Bacterial formate dehydrogenase. Increasing the enzyme thermal stability by hydrophobization of alpha-helices

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Abstract NAD$^+$-dependent formate dehydrogenase (EC 1.2.1.2, FDH) from methylotrophic bacterium *Pseudomonas* sp.101 exhibits the highest stability among the similar type enzymes studied. To obtain further increase in the thermal stability of FDH we used one of general approaches based on hydrophobization of protein α-helices. Five serine residues in positions 131, 160, 168, 184 and 228 were selected for mutagenesis on the basis of (i) comparative studies of nine FDH amino acid sequences from different sources and (ii) with the analysis of the ternary structure of the enzyme from *Pseudomonas* sp.101. Residues Ser-131 and Ser-160 were replaced by Ala, Val and Leu. Residues Ser-168, Ser-184 and Ser-228 were changed into Ala. Only Ser/Ala mutations in positions 131, 160, 184 and 228 resulted in an increase of the FDH stability. Mutant S168A was 1.7 times less stable than the wild-type FDH. Double mutants S(131,160)A and S(184,228)A and the four-point mutant S(131,160,184,228)A were also prepared and studied. All FDH mutants with a positive stabilization effect had the same kinetic parameters as wild-type enzyme. Depending on the position of the replaced residue, the single point mutation Ser/Ala increased the FDH stability by 5–24%. Combination of mutations shows near additive effect of each mutation to the total FDH stabilization. Four-point mutant S(131,160,184,228)A FDH had 1.5 times higher thermal stability compared to the wild-type enzyme.

Key words: NAD$^+$-dependent formate dehydrogenase; *Pseudomonas sp.*101; Thermal stability; Site-directed mutagenesis; Hydrophobization of alpha-helices; Additive stabilization effect

1. Introduction

NAD$^+$-dependent formate dehydrogenase (EC 1.2.1.2, FDH) is widely spread in nature. FDH genes were found in bacteria *Pseudomonas* sp.101 [1], *Mycobacterium vaccae* N10 [2], *Moraxella* C-2 (EMBL Accession O08375), yeast *Saccharomices serevisiae* (EMBL Accession Z75296), *Hansenula polymorpha* [3], *Candida methylica* [4], *Candida boidinii* [5], fungi *Aspergillus nidulans* [6], *Neurospora crassa* [7], potato mitochondria [8] and barley [9]. Parts of FDH genes we also found in mammals, e.g. mouse (GeneBank Accession AA681620) and human (GeneBank Accession N75707). All enzymes have similar kinetic properties, but bacterial FDHs overcome the other enzymes in stability. For example, FDH from *Pseudomonas* sp.101 can be stored in phosphate buffer, pH 7.0, at +4°C at least for 12 months without any loss of its activity, while the enzyme from *C. boidini* under the same conditions loses 50% activity in 2 weeks. The comparison of amino acid sequences of FDH from various sources (Fig. 1) shows that enzymes from bacteria differ from other FDHs by the longer N-termini. Some preliminary experiments with FDH from *M. vaccae* N10 [2] showed that this fact can explain a higher stability of bacterial enzymes, but a systematic detailed study on the nature of FDH stability has not been carried out so far.

The elucidation of molecular determinants of enzyme stability is of fundamental interest. The improvement of FDH thermal stability is also very important for the applied purposes. FDH is the best catalyst for NADH regeneration system in the processes of fine organic synthesis of chiral and physiologically active compounds using NAD$^+$-dependent dehydrogenases [10]. FDH from *C. boidinii* is currently used in the industrial process of L-*tert*-leucine production [11]. The process of large-scale production of the recombinant FDH from *Pseudomonas* sp.101 expressed in *Escherichia coli* has also been developed [12]. Preparation of FDH with a higher thermal stability will facilitate protein purification and results in decrease of the enzyme production cost and FDH consumption per kg of a final product.

This paper reports on our experiments oriented to increase the thermal stability of FDH from *Pseudomonas* sp.101 using an approach based on hydrophobization of protein α-helices.

2. Materials and methods

2.1. Materials

All chemicals used for genetic engineering manipulations were of 'Molecular Biology Grade' (Sigma). T4 polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase and restriction endonucleases *Eco*RI and *Xho*I were from New England Biolabs. Oligonucleotides were prepared with an Applied Biosystems DNA Synthesizer 380B. NAD$^+$ (Grade V, Sigma) and sodium formate (analytical grade, Reachim, Russia) were used in kinetic experiments.

