding domain is not the only element that is conserved in NAD⁺-dependent dehydrogenases. Another topologically conserved unit is an α-helix located in a fixed orientation relative to the plane of the β-sheet of the coenzyme binding domain [8]. This is the αA-helix in ADH, the C-terminal helix in GPDH and the α3G-helix in LDH. Thus the β-sheet of the coenzyme binding domain comprising two Rossmann folds and helix αA (or its equivalent) form a stable supersecondary structural motif specific to the LDH subfamily of NAD⁺-dependent dehydrogenases. This motif is also present in the catalytic domain of FDH [116].

The FDH catalytic domain may be superimposed on the coenzyme binding domain from the same protein, providing 52 structurally equivalent pairs of C_α atoms with a root mean square deviation of about 1.1 Å. This three-dimensional alignment includes the entire β-sheet in the FDH catalytic domain as well as flanking (α8) and internal (α2, α3) helices (Figure 5). The most interesting feature of the alignment is the topological equivalence of two pairs of α-helices: α8–αA and α2–α7. The former provide links between two domains, while the latter facilitates the advance of the main chain from one side of the β-sheet of the respective domain to the other. However, no statistically significant similarity between the primary structures of the two parts of the FDH polypeptide chain is observed.

A number of proteins show remarkable similarity in the folding of constituent domains [117]. FDH provides one more example of a highly symmetrical molecule composed of domains with very similar topology but with quite different physiological functions. However, the question as to whether both domains of FDH evolved from the same ancestral protein, or whether the similarity between domains is a result of convergent evolution to a stable spatial structure, remains to be clarified.

Quaternary structure

In order to describe the quaternary structure of NAD⁺-dependent dehydrogenases, an orthogonal set of co-ordinate axes named P,

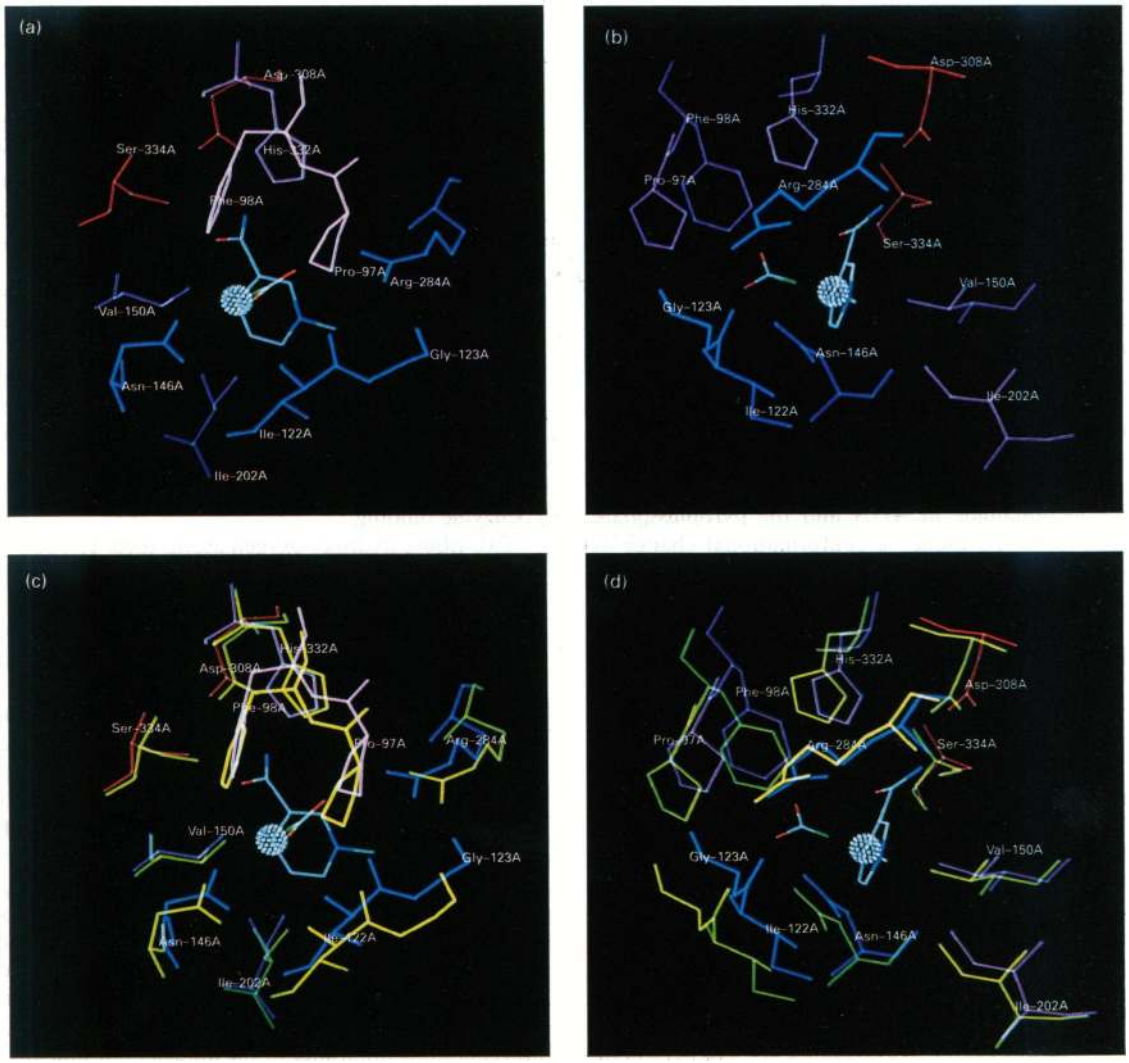


Figure 7 Structure of the FDH active site

The formate anion is built into the position of the azide inhibitor. Only selected residues essential for catalysis and substrate binding are shown. The catalytic C4N position of NAD^+ is marked by dotted sphere. Hydrophobic residues and His-332 are magenta, residues participating in formate binding and Ile-122 are blue, and residues participating in carboxamide binding are red. (**a**) Nicotinamide moiety of NAD^+ viewed perpendicular to the pyridine plane. (**b**) Nicotinamide moiety of NAD^+ viewed along its pyridine plane. (**c**) and (**d**) Displacement of the active site residues upon the transition from the apo (green) to the holo state.

