Site-directed mutagenesis of the essential arginine of the formate dehydrogenase active centre

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Abstract

Sequence alignment shows that residue Arg 284 (according to the numbering of the residues in formate dehydrogenase, FDH, from the methylotrophic bacterium Pseudomonas sp. 101) is conserved in NAD-dependent FDHs and α-specific 2-hydroxyacid dehydrogenases. Mutation of Arg 284 to glutamine and alanine results in a change of the catalytic, thermodynamic and spectral properties of FDH. In comparison to wild-type, the affinity of the mutants for the substrate (K formate m ) or the transition state analogue (K azide i ) decreases and correlates with the ability of the side chain of residue 284 to form H-bonds. In contrast, the affinity for the coenzyme (K NAD d or K NAD m ) is either not affected or increases and correlates inversely with the partial positive charge of the side chain. The temperature dependence of circular dichroism (CD) spectra of the wild-type FDH and its Ala mutant has been studied over the 5–90°C temperature range. Both proteins reveal regions of enhanced conformational mobility at the predenaturing temperatures (40–55°C) associated with a change of enzyme kinetic parameters and a co-operative transition around 55–70°C which is followed by the loss of enzyme activity. CD spectra of the wild-type and mutant proteins were deconvoluted and contributions from various types of secondary structure estimated. It is shown that the co-operative transition at 55–70°C in the FDH protein globule is triggered by a loss of α-helical secondary structure. The results confirm the conclusion, from the crystal structures, that Arg 284 is directly involved in substrate binding. In addition this residue seems to exert a major structural role by supporting the catalytic conformation of the enzyme active centre. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Formate dehydrogenase; Site-directed mutagenesis; Mechanism; Secondary structure; Stability

Abbreviations: FDH, formate dehydrogenase; CD, circular dichroism; MLR, multiple linear regression
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1. Introduction

NAD-dependent formate dehydrogenase (FDH) from the methylotrophic bacterium *Pseudomonas* sp. 101 is one of the most extensively characterised NAD-dependent dehydrogenases. High-resolution structural information \[1,2\] combined with a wealth of kinetic data and physico-chemical studies in solution provided the basis for a tentative FDH mechanism \[1,3\].

FDH belongs to the family of d-specific 2-hydroxyacid dehydrogenases acting on d-stereoisomers of respective substrates \[4\]. Significant sequence similarity has been detected within this family \[4\]. Recent structural studies of d-specific dehydrogenases, d-phosphoglycerate dehydrogenase \[5\], d-glycerate dehydrogenase \[6\], d-lactate dehydrogenases \[7,8\] and d-hydroxyisocaproate dehydrogenase \[9\], revealed strong similarities of protein fold between FDH and these proteins. FDH is a typical NAD-dependent dehydrogenase composed of two identical subunits each comprising two domains: a coenzyme binding domain and a substrate binding domain. Both domains in FDH, as well as in other members of the family, reveal significant topological similarity and are based on Rossman folds (Fig. 1). A topologically conserved part consists of the five-stranded parallel \(\beta\)-sheet with a left-handed twist, one complete and one partial Rossman fold, and a number of \(\alpha\)-helices, including a long helix (about 20 amino acids) which provides a link between two domains \[10\]. Recently another putative FDH from the hyperthermophilic archaeabacterium *Pyrobaculum aerophilum* has been solved at a resolution of 2.80 Å \[11\], revealing a similar structural organisation.

\(\alpha\)-Helices comprise a substantial part (44% according to the X-ray structure analysis) of the overall FDH secondary structure \[1\]. Oriented \(\alpha\)-helices of the Rossman folds contribute substantially (up to 20–30\%), through their dipolar moments, to the electrostatic binding of the coenzyme and substrate in the active centre of NAD-dependent dehydrogenases \[12\]. In FDH specific orientation of the \(\alpha\)-helices relative to the substrate binding cleft creates a unique electrostatic environment in the vicinity of the enzyme active centre. These long-range interactions could be of importance in guiding charged FDH substrates to their respective binding sites and in governing the overall stability of the protein molecule.

Three invariant residues are found in the active sites of a number of d- and l-specific dehydrogenases: arginine, histidine and a carboxylic acid (aspartate or glutamate). The conserved arginine provides an anchor for a substrate by making a H-bond between its guanidinium group and the substrate carboxylate, while the conserved histidine, H-bonded to the carboxylic acid (Asp in l-specific dehydrogenases or Glu in d-specific ones), forms a composite acid–base catalyst and provides a proton shuttle to and from the 2-hydroxyl group of the substrate \[3,13,14\].