2.2. Methods

2.2.1. Preparation of mutants. Uracil single stranded phagemids pFDH6 (wild-type FDH), pFDH6S131A, pFDH6S228A and pFDH6S(131,160,228)A were prepared according to [13]. Site-directed mutagenesis reactions were performed by the method of Kunkel [14]. The following primers were used to obtain mutants: S131A, 5′-CGGTCGATAGCCGCCTGAAGATCG-3′; S131L, 5′-CGGTCGATGGCCACTGAAGATCG-3′; S160A, 5′-GCGCACCAGCGCCA-

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Abbreviations: FDH, NAD$^+$-dependent formate dehydrogenase (EC 1.2.1.2)

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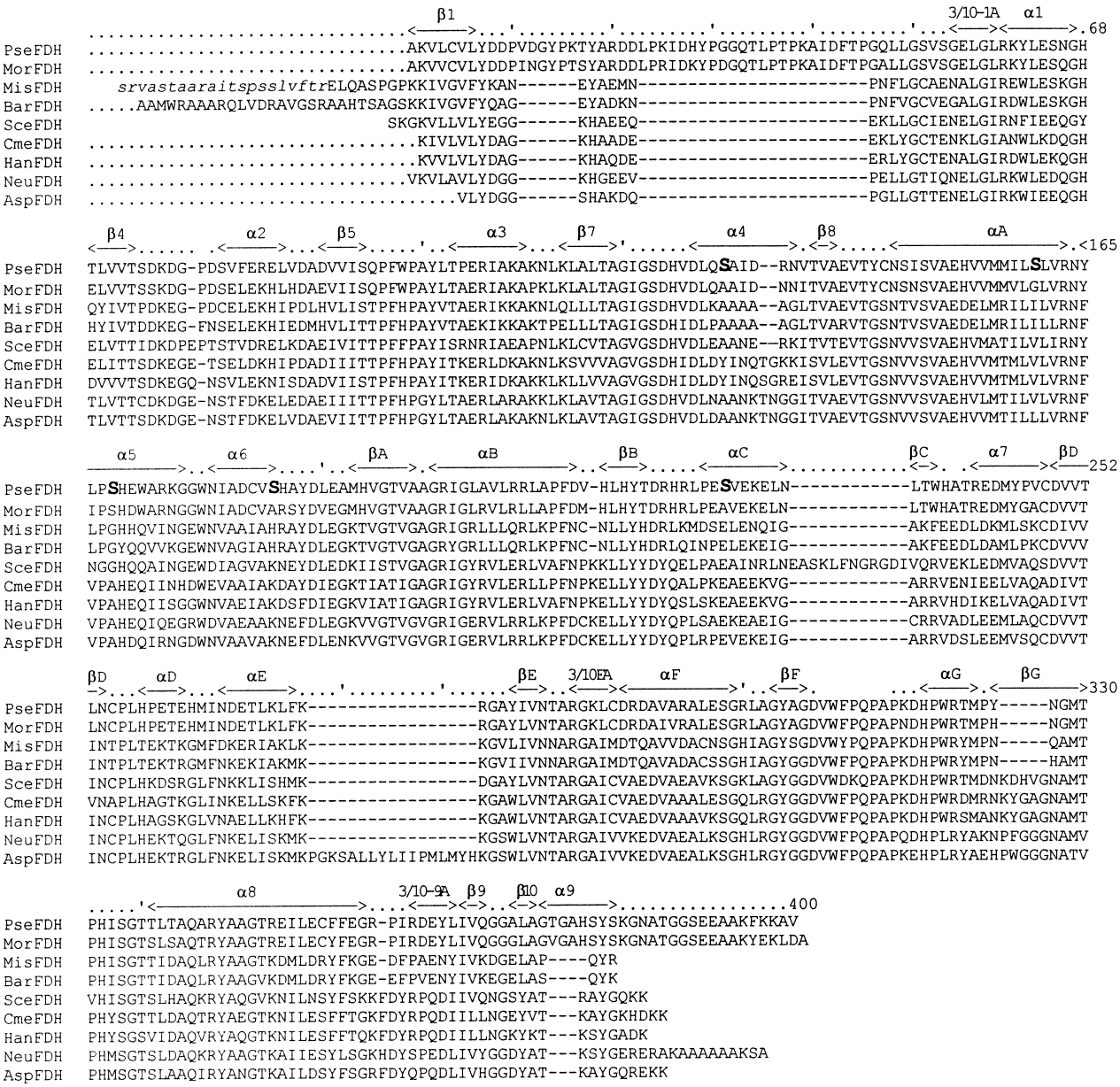