Q and R, related to the orientation of the β-sheet in the coenzyme binding domain of the proteins, is employed [5–8,111]. Three basic types of quaternary structure in dimeric NAD^+-dependent dehydrogenases can be envisaged: the predominant intersubunit contacts occurring across the P axis (when the symmetry axis between β-sheets is parallel to the P axis), or across the Q or R axes, giving rise to 'P-', 'Q-' and 'R-oriented' dimers respectively. MDH was characterized as a Q-dimer, while tetrameric LDH and GPDH were associations of two Q-dimers (Figure 6) [5,111]. The orientation of the coenzyme binding domains in another dimeric dehydrogenase, ADH, relative to its molecular symmetry axis was similar to that in LDH, GPDH and MDH [5].

The orientation of the symmetry axis in FDH is close to the P axis in tetrameric dehydrogenases. The symmetry axis in FDH is parallel to the 'β-sheet plane' and perpendicular to the direction of the β-strands (Figure 6). Thus, according to its quaternary structure organization, FDH constitutes a new subfamily of NAD^+-dependent dehydrogenases, the 'P-oriented' dimers. As a result of subunit association, a large hydrophobic core is formed which is delineated by two planes of adjacent β-sheets of coenzyme binding domains with α-helices in between.

Conformational changes

Conformational changes on transition from the apo (open) to the holo (closed) form on coenzyme binding may be regarded as a general property of NAD^+-dependent dehydrogenases. However, the degree of these conformational changes as well as their origin differ significantly within the family, ranging from relatively small adjustments in dihydrofolate reductase [118] to dramatic changes in LDH resulting in the displacement of certain functional groups of up to 23 Å [119]. The fine details of conformational changes in dehydrogenases differ from enzyme

to enzyme. Proteins which lack a distinct domain structure, e.g. LDH, accomplish conformational changes by the movement of loops and other elements of secondary structure, while in proteins with clear domain structure (ADH, GPDH), conformational changes may be described as domain movements as rigid bodies, i.e. sliding or rotation over hinges.

FDH also undergoes considerable conformational change on cofactor binding [92]. The structures of holo (ternary complex with NAD^+ and azide) and apo FDH-Ps differ in the position of peripheral catalytic domains, which rotate 7.5 ° around hinges connecting residues 146–147 and 340–341 located in the αA and α8 helices respectively. Rearrangements in FDH on coenzyme binding result in substantial changes in the structural organization of the C-terminus, leading to its ordering and formation of a new α-helix (α9) compared with the apo form.

Conformational changes in NAD^+-dependent dehydrogenases are triggered by rather diverse factors. Coenzyme units necessary for conformational changes seem to be the nicotinamide ribose in GPDH, the nicotinamide in ADH and the pyrophosphate moiety in LDH [6–8]. To induce a conformational change in LDH, substrate binding is required. By employing small-angle X-ray scattering it was shown that for FDH-Ps the ADPR fragment of the coenzyme is sufficient to induce major conformational change [120]. Another important finding from small-angle scattering experiments is the critical role of the charge on the nicotinamide ring of the coenzyme. The neutral form of this moiety (FDH–NADH binary complex) or even its entire absence (FDH–ADPR) induces a shift to the closed conformation, while the binary complex with positively charged NAD^+ resembles in its overall conformation the open apo form of FDH [120].

Regardless of the type of conformational changes in NAD^+-dependent dehydrogenases, the net result for catalysis is always the same: closing of the substrate cleft and shielding of the enzyme active centre from the solvent (transition from the open to the closed conformation), compacting of the active site and fine positioning of the functional amino acid residues relative to the catalytic C-4 position of the nicotinamide. Rotation of the catalytic domains results in effective screening of NAD^+ from the bulk solvent by the C-terminal portion of the polypeptide chain. Closing of the second entrance to the active site (the 'substrate channel'; see below) resulting in significant displacements of some catalytically important residues in the immediate vicinity of the C-4 atom of the nicotinamide moiety also occurs, and will be discussed in the following sections.

Active centre

A view of the FDH active site is presented in Figure 7. Two deep channels lead into it; one channel is occupied by NAD^+, while the other provides a pathway for substrate.

(a) NAD^+ conformation. Structural studies have shown that for all dehydrogenases investigated so far, the conformation of the coenzyme bound to the enzyme is very similar, reflecting similarities in the fold in dinucleotide binding domains [8]. The conformation of NAD^+ bound to FDH is similar to the 'standard' coenzyme conformation. NAD^+ binds to FDH in an open conformation (the C2N–C6A distance is 14.7 Å and the angle N9A–OPP–N1N is 108 °); the torsion angle governing the stereospecificity of dehydrogenases is −124 °, in accordance with the type A stereospecificity of this protein [51]. NAD^+ binding follows the same general rules as for other NAD^+-dependent dehydrogenases (Table 4).

1. Adenine subsite. Adenine lies in an extensive hydrophobic pocket and is also sandwiched between two residues, Arg-241 and Cys-255, which occupy structurally equivalent positions in other dehydrogenases [8]. Cys-255 has been identified as the 'essential thiol' (see above). In apo FDH this cysteine is easily accessible to modifying agents.