Recently an additional role has been ascribed to the essential arginine in d-specific dehydrogenases in contrast to the case for l-specific enzymes \[8,9,15\]. It has been hypothesised that this residue is implicated...
in catalysis through polarisation of the carbonyl group of the substrate. According to this assumption arginine of δ-specific dehydrogenases not only anchors the substrate but also fulfills a role similar to that of Arg 109 in L-specific LDH [16]. This implies a different geometry and H-bonding pattern for the essential arginines in the active centres of L- and δ-specific dehydrogenases.

FDH occupies a special place among the other members of the family of δ-specific dehydrogenases because of the structure of its substrate, the formate ion. Firstly, in the case of FDH the reaction centre is located at C1 contrary to C2 for the other 2-hydroxyacid dehydrogenases. Secondly, no proton transfer is implicated in the catalytic mechanism. These features should have a profound effect on the organisation of the FDH active centre.

In FDH Arg 284 is in the position of the conserved arginine in other dehydrogenases [17] and together with Asn 146 and His 332 is the most probable candidate for formate binding. Molecular dynamics simulations performed on the active site of FDH from Pseudomonas [18] are also in favour of the substrate binding pattern as revealed by crystallographic studies between Arg 284 and Asn 146. The same studies implicate His 332 in both substrate binding and transition state formation.

Introduction of point mutations in the enzyme active site is widely used in modern enzymology to probe catalytic mechanisms. However, point mutations can affect not only the catalytic properties and stability of the enzyme, but also the conformation or even the folding. It is important to discriminate between these possibilities. Circular dichroism (CD) spectra of proteins are instrumental in evaluating contributions of various types of secondary structure to the overall protein fold in solution [19]. Continuous monitoring of the protein structural properties with temperature can provide additional insight into details of the protein denaturation process and reveal contributions of the various parts of the structure in maintaining the integrity of the protein globule.

The goal of the present work is to verify assumptions concerning the role of Arg 284 in FDH substrate binding and catalysis using site-directed mutagenesis.

2. Materials and methods

2.1. Materials

The following chemicals were used: NAD (grade V), EDTA, TRIZMA-Base (Sigma, USA). Reagents for genetic engineering experiments were ‘Molecular Biology Grade’ (Sigma) or ‘DNA Purification Grade’ (Applied Biosystems). All other chemicals were of ‘Analytical Grade’ from Reachim (Russia).

2.2. Site-directed mutagenesis

28-mer primers 5'-CGTCAACACCGCCCAGGG-CAGCTGTGC-3' and 5'-CGTCAACACCGCCGCCGCAAGCTGTGC-3' were used to obtain Arg284Gln and Arg284Ala FDH mutants, respectively. Synthesis of the primers, site-directed mutagenesis reactions, the expression and purification of the mutants were carried out as described previously [20]. Both strands of the DNA for each of the FDH mutants were sequenced with the dye-labelled terminator sequencing kit on an Applied Biosystems Automated DNA Sequencer model 370A and only the expected differences at the mutation sites were observed. No other differences from that of the wild-type have been detected.

2.3. FDH purification

The wild-type enzyme from Pseudomonas sp. 101 and its mutants were purified as described in [21]. The yield of inactive Arg284Ala FDH during purification was checked by ELISA [22]. Enzyme preparations were homogeneous according to electrophoresis in SDS-polyacrylamide gels [23].

2.4. Protein concentration

To determine the protein concentration of the FDH preparations the extinction coefficient of 1.32×10⁵ M⁻¹ cm⁻¹ at 280 nm has been used. Protein concentration has also been determined by measuring the absorbance at three additional wavelengths, 210, 215 and 220 nm [24], as well as at 280 nm. Values derived from the measurements at different wavelengths were compared. The errors in protein concentrations thus determined were in the order
of 3%. For processing CD and fluorescent spectral data protein concentrations have been verified by the biuret method [25] as recommended by Greenfield [19].

The concentration of the wild-type enzyme active centres has been evaluated from the quenching of the protein tryptophan fluorescence by azide in the presence of NAD as described in [26]. From these experiments the content of the active enzyme in the preparations obtained was shown to be not less than 95%.

2.5. Assay of enzyme activity

Enzyme activity was routinely measured spectrophotometrically using a Hitachi 557 spectrophotometer by monitoring the increase of NADH concentration \( V_{340} = 6220 \, \text{M}^{-1} \, \text{cm}^{-1} \) in 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM EDTA, 200 mM sodium formate and 1 mM NAD at 37°C. The reaction was initiated with an aliquot of enzyme. The total volume of the reaction mixture was 2 ml.