Fig. 1. Alignment of amino acid sequences of formate dehydrogenases from bacteria *Pseudomonas* sp.101 (PseFDH), *Moraxella* C-2 (MorFDH); potato mitochondria (MisFDH), barley (BarFDH); yeast *S. cerevisiae* (SceFDH), *H. polymorpha* (HanFDH), *C. methylica* (CmeFDH); fungi *N. crassa* (NeuFDH) and *A. nidulans* (AspFDH). Residue numeration and structural elements are shown for the FDH from *Pseudomonas* sp.101 [25]. Bold letters S in the amino acid sequence of FDH from *Pseudomonas* sp.101 indicate serine residues selected for mutagenesis.

GGATCATCATC-3′; S160V and S160L, 5′-GCGCACCAGGA(**G**-**C**)CAGGATCATCATC-3′; S184A, 5′-GATGGCGTGGG**C**GACG-CAGTCGG-3′; S228A, 5′-CCTGCCGGAA**G**CGGTCGA**C**AAGG-3′.

The *E. coli* TG1 cell line was used for transformation by the reaction products. Double mutants S(131,160)A and S(184,228)A FDH were prepared using uracil single stranded phagemids pFDH6S131A and pFDH6S228A, respectively. The three-point mutant was obtained by cloning the *Xho*I-*Eco*RI fragment containing a part of FDH gene with the mutation S228A from phagemid pFDH6S228A to phagemid pFDH6S(131,160)A digested with the same restrictases. The final four-point mutant S(131,160,172,228)A FDH was prepared using uracil single stranded phagemid pFDH6S(131,160,228)A. Screening of mutants was performed by DNA sequencing with an Applied Biosystems Automated DNA Sequencer 370A and a 'ABI PRISM' dye-labeled terminators DNA sequencing kit. Positive mutants were selected, and both DNA strands of the complete FDH gene were sequenced to prove only necessary mutations. The mutants were expressed in *E. coli* TG1 cells and purified similar to the wild-type recombinant enzyme [15]. The purity of mutant enzymes (95–98%) was judged by SDS-PAGE. Two preparations of each mutant were made from two different clones and studied for thermal stability.

2.2.2. Characterization of mutants. Enzyme thermal stability was determined at 60°C in 0.1 M K-phosphate buffer, pH 7.0. To provide the same buffer conditions, wild-type and mutant FDHs were passed through column with Sephadex G-25 equilibrated with the buffer mentioned above. Aliquots of 100 μl of enzyme (0.2 mg/ml) were added in 1.5 ml Eppendorf tubes, and a rack with tubes was placed in a water thermostat (60°C ± 0.1°C). In definite time intervals, one tube was removed from the thermostat and cooled for 3 min in water at 15°C. Then the tube was centrifuged for 3 min in an Eppendorf 5415 centrifuge (14 000 rpm) to remove a possible precipitate, and 25 μl aliquots were used to determine the enzyme residual activity.

The enzyme activity was measured spectrophotometrically with a

Beckman DU8B spectrophotometer equipped with a thermostatted cell holder at 340 nm in 0.1 M K-phosphate buffer, pH 7.0, at 37°C. The concentrations of NAD^+ and sodium formate were 1.5 mM and 0.3 M, respectively. V_{max} and K_m for the wild-type and mutant FDHs were measured in 0.1 M sodium phosphate buffer, pH 7.0, varying the concentration of one of the corresponding substrates at the saturating concentration of the second as described earlier [16].

3. Results and discussion

3.1. Selection of residues for mutagenesis

One of the most successful approaches to improve protein thermal stability is based on comparison of amino acid sequences of the protein of interest with those from thermophilic microorganisms. If a ternary structure of one of the proteins of the family is available, the identification of amino acid residues responsible for the globule stability becomes an easier task. This approach can be illustrated by the works of Oshima et al. [17–20] who enhanced the stability of isopropylmalate dehydrogenase from a moderate thermophilic organism using amino acid sequences and the structure of the analogous enzyme from an extremal thermophilic organism. However, in the case of *Pseudomonas* sp.101 FDH, this approach cannot be used since the enzyme exhibits the highest stability among all other FDHs studied so far. Thus, we decided to use general approaches to protein stabilization based on the analysis of general features of the sequence and structural differences between thermophilic and mesophilic proteins belonging to several families [21,22]. One of the approaches is based on hydrophobization of α-helices, i.e. the replacement of polar amino acid residues with more hydrophobic ones, for example, Lys → Arg, Asp → Glu, Ser → Ala. The Ser → Ala replacement usually gives the highest and most predictable stabilizing effect. The search for possible Ser replacements in *Pseudomonas* sp.101 FDH molecule was performed using the following criteria: (1) the residues should be located in α-helices; (2) the residues should not belong to the conserved ones; (3) the residues should not be adjacent to the active site of the enzyme. The amino acid sequences of FDHs from different sources with the marked structural elements of *Pseudomonas* sp.101 holo-FDH are presented in Fig. 1. The *Pseudomonas* sp.101 FDH contains six non-conserved Ser residues located in α-helices, e.g. Ser-131, Ser-147, Ser-160, Ser-168, Ser-184 and Ser-228. Since Ser-147 residue is adjacent to Asn-146 located in the enzyme active site, it was excluded from those to be replaced by site specific mutagenesis.