Introduction of any bulky substituent in place of the SH group as a result of modification or simple oxidation catalysed by transition metal ions would result in steric hindrance between this substituent and adenine, and thus prevent productive binding of NAD^+. In holo FDH, Cys-255 is protected by bound cofactor and is inaccessible to modifying agents.

The 'essential' cysteine is conserved in the structure of *Hansenula polymorpha* FDH, but is replaced by threonine in the potato enzyme. FDHs vary considerably in the number of cysteines (from three per subunit for *Pichia pastoris* to seven for *Pseudomonas* sp. 101). However, all FDHs are inhibited by SH-modifying reagents (Table 1). Based on structural data, this might be attributed in most cases to the blocking of the cysteine residue at the adenine binding subsite, preventing productive coenzyme binding.

Two ribose hydroxy oxygen atoms form H-bonds to Asp-221. This aspartate residue is a part of the $GXGXXG_{17}XD$ consensus sequence [102] and is responsible for a preference for NAD^+ over $NADP^+$. Its spatial position (end of βB) is highly conserved [5–8]. However, FDH-Ps shows considerable activity towards $NADP^+$ (see above), which is easily explained by taking into account the environment of the hydroxy groups of the adenine ribose. A number of positively charged amino acids (Arg-222, His-223, His-379) located in this region provide a suitable environment for the bulky negatively charged phosphate group of $NADP^+$.

2. Pyrophosphate binding. In FDH, as in other NAD^+-dependent dehydrogenases, the pyrophosphate part of NAD^+ lies close to the residues from the turn between βA and αB belonging to the GXGXXG template. The dipole moment of the αB helix provides a positive charge to allow binding of the pyrophosphate moiety of the coenzyme. A specific role has been ascribed to each of the glycine residues in this fingerprint. The first glycine is critical for the tightness of the turn, the second prevents steric hindrance of the dinucleotide and amino acid side chain of the protein backbone at this position, and the third is essential for proper interactions between the β-strand and overlying α-helix [102,121,122].

In FDH-Ps the first glycine from the consensus sequence is replaced by Ala-198. However, the main chain dihedral angles of this alanine correspond to those for the first glycine in the fingerprint [92]. In other dehydrogenases, as well as in FDHs from the yeast *Hansenula polymorpha* [85] and potato [42], and in the putative FDH from *Aspergillus nidulans* [87], this position is occupied by glycine. Thus glycine does not need to be the first residue of the consensus sequence in NAD^+-dependent dehydrogenases, but it is certainly the preferred residue at this position.

Another important glycine residue that is often found in NAD^+-dependent dehydrogenases at the end of the βD strand, whose role is considered to be to provide a close fit of the coenzyme to the edge of the β-sheet [2], is replaced in all FDHs (as well as in the homologous D-specific 2-hydroxyacid dehydrogenases) by proline (Pro-256).

A positively charged residue(s) is usually provided by NAD^+-dependent dehydrogenases to compensate for the negative charge of the pyrophosphate group of the coenzyme (Table 4). However, these residues are not conserved. In FDH Arg-201 fulfills this role.

3. Nicotinamide subsite. The nicotinamide moiety of NAD^+ in FDH (Figure 7) points to the substrate binding site on its *pro-R* side, thus accounting for the A-type stereospecificity [51]. The carboxamide group with its O7N facing C4N (*cis* con-

Table 4 Dehydrogenase-coenzyme interactions

Adapted from ref. [8].

	ADH	GPDH	LDH	MDH	DHFR	FDH
Side chains between which the adenine rings bind						
Side 1	Ile-224	Leu-34	Val-54	Val-42	His-64	Arg-222
Side 2	Ile-269	Thr-96	Ala-98	Ile-102	Leu-62	Cys-255
Hydrogen bonds to adenine atoms						
N-1	Water	Water				Water
N-6	Water					Glu-260
						Water
N-7	Water	Arg-98			Water	His-258
Hydrogen bonding residues to adenine ribose						
O-2′	Asp-223	Asp-33	Asp-53			Asp-221
O-3′	Asp-223	Asp-33		Asp-41	Water	Asp-221
	Lys-228	Water				Water
O-2′-PO_3 (NADPH)					Arg-43	
					Gln-65	
					-NH-64	
Hydrogen bonds to phosphate oxygen atoms						
OP-1-A	Arg-47		Arg-101	Arg-88	-NH-99	Ser-380
						Water
						Water
OP-2-A		Asn-182		Arg-88	-NH-102	Arg-201
		Water			-NH-45	-NH-201
					Thr-45	
OP-1-N	Arg-369	Water	Arg-101		-NH-101	-NH-201
	-NH-47	Water				-NH-202
						Water
OP-2-N	-NH-202	-NH-11	-NH-32	Asn-223	Arg-44	Ser-147
	-NH-203	-NH-12			Water	Water
Hydrogen bonds to the NMN-ribose						
O-2′	Ser-48	SO_4^{2-}	Gln-102	Asn-125	C-O-18	Water
	His-51		C-O-101			
O-3′	His-51	Water		-NH-125	C-O-18	Water
	C-O-269	Water			Water	
Hydrogen bonds to carboxamide						
N-7	C-O-317	Water		C-O-149	C-O-6	C-O-282
	C-O-292	Water		His-181	C-O-13	Ser-334
						Asp-308
O-7	-NH-319	Asn-314		C-O-123	-NH-6	-NH-335
				C-O-149		Ser-334

formation) is twisted by 25 ° from the pyridine plane through a complicated set of interactions with a number of negatively charged amino acids (Thr-282, Asp-308, Ser-334). The carboxamide binding site of FDH properly orients the catalytic C4N position of the nicotinamide moiety, provides its necessary polarization and forces the carboxamide group to be out of plane with the pyridine.