2.6. Kinetic and equilibrium constants

For each enzyme preliminary estimates of \( V_{\text{max}} \) and saturating substrate concentrations were obtained by increasing sequentially the concentration of each of the reactants by a factor of two from initial values of 80 \( \mu \)M 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 \( V_{\text{max}} \) and the concentration of the reactant last varied was assumed to be saturating.

The ‘saturating’ concentrations of formate for the wild-type FDH and Arg284Gln mutant (pH 7.0) thus obtained were 0.15 M and 1.5 M, respectively. The ‘saturating’ concentrations of NAD obtained were 1.0 mM and 2.0 mM for the wild-type FDH and Arg284Gln mutant (pH 7.0), respectively.

When determining kinetic parameters the concentration of one of the substrates was kept constant at saturation, while the concentration of the other substrate was varied from ‘saturating’ to close to \( K_m \) \( (V = V_{\text{max}}/2) \). At least eight data points for each data set were obtained. \( K_m \) and \( V_{\text{max}} \) values were obtained by non-linear regression to the Michaelis–Menten equation (Eq. 1):

\[
v = \frac{V_{\text{max}} S}{(K_m + S)}
\]

The inhibition constants for azide (\( K_{\text{azide}} \)) 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 10 \( K_i \), in 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 using a weighted least-squares method, assuming equal variances for \( v \). The slopes then were plotted versus inhibitor concentrations (secondary plot) and fitted to Eq. 2 with the statistical weight for each data point proportional to \( 1/\sigma^2 \) obtained from the primary plot.

\[
slope = \frac{K_m}{V_{\text{max}}} \left( 1 + \frac{|I|}{K_i} \right)
\]

The NAD dissociation constants (\( K_{\text{NAD}}^d \)) for the enzyme–substrate complexes FDH–NAD were obtained from the experiments on the quenching of the tryptophan fluorescence of FDH in the presence of NAD [26] by linear regression of Eq. 3:

\[
F_0/(F_0−F) = (1−\alpha)^{-1} \times K_{\text{NAD}}^d/|\text{NAD}| + (1−\alpha)^{-1}
\]

where \( F_0 \) is the initial fluorescence, \( F \) the fluorescence in the presence of the quencher, NAD, and \( \alpha \) is the relative quantum yield of the binary complex FDH–NAD.

2.7. Temperature dependence of kinetic parameters (\( K_{\text{NAD}}^m \) and \( K_{\text{form}}^m \))

Michaelis constants for NAD (\( K_{\text{NAD}}^m \)) and formate (\( K_{\text{form}}^m \)) were determined at various temperatures following the procedure described above. The FDH sample and a spectrophotometric cell containing all the other components of the reaction were preincubated at a given temperature. The cell was moved to a cell compartment of a Hitachi 557 instrument adjusted to the same temperature, the reaction was initiated with an aliquot of enzyme and the initial reaction rate was monitored.
2.8. Thermal inactivation

Thermal inactivation experiments were carried out in 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM EDTA. The FDH sample was preincubated at high temperatures (45–70°C) and the aliquots were regularly withdrawn within 1–30 min in order to evaluate the residual enzymatic activity. The enzymatic activity was measured as described above. For each of the temperatures studied the value of the corresponding first order inactivation rate constant ($k_{\text{in}}$) was obtained. The logarithmic form of Eq. 4:

$$k_{\text{in}} = \frac{k \times T}{h} \times \exp\left(\frac{\Delta S^\circ}{R}\right) \times \exp\left(-\frac{\Delta H^\circ}{R \times T}\right)$$

was used to estimate $\Delta H^\circ$ and $\Delta S^\circ$ [20].

2.9. CD spectra

For each of the proteins 20 CD spectra were obtained within the 5–90°C temperature range in 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA using a 1mm programmed thermostatted cell with a Jasco 715 instrument. For the mutant enzyme the potassium phosphate concentration was reduced to 6.4 mM. The temperature was increased at a rate of $<0.3^\circ$C/min. The temperature was kept constant whilst spectra were recorded. Spectra were recorded twice for about 2 min at each of the temperatures and the data obtained were averaged. The error of the instrument was within ±1 mdeg for the 200–250 nm range at a sensitivity of 50 mdeg (≈2%) and increased up to 5% for the 195–200 nm range. The data obtained were presented as the ellipticity units per mole of an average amino acid residue assuming the average molecular weight of the FDH residue to be 109.7 using software supplied with the instrument.

The concentration of the wild-type FDH was 0.14 mg/ml or 1.29 mmole of amino acid residues per litre. For the Arg284Ala mutant the values were 0.15 and 1.37, respectively. The resultant error of the CD measurements was assumed to be a combination of the instrumental and concentration errors estimated by:

$$\Delta \Theta = [\Theta] \sqrt{\left(\frac{\Delta \Theta}{\Theta}\right)^2 + \left(\frac{\Delta \Theta}{c}\right)^2}$$

giving an overall accuracy of measurements better than 3–6% (depending on wavelength).