3.2. Single point mutations of FDH residues S131, S160, S168, S184 and S228

Plots of residual enzymatic activity versus the inactivation time for wild-type and mutant FDHs at 60°C are presented in Fig. 2. The linear dependence of the plots in semi-logarithmic scale indicates the first-order kinetics of the inactivation process for both native and mutant FDHs. Ser131Ala and Ser160Ala mutations caused the enzyme stabilization (1.2 and 1.24 times, respectively). The Ser160Ala replacement results in a slightly higher stabilization effect than Ser131Ala. This could be predicted since Ser-131 is located on the protein surface (helix α4) and is exposed into the solution (Fig. 3A). Moreover, hydroxyl oxygen of Ser-131 is located 2.8 Å apart from carboxyl oxygen over Asp-128 side chain and the angle between C^{β}(Ser-131), O^{OH}(Ser-131) and O^{COOH}(Asp-128) is 106° that allows the existence of a hydrogen bond between Ser-131 and Asp-128 to be proposed. Thus, the replacement of Ser-131 with Ala destroys the hydrogen bond and decreases the hydrophilicity of protein surface that should result in the protein destabilization. However, the increase in general hydrophobicity of α4-helix is so significant that the final effect of the replacement is positive. We note that Asp-128 forming the hydrogen bond is also in α4-helix, and thus, the removal of this bond in the Ser131Ala mutant has no effect on stability of the other structural elements in FDH globule unlike the Ser168Ala mutation (see below).

The Ser-160 residue is located in αA-helix in the region of subunit contact (Fig. 3A). The Ser160Ala replacement has a double positive effect due to the removal of a hydrophilic residue from the hydrophobic core of the protein and the increase in stability of αA-helix. The insignificant difference in stabilization effects caused by Ser131Ala and Ser160Ala

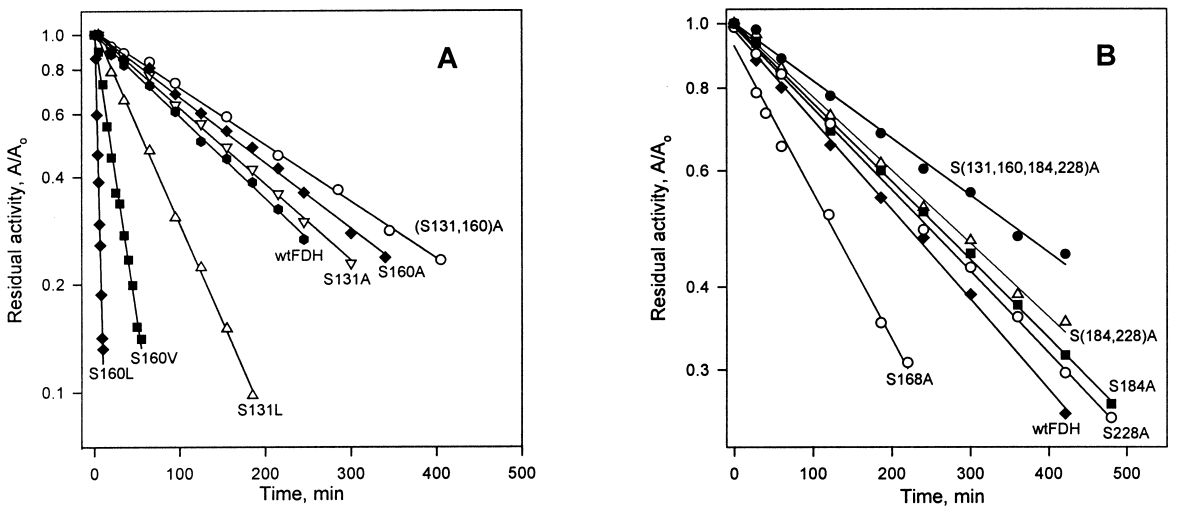

Fig. 2. Semi-logarithmic plots of dependence of enzyme residual activity on time for wild-type (wtFDH) and mutant FDHs at 60°C in 0.1 potassium phosphate buffer, pH 7.0. Protein concentration 0.2 mg/ml. Data for each line are the mean value from two independent experiments for two separate enzyme preparations of each mutant (total four measurements).