(b) Substrate channel. The active site is deeply buried about 15 Å inside the FDH subunit and is accessible to formate anion, either through the NAD^+ binding site if NAD^+ is absent or through a wide channel running from the active centre to the surface [92]. Three side chains comprising part of the FDH active site, Pro-97, Arg-284 and His-332, form the inner neck of the channel, which is the gateway for the substrate to proceed to the active centre. In holo FDH the channel is additionally shielded from solvent by the loop Asn-385–Ser-390, and the inner neck separating the interior of the active centre from the channel becomes narrower.

The substrate channel is large enough to accommodate a molecule bigger than formate but not as big as NAD^+. Modelling studies indicate that molecules of glutathione or GSF may be inserted into the substrate channel of apo FDH with minor adjustments of side chains composing the channel walls. GSF may be oriented in such a way that its formyl group points to the active centre through the inner neck. Thus crystallographic studies do not conflict with the observations concerning broad substrate specificity of FDH-Ps.

If GSF is a true substrate for bacterial FDH as well as for enzymes from other sources, then the molecular mechanism of these enzymes with GSF would be much more complicated than that with formate. Possibly the reaction with GSF is not a concerted one but has two stages: binding and hydrolysis of the thioester somewhere in the substrate binding channel, and subsequent NAD^+-dependent oxidation of the resulting formate ion in the normal way. However, the question as to whether GSF might bind to the protein and be a true substrate of FDH remains open and needs further verification by crystallographic analysis of complexes with glutathione or its derivatives.

The presence of the special substrate channel accounts for the inhibitory effect of pyridoxal and related compounds (see above) with linear dimensions much larger than the formate binding subsite can accommodate. It is assumed that these substances

Destabilizing	Stabilizing
Twist of the carboxamide group enhances polarization of C4N	'Negative cluster' (Thr-282, Asp-308, Ser-334) enhances polarization of C4N
Hydrophobic wall (Val-150, Ile-202) perturbs the nicotinamide moiety of NAD^+	Hydrophobic wall (Val-150, Ile-202) stabilizes neutral NADH and forces bending of the nicotinamide moiety towards the substrate ('boat' conformation)
Ile-122 perturbs the nicotinamide moiety of NAD^+	Ile-122 prevents unproductive substrate binding
Positive charge of Arg-284 perturbs the nicotinamide moiety of NAD^+	Arg-284 stabilizes the negative charge on migrating hydride ion
Formate anion is destabilized by the hydrophobic wall (Pro-97, Phe-98)	Ile-122 oxygen stabilizes the positive charge on the formate carbon

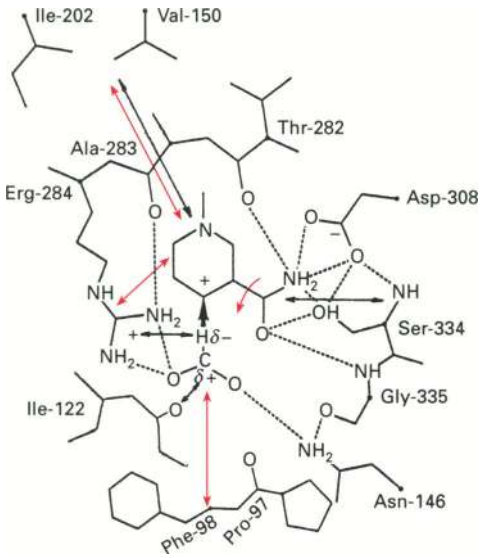


Figure 8 Scheme of the FDH active centre near the point of catalysis

H-bonds are indicated by dashed lines. The pointer shows the direction of hydride anion transfer. Black arrows show stabilizing interactions, whereas red arrows show destabilizing interactions within the active centre. See the text for further details.

bind within the channel, preventing access of substrate to the active site. In this respect pyridoxal might be considered as an allosteric inhibitor. Lys-286 near to the active centre is located more than 15 Å away from the presumed formate binding site, near the outlet of the substrate channel to bulk solvent. It is the most probable site of pyridoxal binding.

(c) Substrate binding site. The active centre of FDH-Ps is presented in Figures 7 and 8 with formate anion occupying the azide binding site revealed by crystallographic studies [92]. Oxygen atoms of formate form H-bonds to Arg-284 and Asn-146, while the direction of the presumed hydride ion transfer makes an angle of about 90 ° with the pyridine plane (similar to other dehydrogenases). The H atom of the substrate is within 1.4 Å of the C4N position of the coenzyme.

The active site of FDH comprises negatively and positively charged clusters of residues as well as hydrophobic patches. The 'negatively' charged region (Figure 8) is made up of residues Thr-282, Asp-308 and Ser-334 holding in place the carboxamide group of NAD^+. They belong to the coenzyme binding domain, are already properly oriented in the apo enzyme and do not undergo considerable shifts on holo complex formation.

Another cluster of residues can be designated as 'positively' charged and comprises the Arg-284 and Asn-146 side chains and the imide nitrogen of Ile-122. All three residues undergo substantial displacement on the transition from apo to holo. Arginine and asparagine move about 0.7–0.8 Å towards the substrate binding site in holo FDH, while the wall comprising the Ile-122–Gly-123 pair as well as several neighbouring residues advances about 1.8 Å towards the substrate. The role of the positively charged cluster is mostly to bind formate anion and to fix it in the proper position relative to NAD^+.