2.10. Spectrum deconvolution

A number of methods belonging to various classes of deconvolution approaches (multiple linear regression (MLR), self-consistent, ridge regression, etc. (for review see [19])) were applied to the spectrum of the wild-type FDH recorded at 25°C. To discriminate between various methods the results obtained were compared with the known content of each type of the secondary structure derived from the X-ray data. A deconvolution method which showed the best result (Table 1) was selected and further used for processing data obtained at various temperatures both for the wild-type enzyme and for its point mutant. All other methods used gave unsatisfactory results when applied to FDH.

The modified method of Bolotina and coworkers [27–29] used in the present work is a representative of the MLR group of methods and takes into account the impact of the aromatic residues absorbing in the peptide region (<250 nm) on the resulting protein CD spectrum. The software for implementation of the MLR method was provided by one of the authors (N.P.B.).

2.11. Fluorescence

Fluorescence measurements were carried out at 20°C using a Hitachi 3MF spectrofluorimeter with protein concentration of (0.4–0.8)×10^{-6} M (excita-
tion wavelength 300 nm). Prior to spectroscopic measurements, protein samples were filtered through a 0.22 μm membrane filter.

Relative quantum yields of the apo forms of the FDH mutants were obtained by normalising the fluorescence of the protein preparations by the protein concentration determined as described above.

2.12. Differential scanning calorimetry

Differential scanning calorimetry experiments were performed on a DASM-4M microcalorimeter (Institute for Biological Instrumentation, Pushchino, Russia) with a cell volume of about 0.5 ml, interfaced with a personal computer. Experiments were performed at a scanning rate of 0.25 K/min. Prior to measurements, the FDH samples were dialysed against 0.1 M phosphate buffer at pH 7.0 containing 10 mM EDTA. Before measurements were made, samples were degassed by stirring in an evacuated chamber for 10 min at room temperature and then immediately loaded into the calorimeter cells. The final dialysis buffer was used (after the same degassing procedure) as a reference solution. A pressure of 2.0 atm of nitrogen was always maintained over the calorimetric cells throughout the scans to prevent any gas release artifacts during heating. The reversibility of the thermal transitions was checked by a second heating period immediately after the sample had cooled following the first scan. The thermal transition was found to be completely irreversible under the conditions used. The calorimetric traces were corrected for variations in the instrumental baseline by subtraction of the scans obtained from the second heating of the samples. The temperature dependence of the molar heat capacity was further analysed and plotted using the Origin software package (Microcal, Northampton, MA, USA). The thermal stability of the FDH was characterised by the temperature of the maximum of thermal transition ($T_m$). For evaluation of the relative co-operativity of the thermal transition, the value of the width at the half-height of the excess heat capacity traces ($\Delta T_{0.5}$) was used.

3. Results and discussion

Arg 284 is one of the key residues in the FDH active site (Fig. 2) and in the FDH–NAD–azide ternary complex it directly participates in the binding of azide anion as revealed by X-ray structural studies [1]. Azide is the closest structural and electronic analogue of CO$_2$, the product of the reaction [30], and forms a tight ($K_i \approx 1.5 \times 10^{-7}$ M, Table 2) dead-end ternary complex that is considered to be a stable analogue of the reaction transition state [1,3,31].

To investigate the role of Arg 284 substitutions by glutamine (a polar residue, about 2 Å shorter than arginine and capable of H-bonding) and alanine (a small residue with a hydrophobic side chain which is unable to form H-bonds with the substrate) were made. Table 2 summarises the kinetic properties of

Fig. 2. Stereo view of the active centre of FDH from Pseudomonas sp. 101. Formate is supposed to overlap the azide binding site. AZI, azide.
the mutants and the wild-type enzyme. In the case of FDH from *Pseudomonas* sp. 101 the steady state kinetic parameters, $k_{\text{cat}}$ and $K_m$, are easy to interpret. The catalytic constant is governed by the rate of hydride transfer [3,31^33], while $K_m$ refers to the dissociation constant of the respective substrate from the central ternary complex FDH-NAD-formate [3].

Mutation to glutamine results in a nearly 10-fold decrease ($v_{G} = 5.8 \text{ kJ/mol}$) in substrate affinity and a 32-fold decrease ($v_{G} = 8.9 \text{ kJ/mol}$) in the catalytic constant. Affinity for azide, a transition state analogue, is affected most of all and reduced 160-fold ($v_{G} = 13.1 \text{ kJ/mol}$). The Arg284Ala mutant is completely inactive (Table 2). This is attributed to the lack of substrate binding as the ability to bind the cofactor molecule is still retained. The mutant does not form a ternary dead-end complex with NAD and azide either. Formation of this complex, if it were formed, would be observed by quenching of the tryptophan fluorescence of the protein [26].