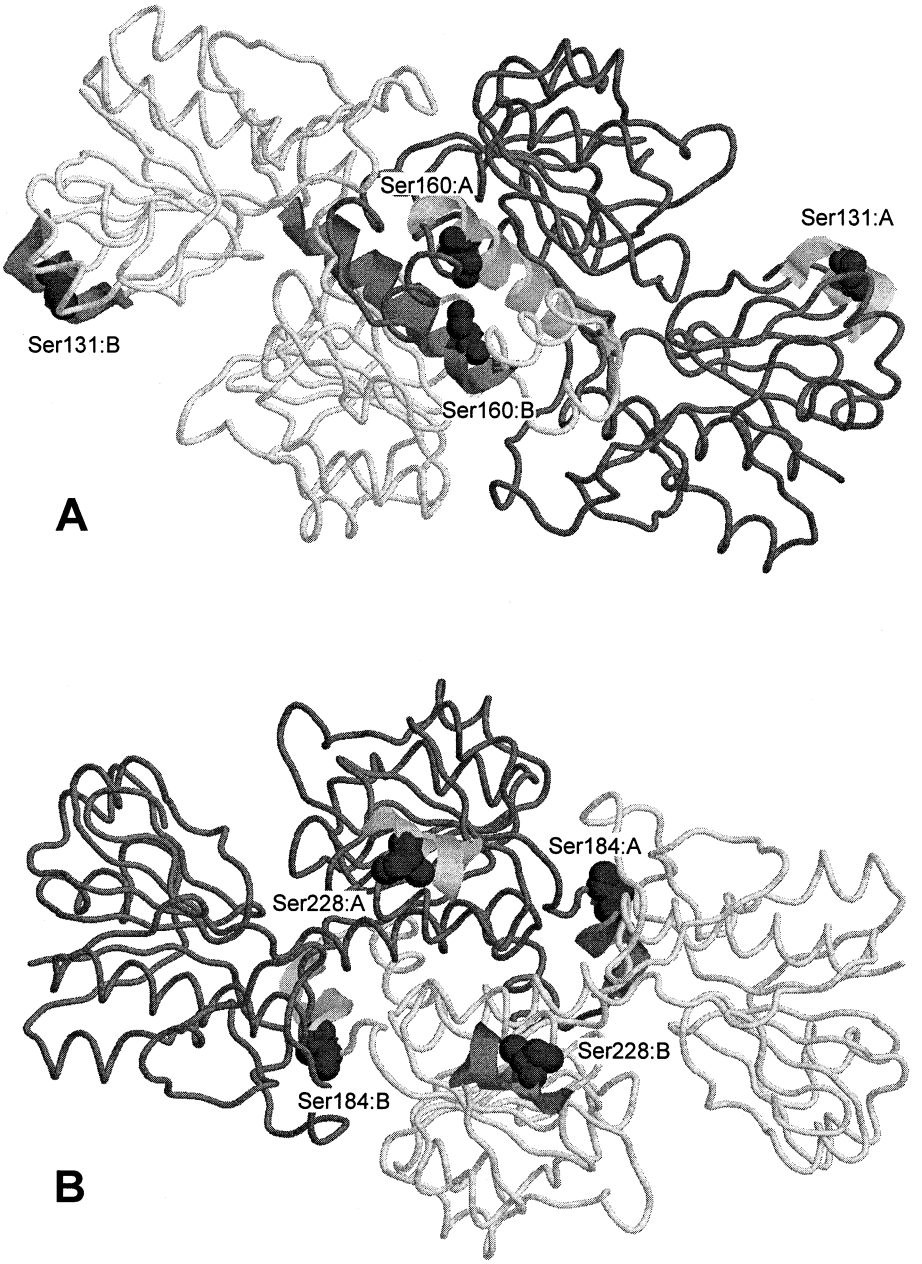

Fig. 3. Positions of Ser residues mutated in the FDH globule. A: Positions of Ser-131 and Ser-160. B: Positions of Ser-184 and Ser-228. Pictures prepared using RasMol ver.2b software and PDB2NAC.ENT file for the structure of apo-FDH [25].

replacements (4%) indicates that the effect of general hydrophobicity is lesser than that of α-helices hydrophobization.