There are several hydrophobic clusters in the active site. The first is composed of residues Val-150 and Ile-202, and provides a hydrophobic environment for one face of the NAD^+ pyridine ring. The position of these residues in holo FDH differs by less than 0.4 Å from that in the apo form. The second cluster comprises Pro-97 and Phe-98. Their side chains in holo FDH move more than 1 Å and point to the formate binding site. The third hydrophobic region is the Ile-122 side chain hanging over the edge of the pyridine ring. The fourth hydrophobic area is the Val-309 side chain that supports the proper conformation of the NAD^+ carboxamide.

His-332 is another key residue of the active centre. Its side chain moves 0.6 Å closer to C4N in holo compared with apo FDH. The role of this histidine is not well understood. Formate anion might be placed in the active site between this histidine and Asn-146, providing good geometry for the anticipated transition state [92]. However, His-332 makes an H-bond with Gln-313 and thus seems to be locked in the unprotonated state that is unfavourable for binding of negatively charged formate.

MOLECULAR MECHANISM

High-resolution crystallographic studies of FDH-Ps have shown the detailed architecture of the active centre. Combined with the wealth of information on the solution properties of FDHs from various sources accumulated over the past decades, they provide a firm basis for hypotheses of the molecular mechanism. Moreover, the molecular mechanism of FDHs can be regarded as a 'case study' for the whole class of NAD+-dependent dehydrogenases.

Alignment with D-dehydrogenases

An important insight into the role of the various parts of the active centre of FDH in the molecular mechanism of the enzyme can be acquired by comparing homologous regions of FDH and D-specific dehydrogenases at the FDH active site (Figure 3). Formate is the smallest substrate for enzymes of the D-specific 2-hydroxyacid dehydrogenase family. In the FDH-catalysed reaction, hydride anion leaves the first carbon atom, in contrast to 2-hydroxyacids where hydride leaves the second carbon. This suggests that the active sites of enzymes acting on 2-hydroxyacids should differ from that of FDH. Among the FDH active site residues, Ile-202, Arg-284, Asp-308 and His-332 appear to be essentially conserved in the alignment, whereas some areas are conserved in terms of hydrophobicity and some regions (Pro-97–Phe-98, Asn-146, Gln-313, Ser-334) are specific to FDHs (Figure 3).

The alignment emphasizes the importance of Arg-284 for substrate binding in both FDH and related dehydrogenases. Arginine is a well known anchor group in the active centres of L-specific LDH and MDH [1,4]. In these enzymes it forms a bifurcated H-bond with the carboxylic acid group of the substrate. The same role is ascribed to Arg-284 in FDH; however, it seems to H-bond to only one of formate's oxygens. Moreover, the FDH active centre prevents formation of a fork-shaped structure with formate carboxyl, which would result in unproductive complex formation. As anticipated, the second residue taking part in substrate binding, Asn-146, is specific to FDHs, because of the stereochemical and geometrical differences between dehydrogenation of formate anion and 2-hydroxyacids.

The role of the other conserved residue, Asp-308, is unambiguous. It is an anchor for coenzyme carboxamide function, ensuring proper positioning and polarization of the nicotinamide moiety in the active centre. Thr-282 and the Ser-334–Gly-335 pair in FDH are a part of the negatively charged zone which coordinates the NAD+ carboxamide. Thr-282 and Gly-335 contribute to binding through their main-chain atoms and thus need not be conserved in the alignment. However, Ser-334 is specific to FDHs.

Three out of the four hydrophobic sites of the FDH active centre are conserved in the alignment in terms of hydrophobicity. Val-150 and Ile-202 provide a hydrophobic environment from one side of nicotinamide. The Ile-122–Gly-123 pair plays a dual role. Firstly, these residues participate in substrate binding: Ile-122 by its main-chain atoms, and Gly-123 oxygen maintains proper positioning of the Arg-284 residue in the active centre. Secondly, the hydrophobic side chain of Ile-122 is pressed over the edge of the nicotinamide. Another hydrophobic cluster, Pro-97–Phe-98, is specific to FDHs. In holo FDH these residues are pressed over the substrate from the side opposite to the hydride ion to be transferred and create an unfavourable environment for the charged formate anion.

Special consideration should be given to the conserved residue His-332. In general, both L- and D-specific dehydrogenases require an acid–base pair for catalysis (proton abstraction or uptake from/to the hydroxy group of the substrate). Histidine is the most probable candidate for this role. A His–Asp pair linked by an H-bond and functioning as a 'charge relay system' was found in some NAD+-dependent dehydrogenases [123] as well as other enzymes, e.g. serine proteases [124], that require assistance in proton transfer. However, according to the alignment, in D-specific dehydrogenases the His–Asp pair is replaced by a His–Glu pair which fulfills the same functional role. This was confirmed by spatial alignment of the key residues constituting the FDH active site and participating in the substrate coordination (the C4N position of NAD+, Arg-284, His-332 and Gln-313) with the corresponding residues of L-specific enzymes [125]. It appeared that these residues in FDH make a 'mirror', inverted, image of the respective residues in L-specific enzymes, thus matching the stereochemical requirements of the substrate.

In FDHs the catalytically important Glu comprising the His–Glu charge relay system is replaced by Gln. One probable reason for such a substitution in FDH, whose substrate lacks a hydroxylic function, is trapping of the catalytic histidine in an unprotonated state, thus blocking its ability to switch between protonated and unprotonated forms. However, the final conclusion concerning the role of His-332 and Gln-313 in catalysis by FDH (if any) should await the results of site-directed mutagenesis experiments.