Assuming that the Arg 284 guanidinium group is directly H-bonded to one of the formate oxygen atoms (Fig. 2), mutation to glutamine is expected to result in a considerable decrease in substrate affinity: the H-bond distance to formate is increased while the partial positive charge of the anchoring group is decreased. This implies some conformational changes in the active site and possible movement of glutamine and/or formate towards each other thus affecting fine positioning of the reactants for hydride transfer.

The change of the free energy of substrate binding by 5.5–8.9 kJ/mol in the Arg284Gln mutant corresponds to the loss of 1–2 hydrogen bonds. The larger effects observed for azide indicate that the mutation affects the transition state to a greater extent than the initial ground state and supports the conclusion that a vital role of Arg 284 is not only in substrate binding but in catalysis as well. Some interactions essential for transition state stabilisation in the mutant enzyme either do not occur or are incomplete.

Arg 284 is one of the main structure-supporting residues of the FDH active centre (Fig. 2). In the apo FDH this residue is H-bonded only to the main chain carboxyl of Ala 283 (distance 2.8 Å) and to the carboxylic group of Asp 125 (distance 3.5 Å). On transition from the apo to the holo state caused by coenzyme (and substrate) binding, Arg 284 shifts by 0.7–0.8 Å towards the presumed substrate binding site [1]. In the FDH–NAD–azide ternary complex, Arg 284 already makes five H-bonds: with the main chain carboxyl of Ala 283 (2.9 Å), the carboxylic group of Asp 125 (2.7 Å), the distal nitrogen of the azide anion (3.2 Å) and the main chain carboxyls of Ile 122 and Gly 123 (2.9 and 3.1 Å, respectively). This structuring is reflected by the decrease of the temperature factor of the CZ atom of Arg 284 from 48–56 in the apo enzyme to 11–12 in the ternary complex.

Computer modelling shows that the Arg284Gln mutant should still be able to make two H-bonds with the carboxylate of Asp 125. Interaction with the main chain carboxyl of Ala 283 (now unfavourable) is also maintained. Thus, the Arg284Gln mutation results in partial preservation of the H-bonding network in the apo enzyme, while considerable rearrangement of the H-bonding pattern is expected for the holo enzyme due to unfavourable close contact between Gln 284 OE1 and Ala 283 O and increased H-bonding distances. More profound changes are expected for the Arg284Ala mutation which completely change the H-bonding network in the active site.

Substitutions in the sequence Arg→Gln→Ala should result in considerable rearrangements within the FDH active centre and lead to (1) a change in the electrostatic balance (positively charged Arg, neutral polar Gln, non-polar Ala), (2) an alteration of the H-bonding pattern (glutamine in contrast to alanine

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$k_{\text{cat}}^{\text{formate}}$ (mM)</th>
<th>$k_{\text{cat}}^{\text{azole}}$ (µM)</th>
<th>$K_{\text{NAD}}$ (mM)</th>
<th>$K_{\text{NAD}}^{d}$ (mM), 20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>10</td>
<td>15 ± 2</td>
<td>0.15 ± 0.02</td>
<td>0.11 ± 0.01</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td>Arg284Gln</td>
<td>0.31 ± 0.02 (32)$^a$</td>
<td>145 ± 18 (9.7)$^a$</td>
<td>24 ± 5 (160)$^a$</td>
<td>0.17 ± 0.06 (1.5)$^a$</td>
<td>0.53 ± 0.03 (1.02)$^a$</td>
</tr>
<tr>
<td>Arg284Ala</td>
<td>not detected</td>
<td>–</td>
<td>no binding</td>
<td>–</td>
<td>0.08 ± 0.03 (6.5)$^a$</td>
</tr>
</tbody>
</table>

$^a$Change of the parameter.
still preserves the ability of H-bonding) and (3) possible changes in protein conformation sensed by appropriate physico-chemical techniques.

The temperature stability of FDH has been studied to investigate the changes occurring on mutation of Arg 284. In apo FDH the uncompensated positive charge of Arg 284 placed within the interior of the protein globule is thermodynamically disadvantageous. Removal of this charge dramatically enhances the temperature stability of FDH. The rate of inactivation of the Gln mutant at 65°C is decreased 6-fold compared to the wild-type (data not shown). The activation energy of FDH inactivation are significantly reduced for the mutant enzyme (from 420 to 120 kJ/mol for $\Delta H^*$ and from 950 to 40 J/(mol×K) for $\Delta S^*$), suggesting that some conformational changes may have occurred.