Catalytic mechanism

According to the 'central dogma' of catalysis, for a protein to be an efficient catalyst it should accomplish two tasks: stabilize the transition state of the reaction and/or destabilize the initial states of the reactants. The net result is lowering of the reaction barrier and enhancement of the rate. In the transition state of the reaction catalysed by FDH, a hydride anion leaves formate and attacks the electrophilic C4N of the positively charged nicotinamide moiety of NAD+. As a result two neutral species, CO_2 and NADH, are produced, while NAD+ C4N changes its hybridization from sp_2 to sp_3 and the nicotinamide moiety becomes uncharged.

The mechanism of hydrogen transfer in the FDH-catalysed reaction is mainly governed by electrostatic effects. The positively charged nicotinamide unit of NAD+ is properly oriented in the active centre by multiple interactions with a 'negatively' charged cluster and hydrophobic side chains, whereas formate is bound and oriented by multiple H-bonds with a 'positively' charged cluster. Several stabilizing and destabilizing interactions take place in the active centre in the course of catalysis (Figure 8).

An important factor for catalysis by all NAD+-dependent dehydrogenases, including FDH, is enhancement of the electrophilic properties of C4N of the nicotinamide moiety of the coenzyme. This might be achieved through sufficient polarization of the NAD+ carboxamide group via interactions with negatively charged ligands (see above) and perturbation of its ground state due to the twist of the carboxamide with respect to the pyridine plane. Such out-of-plane positioning of the carboxamide group of the coenzyme is quite common among NAD+-dependent dehydrogenases. Most of these have NAD(P)+/NAD(P)H bound with carboxamide in the *cis* conformation, supporting the polarization of the C4N atom with the torsion angle in the range from $-27°$ to $+34°$ [8,126]. For FDH the value is $+25°$.

Three hydrophobic walls comprising Pro-97–Phe-98, Ile-122 and Val-150/Ile-202 are pressed over the active site as a result of the conformational change in the course of the reaction. The Pro–Phe pair may perturb the formate ground state and provide a more suitable environment for neutral CO_2, thus accounting

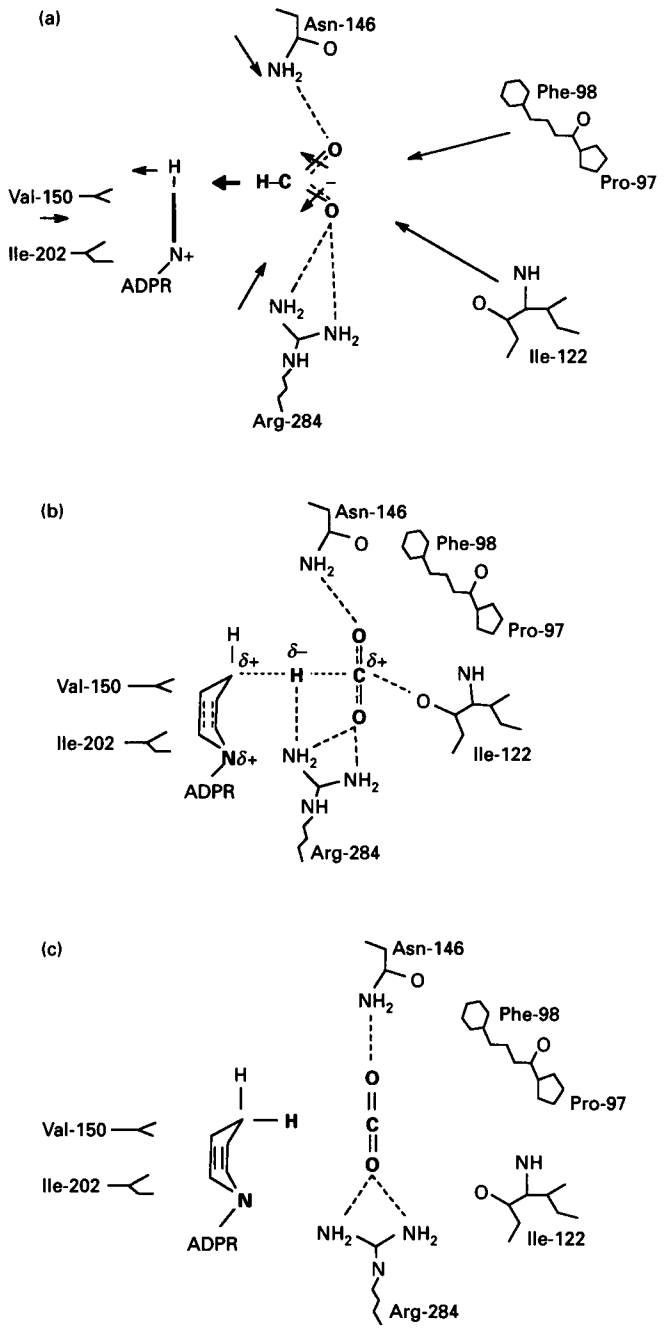


Figure 9 The main steps of the FDH molecular mechanism

Pre-transition (**a**), transition (**b**) and post-transition (**c**) states of the reaction. Arrows indicate the movements of parts of the enzyme active centre and the reactants during the course of the catalytic transformation. The size of the arrows represents the relative displacement of the respective amino acid residues on the transition of FDH from the apo to the holo state.

for better binding of the latter compared with the charged formate anion. The Val-150 and Ile-202 side chains face the other side of the pyridine ring and thus favour better binding of neutral NADH compared with charged NAD^+. This wall also facilitates possible bending of the nicotinamide moiety towards the substrate, forcing it to acquire the 'boat' conformation assumed to result in lowering of the activation barrier [127]. However, the hypothesis of changing the pucker of the nicotinamide in the transition state is in conflict with recent observations of a lack of change in the N-1 hybridization state in the FDH and ADH reactions.

Besides being an anchor group for the substrate, Arg-284 can play two additional roles. First, the positive charge on Arg-284 may stabilize a partial negative charge on the migrating hydride ion and, secondly, it may perturb positively charged nicotinamide. The crucial role of Arg-284 in the architecture of the active centre is corroborated by site-directed mutagenesis. When this residue was replaced by Gln it resulted not only in a 25-fold decrease in affinity towards the substrate and lowering of the catalytic constant (1.5% of that of the wild-type enzyme), but also in a 30-fold decrease in affinity towards NAD^+ and general destabilization of the protein [24]. Substitution of Arg-284 with alanine abolishes enzyme activity completely (V. I. Tishkov and A. V. Mezentzev, personal communication).

An important role in the mechanism is ascribed to Ile-122, which together with the flanking parts of the polypeptide chain moves by nearly 2 Å on transition from apo to holo. This movement is essential for several reasons: (1) it prevents unproductive substrate binding due to pairing of the guanidinium group of Arg-284 and the formate carboxylic acid group; (2) the main chain carbonyl of Ile-122 stabilizes the partial positive charge on the carbon atom of the formate; and (3) the hydrophobic side chain of Ile-122 might perturb positively charged NAD^+.

By combining all these features, FDH provides a very effective machinery for abstraction of the hydride ion from formate. The C4N position of NAD^+ in the active centre is an at least 10^5-fold better acceptor of hydride than stable thriaryl methylcarbonium cations in model reactions with formate anion proceeding via a similar mechanism of hydride ion transfer [128–130].

The main steps of the proposed FDH molecular mechanism which accounts for the majority of the crystallographic and solution data are presented in Figure 9. When compared with the mechanism suggested earlier for some other NAD^+-dependent dehydrogenases [1–8], the FDH mechanism has both common and specific features. It seems that dehydrogenases may employ a common mode of coenzyme activation (at least this might hold true for the LDH subfamily of dehydrogenases with closely related spatial organization of coenzyme binding domains): (1) the nicotinamide ring is placed in an anisotropic environment with one side facing a hydrophobic wall and the other facing the hydrophilic substrate binding site, and (2) the electropositive character of C4N is enhanced through the out-of-plane *cis* conformation of the carboxamide group and its interaction with electron-withdrawing ligands. However, the mode of activation of the C–H bond in the substrate appears to be specific to the enzyme. For inert substrates, e.g. alcohols, it may be achieved through formation of a strong inner sphere complex with a Lewis base (ADH). In the case of LDH/MDH or GPDH (activated β-carbonyl compounds), H-bond formation between the 2-hydroxy group of a substrate and the histidine residue (acid–base catalyst) supported by additional interaction with a positively charged arginine is sufficient.

The charge relay system present in LDH/MDH and ADH is not required for FDH. This might be a reason for locking His-332 of the active centre in only one (unprotonated) state. Moreover, there is no obvious activator of the C–H bond in FDH. Activation is likely to be a result of a concerted effect of a number of interactions, including hydrophobic (Pro-97–Phe-98 wall) and electrostatic (Ile-122). Compared with other related dehydrogenases, FDH appears to be a relatively slow enzyme. At least one of the reasons might be partial deactivation of the C–H bond in the substrate because of the strong electrostatic interactions with positively charged substituents of the active centre

(Asn-146, Arg-284). Thus slow catalysis might be a cost of proper binding of the substrate.

PRACTICAL APPLICATIONS

NADH regeneration systems

NAD+-dependent dehydrogenases have strong potential for practical applications. However, their use is still limited because of the requirement for stochiometric amounts of rather expensive coenzymes to perform catalytic transformations. The high price of NAD(H) demands its continuous regeneration throughout the process. Several approaches for regeneration of nicotinamide coenzymes have been suggested, including chemical, photochemical, electrochemical and enzymic methods [11]. While the problem of NAD+ recycling might be reasonably solved by various means, e.g. direct oxidation on carbon or modified electrodes, regeneration of NADH is a more challenging task. Among the methods suggested, enzymic means of NADH regeneration are most promising [11].

FDH has been extensively studied as a candidate for developing industrial NADH regeneration [9–13,131–146]. The advantages of FDH are: availability and low cost, a favourable thermodynamic equilibrium, the inertness of CO_2 and its ready removal from the main product, and the low cost of the auxiliary substrate (formate). The disadvantages of FDH reside in its relatively low specific activity and limited coenzyme specificity (towards NAD+ only).

Co-immobilized preparations of D-LDH and FDH were employed for production of D-lactate from pyruvate [131]. The optical purity of the product was above 92%, the yield was about 80% and the NAD+ cycling number was about 1500. Carrea and co-workers [12] used FDH to regenerate NADH for production of 12-oxochenodeoxycholic acid from dehydrocholic acid in a system comprising two hydroxysteroid dehydrogenases. The concentration of the final product reached 0.9% (w/v), with 100% conversion of the starting material. The coenzyme was recycled 1200 times.

Continuous production of malate from oxaloacetate over a period of 20 days was obtained in a column containing a polyacrylamide gel for entrapping FDH, thermostable MDH and a polymerizable NAD+ derivative [132]. FDH–MDH and FDH–leucine dehydrogenase entrapped in a polyacrylamide gel were used for continuous production of malate from oxaloacetate [133] and of leucine from α-oxoisocaproate and ammonia [134] in column reactors with soluble NAD+ as a cofactor. A system for leucine production with a bienzyme system, i.e. leucine dehydrogenase–FDH encapsulated in the membrane emulsions of surfactants, has been described [135]. FDH was used together with methane mono-oxygenase to produce alcohols from alkanes [136], and together with ADH for preparation of isotopically labelled alcohols [137].