The observed stabilisation effect upon neutralising the uncompensated positive charge of the active site arginine has a universal origin in dehydrogenases. Significant stabilisation against temperature induced inactivation has also been observed for l-LDH from *Bacillus stearothermophilus* when active site arginine (Arg 171) was substituted for Tyr or when its positive charge was simply counterbalanced by negatively charged sulphate ions [34].

Two spectral methods, CD and tryptophan fluorescence, have been used to probe how the mutations affect the FDH conformation. Tryptophan fluorescence is sensitive to changes in the local protein conformation because the quantum yield, maximum and half-width of the spectrum are affected by subtle changes in the environment. The FDH subunit contains seven tryptophans. Two are located within 10 Å of the catalytic NAD C4 position. The closest, Trp 99, together with Pro 97 and Phe 98 constitute a part of the hydrophobic wall of the enzyme active centre. Trp 310 is located in the main chain between two important active site residues, Asp 308 and Gln 313.

Replacement of Arg 284 by glutamine and alanine affects the fluorescent properties of the mutant forms (Fig. 3). The most significant changes in the environment of the tryptophan residues are observed for the Arg284Ala mutant. The mutation is accompanied by a 2-fold decrease in the relative quantum yield of tryptophan fluorescence. The form of the spectrum (i.e. the maximum and half-width), however, remains unaltered. It is worth noting that the magnitude of the changes on arginine substitution are significant but much less than the quenching of tryptophan fluorescence in the wild-type FDH upon NAD binding, which reaches 85% [26].

CD spectra of the wild-type FDH and its mutants are presented in Fig. 4. At predenaturing temperatures spectra of the wild-type FDH reveal two minima at around 208 and 222 nm (Fig. 4A). These minima are characteristic of $\alpha$-helices and this form of CD spectra is typical for the majority of proteins with a large $\alpha$-helical content [19,35,36]. FDH also falls into this class because, according to X-ray data (Table 1), it has about 44% of its secondary structure in the form of $\alpha$-helices.

The change of the FDH CD spectra with temperature follows a complex pattern. Four regions related to various states of protein conformational stability can be recognised (Fig. 5). The spectra remain virtually unchanged in the 5–30°C temperature range. However, already at predenaturing temperatures in the 30–55°C range, where the enzyme retains its activity for hours, substantial changes in the intensity of the spectra (up to 20%) occur. For the wild-type FDH this region is characterised by a slow decrease in $\alpha$-helical content (from 44 to about 35%) and a concomitant increase in the content of a random
structure (from 25 to 30%), as predicted by the deconvolution method used (Fig. 6).

The gradual structural transition is accompanied by a change of the enzyme kinetic parameters. While $K_m$ values for both substrates show only a minor change with temperature in the 30–40°C range (region I on the CD spectra) they increase considerably (6–7-fold) at temperatures of 40–57°C (region II on the CD spectra). Thus the affinity of both substrates towards the enzyme active centre is reduced simultaneously with temperature increase and correlates with a change of protein CD properties (Fig. 5). The structural rearrangement in the 40–60°C range affects mainly substrate binding but not the transition state. Plots of $\ln(K_m)$ vs. $1/T$ are non-linear in the 30–60°C temperature range and show two regions with a different $\Delta H$ and a breakpoint at around 45°C which could be indicative of some conformational change taking place. In contrast, the $\ln(V_{max})$ vs. $1/T$ plot (the FDH catalytic constant is governed by the rate of hydride transfer [3]) is linear and provides a single estimate for the activation energy of the enzyme reaction of 38.9 ± 3.3 kJ/mol (data not shown). It can be assumed that FDH as a molecule and its active centre undergo some conformational transitions in the 40–60°C temperature range which
are accompanied by disintegration/rearrangement of a part of the protein secondary structure which, in turn, is reflected in the binding parameters of the substrates.

A major co-operative transition in wild-type FDH as followed by CD is observed around 60–70°C with a midpoint around 65°C (region III on the CD spectra, Figs. 5 and 6) and is accompanied by disintegration of a substantial part of the protein secondary structure. Deconvolution reveals that this transition is mainly accompanied by disruption of \( \alpha \)-helices (from \( \sim 35 \) to \( 10-5\% \)) (Fig. 6).