The most ambitious project employing FDH was developed in Germany for production of amino acids from oxoacids [12,138–144]. The concept of the membrane reactor system is used. NAD+ is coupled to water-soluble dextran [145] and is retained in the reactor together with FDH and an amino acid dehydrogenase (e.g. leucine dehydrogenase) with the help of an ultrafiltration membrane. The performance of the membrane reactor has been optimized [146]. Continuous production of L-leucine for a period of 48 days was obtained, with the conversion ratio reaching 99.7%. The NAD+ cycling number was about 35000 to 50000 [139,144], pointing to the economic feasibility of the process.

Purified FDH or whole cells with FDH activity may be employed for the production of NADH as the final product. FDH-Ps immobilized on silica supports has been used for quantitative conversion of NAD+ to NADH [24]. Cells of *Arthrobacter* sp. KM62 containing a high level of FDH activity in freeze–thawed, air-dried or acetone-dried form [147] or entrapped in various supports [148] were used to obtain NADH; 90–100% conversion was obtained and the isolated NADH was 83% pure.

Several attempts have been made to transfer NAD(H) into some kind of prosthetic group in FDH and thus overcome one of the principal limitations of NAD+-dependent dehydrogenases [149,150]. Conjugates of NAD+ and a water-soluble co-polymer of vinyl pyridine and acrolein form stoichiometric complexes with FDH-Ps that show high intrinsic activity in the presence of formate without any exogenous coenzyme [149]. Continuous recycling of the cofactor bound in the vicinity of the active centre was obtained in the presence of dichlorophenol indophenol and diaphorase as second enzyme. NAD+ modified at the N-6 position may be covalently attached to yeast FDH [150]. The FDH–NAD+ complex thus obtained was active in reactions with LDH, MDH, alanine dehydrogenase and leucine dehydrogenase.

Bioenergetics and analysis

Due to its low redox potential ($E_0^h = -200$ mV), formic acid is of clear interest for bioenergetics. It may be regarded as potential fuel for biochemical fuel cells [151]. Sukhno and co-workers [152] showed that formate can be easily oxidized on a pyrographite electrode in the presence of FDH, diaphorase and methyl viologen as a mediator. A current density of 12 mA/cm² was obtained. Prospects for formic acid as a convenient energy carrier were investigated. Storage of the hydrogen gas obtained, e.g. through biophotolysis of water in the form of formic acid, has been discussed [151,153]. Klibanov and co-workers studied the bienzyme system comprising *Pseudomonas oxalaticus* FDH and hydrogenase isolated from *Alcaligenes eutrophus* or whole cells containing hydrogenase activity for production of formate from CO_2 and H_2 [153]. Several multienzyme systems for hydrogen production employing FDH and hydrogenases from various sources were studied [154]. In all systems, equilibrium hydrogen pressure was obtained.

FDHs from various sources are used for quantification of formate and formic acid in solutions and physiological fluids [155–157].

FUTURE PROSPECTS

The priorities in studies of FDH are clear and distinct. Crystallization and structural characterization of various binary and ternary complexes of wild-type FDH-Ps as well as its genetically engineered mutants is under way. The structure of the FDH–ADPR complex has been recently refined at 1.5 Å resolution (V.S Lamzin, A. V. Mezentzev, T. B. Ustinnikova, E. G. Harutyunyan, Z. Dauter, K. S. Wilson and V. O. Popov, unpublished work). The first crystals of a closely related yeast (*Hansenula polymorpha*) FDH have been obtained (E. Blagova, personal communication). The proposed molecular mechanism of FDH action is being tested through site-directed mutagenesis. A number of mutants will be obtained in order to clarify the role of individual amino acid residues in catalysis and substrate binding.

Protein engineering of FDH seems to be both of academic and practical interest. One challenge is to transform FDH into a D-specific dehydrogenase that is active towards 2-hydroxyacids via multiple mutations in the vicinity of the active centre affecting both its specificity towards the substrate and general folding of the protein. The other is to obtain a versatile biocatalyst for NADH and NADPH regeneration. The first steps in this direction

have been already taken. For FDH-Ps, series of mutations in the adenine binding region of the enzyme active centre were introduced, resulting in changed coenzyme specificity [24]. The double mutant D221A/G203A has catalytic parameters for $NADP^+$ reduction comparable with that for NAD^+ for the wild-type enzyme. To make FDH an ideal enzyme for cofactor regeneration, an increase in turnover rate is required.

Thus FDH is currently emerging as one of the most extensively studied enzymes among NAD^+-dependent dehydrogenases. Comprehensive biochemical information, the availability of high-resolution structures of two enzyme forms, cloned and expressed structural genes of FDHs from various organisms and a wide potential range of biotechnological applications provide a sound basis for FDH to become a model enzyme for mechanistic studies and practical use.

Note added in proof (received 21 June 1994)

Since this Review was submitted, two more FDHs have been sequenced: from *Candida methylica* (J. J. Holbrook, personal communication) and from *Neurospora crassa* [158]. Both FDH sequences are very similar to the sequence of FDH-Ps.

We would like to pay tribute to our late teacher, Professor Iliya Berezin, for his constant interest and support of our research for more than a decade. We acknowledge the decisive contribution of Professor A. Egorov (Moscow State University) in initiating the studies on FDH, and thank him for long-term co-operation and stimulating discussions. The structural part of this research would never be accomplished without the help and co-operation of Dr. K. Wilson (EMBL, Hamburg) and Professor E. Harutyunyan (Shubnikov Institute of Crystallography, Moscow). We thank Dr. V. Tishkov (Moscow State University) for co-operation in the protein engineering studies. V.O.P. thanks all former and present colleagues from his laboratory in the A. N. Bakh Institute of Biochemistry for their contributions.

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