The co-operative transition manifested by a change in the secondary structure is accompanied by a loss of enzymatic activity. Temperature profiles of both the first order inactivation rate constant \( (k_{in}) \) and the molar ellipticity at 222 nm (representing mainly a change in protein \( \alpha \)-helical content) follow generally the same pattern (Fig. 5). The structural transitions revealed by the CD spectra and accompanied by disintegration of protein \( \alpha \)-helices and a loss of catalytic activity coincide with the process of overall melting and randomisation of the protein globule as revealed by scanning microcalorimetry (Fig. 7). The value of the denaturation temperature \( (T_m) \) of 65.9°C and \( \Delta T_{0.5} \) of 4.2°C obtained are in good agreement with the midpoint value obtained from CD measurements.

Thus four major phases determining conformational mobility of FDH with temperature can be outlined. In the 5–30°C temperature range conformation of the protein remains intact as revealed by CD and enzyme activity remains stable (region I). In the intermediate temperature range of 30–55°C (region II) some \( \alpha \)-helices of the FDH become disrupted and this is manifested by an alteration of CD spectra and results in a gradual change of the enzyme kinetic parameters. FDH is, however, highly stable in this temperature range and does not undergo considerable heat inactivation. Consequently it should retain integrity of the active site. The third phase (region
in the 55–70°C region is manifested by an abrupt change of CD properties, melting of the major part of the protein \( \alpha \)-helices, and loss of enzymatic activity. In the fourth phase (region IV) over 70°C wild-type FDH is present as a denatured structure. CD spectra become featureless and difficult to interpret.

Overall a similar picture upon temperature change is observed for the Arg284Ala FDH mutant. Temperature profiles of molar ellipticity at 222 nm for both the wild-type enzyme and its point mutant are very similar (Fig. 7) and reveal two temperature transitions. These are a ‘predenaturing’ transition at 30–55°C and a co-operative one at around 60–65°C. However, the midpoint temperature characterising the co-operative transition is shifted from 65°C for the wild-type to 61–62°C for the mutated protein, manifesting a decreased resistance to heat denaturation. Microcalorimetry studies of the Arg284Ala mutant also reveal parameters somewhat shifted from that of the wild-type FDH: a \( T_m \) of 61.8°C and \( \Delta T_{0.5} \) of 5.5°C (data not shown).

While CD data on the Gln mutant are consistent with relatively small alterations in protein conformation, the Ala mutant seems to undergo significant conformational changes. In spite of rather similar temperature behaviour considerable differences in the overall form of CD spectra of the wild-type FDH and its Arg284Ala mutant are observed (Fig. 4B). The Ala mutant shows only about 50% of the intensity of the spectra of the wild-type protein. A minimum at 208 nm is absent in the spectra of the mutant and the amplitudes of changes in the secondary structure content with temperature are not as pronounced as with the wild-type enzyme. Deconvolution of the CD spectra of the mutant (data not shown) reveals that already in the physiological temperature range the \( \alpha \)-helical content of the mutant is substantially decreased compared to the wild-type enzyme. The mutant appears to have lost about 16–20% of the \( \alpha \)-helical content of the wild-type FDH while the content of the \( \beta \)-structure remains virtually unchanged.

From the data presented it could be assumed that the overall fold of the Arg284Ala FDH mutant should be close to that of the wild-type protein and similar core structures (structural motives) are contributing to the process of melting in both proteins. However, considerable changes in the structure of the mutant enzyme affecting predominantly its \( \alpha \)-helices could have occurred.

Thus, both the thermodynamic and spectral properties of FDH mutants show changes in the parameters directly associated with protein conformation. This proves the crucial role of Arg 284 in maintaining the integrity of the enzyme active site and supporting the catalytic enzyme structure.

Despite the significant conformational changes in FDH upon arginine substitution, the coenzyme binding properties of the protein are not abolished. The affinity towards NAD in the series \( \text{Arg} \rightarrow \text{Gln} \rightarrow \text{Ala} \).
even increases somewhat ($\Delta G \approx 4.8$ kJ/mol, Table 2). This is in agreement with the hypothesis that the positively charged guanidinium group of Arg 284 perturbs the positively charged nicotinamide moiety of NAD [3]. In the ternary complex FDH–NAD–azide, the minimum distance between the arginine residue and the nicotinamide moiety of the coenzyme is about 3.3 Å (Arg 284 NH1 to NAD C2N). A change of the positively charged guanidinium group of the arginine residue to the electroneutral carbamido side chain of glutamine does not affect the affinity for NAD, while replacement by alanine enhances coenzyme binding 6.5-fold (Table 2). These data further corroborate the observation that many dehydrogenases bind neutral NAD considerably better than the positively charged oxidised form of the coenzyme (e.g. FDH binds NAD an order of magnitude better than NAD$^+$ [3]). Moreover, the dead-end inhibitor ADPR that has no nicotinamide moiety at all binds to the wild-type enzyme with the same affinity as NAD [3]. Thus we may speculate that Arg 284 may contribute to catalysis not only through transition state stabilisation as revealed by a change of the FDH catalytic constant and affinity towards the transition state analogue, but also by destabilising the ground state of the positively charged nicotinamide moiety of NAD. Maintenance of the NAD binding properties in the mutant forms of the enzyme upon Arg 284 substitution supports the conclusion concerning preservation of the overall protein conformation. It is rather unlikely that a very specific pattern, ensuring tight and specific coenzyme binding in FDH and comprising more than 20 specific interactions with the protein (either directly or through tightly bound water molecules [1]), would be preserved if the protein became partially disordered or even unfolded.

Essential arginine residues have been mutated both in L- [16,37–39] and D-specific dehydrogenases [15] catalysing oxidation of 2-hydroxyacids. Results obtained for L-LDH from *B. stearothermophilus* [37–39] unambiguously support conclusions drawn from crystallographic studies and ascribe Arg 171 a role as a substrate ‘anchor’. Arg 171 mutations to lysine, tryptophan or tyrosine resulted in a dramatic (1000–3000-fold) decrease in substrate $K_m$ while exerting less profound effects on the turnover rate (3–10-fold reduction). It is interesting to note that preservation of the positive charge at the substrate binding site (arginine to lysine substitution) resulted in less active mutants than exchange of arginine to aromatic residues (tryptophan or tyrosine). This was interpreted to mean that some interactions with other parts of the enzyme active site were compensating for proper positioning of the substrate [39].

Arg 109 is another essential arginine of the l-LDH active centre. It is located on the mobile loop that closes the enzyme active centre on formation of the productive ternary complex, thus shielding it from the solvent [14]. This ‘mobile arginine’ plays a crucial role in the catalytic mechanism of l-LDH and related enzymes through additional polarisation of the substrate carbonyl bond [40]. Its role in stabilisation of the resulting transition state, originally deduced from structural studies, has been confirmed by site-directed mutagenesis [16].

D-Specific dehydrogenases of 2-hydroxyacids possess only one arginine in their active centres that can be structurally aligned with the ‘anchoring’ arginine of l-specific enzymes [3,5–9]. To compensate for the absence of the ‘mobile’ arginine while maintaining the basics of the catalytic mechanism formulated for l-specific enzymes, additional functions have been ascribed to this residue. It is suggested that in D-specific dehydrogenases the active site ‘anchor’ arginine assumes a different conformation from that in l-specific enzymes and, in addition to binding the substrate, participates in catalysis through polarisation of the carbonyl group of the substrate [8,9,15]. Substrate binding functions in D-specific enzymes could also be performed, in part, by a segment of the main chain containing the conserved glycine residue (Gly 123 in FDH). In line with these assumptions on a dual role of essential arginine in D-specific dehydrogenases substitution of arginine for lysine or glutamine in D-LDH from *Lactobacillus pentosus* results in a substantial (100–600-fold) decrease in both substrate affinity and turnover rate [15]. The effects of Arg to Gln substitution in the FDH catalysed reaction (10–30-fold) are much smaller than those in the case of D-LDH. The relative insensitivity of FDH to this mutation compared to D-LDH may result from the inferior original catalytic properties of FDH and/or significant contributions from the other amino acid residues of the FDH active centre (e.g. His332) in substrate binding.
Recently mutagenesis of the essential arginine was reported for the FDH from the methylotrophic yeast *Candida boidinii* [41], an enzyme highly homologous to FDH from *Pseudomonas*. In agreement with our results an exchange of arginine for alanine resulted in inactive enzyme preparations.

The role of Arg 284 in substrate/product/transition state analogue co-ordination in the FDH active site is directly visualised in the FDH-NAD-azide ternary complex [1]. The role of Arg 284 as one of the sites of formate ion binding is further confirmed by the results of site-directed mutagenesis obtained in the present paper. Participation in the transition state stabilisation is also verified as Arg284Gln mutation produces more profound effects on the catalytic constant and azide (transition state analogue) binding than on substrate affinity. As also demonstrated in this work, Arg 284 can also affect FDH catalysis in another way, namely by destabilising the positively charged oxidised coenzyme. The allocation of the roles among active site residues of FDH remains rather speculative as direct evidence, the structure of the complex of FDH with its substrate, the formate anion, is still lacking. We are working on obtaining crystals of binary and ternary complexes with formate and hope to clarify this issue in the near future.

The present study confirms the key role that Arg 284 plays in substrate binding and catalysis. Both the thermodynamic and spectral properties of FDH mutants show changes in the parameters directly associated with protein conformation. This also proves the crucial role of Arg 284 in maintaining the integrity of the enzyme active site and supporting the catalytic enzyme structure.

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