The positive results for Ser131Ala and Ser160Ala mutants inspired us to substitute these amino acid residues by more hydrophobic Val and Leu. The literature shows the example of stabilizing replacement of this with Leu on the surface of chicken lysozyme [23]. The authors explain the stabilization effect of Leu by its rotation around the βC-γC bond resulting in the insertion of the Leu site chain inside the hydrophobic core. In the case of the mutation of *Luciola cruciata* luciferase, the stabilizing effect increased in the order Ala, Val, Leu and Ile [24]. However, the Ser131Leu mutation in FDH led to 2.45-fold increase in the rate of enzyme thermal inactivation compared to the wild-type enzyme (Fig. 2). It is likely that the Leu-131 side chain is not transferred inside the protein globule and stays on the surface, and the contact of the hydrophobic side chain with water causes the enzyme destabilization. The Ser160Val and Ser160Leu mutations also destabilize FDH, and the destabilizing effect increases with the size of the side chain. This could reflect the steric hindrance although analysis of the FDH ternary structure shows that there is enough room even for Leu and Ile residues (Fig. 3A). In other FDHs the whole spectrum of hydrophobic amino acid residues is presented in this position (Fig. 1) that could be reason for their lower stability compared to the bacterial enzymes.

Ser184Ala and Ser228Ala mutations result in the increase in FDH stability compared to the wild-type enzyme by 1.13 and 1.09 times, respectively. The lower stabilization degree in the case of the above mutations, compared to the Ser160Ala replacement, can be explained by their exposure to the solution.

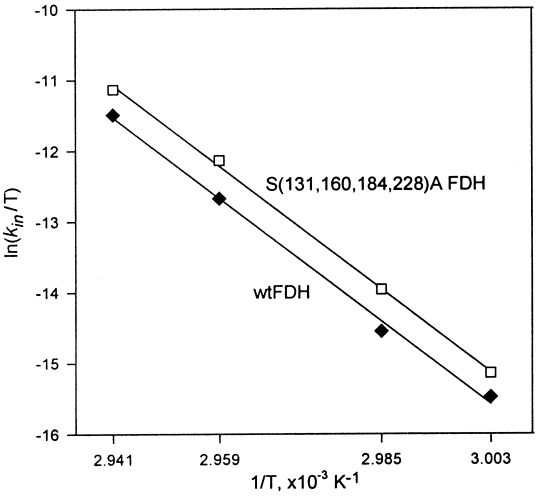

Fig. 4. Dependence of the first-order inactivation constant on absolute temperature in coordinates $\ln(k_{in}/T)-1/T$ for wild-type (wtFDH) and four-point mutant S(131,160,184,228)A FDH.

Ser-184 residue is in α6-helix (Fig. 3B). The hydroxyl group of this residue has no hydrogen binding to other amino acid residues but it interacts with two tightly bonded water molecules located 2.8 and 2.9 Å from O^{OH}(Ser-184) (not shown in Fig. 3). The Ser-228 residue is located in αC-helix (Fig. 3B) and contacts the solvent. The distance between O^{OH}(Ser-228) and $O(H_2O)$ atoms is 2.8 Å according to the X-ray data [25].

The Ser-168 residue in wild-type *Pseudomonas* sp.101 FDH is in α5-helix and it is not among conserved residues (Fig. 1). It is replaced by Gly or Ala in non-bacterial FDHs. The analysis of *Pseudomonas* sp.101 FDH three-dimensional structure points to the hydrogen bond between the Ser-168 OH-group and the Asn-164 basic chain carboxyl oxygen. The distance between O^{OH}(Ser-168) and $O^{-CO-NH-}$(Asn-164) is 2.8 Å and the angle between C^{β}(Ser-168), O^{OH}(Ser-168) and $O^{-CO-NH-}$(Asn-164) is 104°. The fact that Ser168Ala mutant is inactivated 1.7 times faster than the wild-type *Pseudomonas* sp.101 FDH at 60°C (Fig. 2B) indicates the importance of the Ser-168-Asn-164 hydrogen bond for the enzyme stability. This probably reflects the special position occupied by Asn-164. The later is located in a 'non-structured' region between helices αA and α5 (Fig. 1). The 'non-structured' regions are known to be often those of 'weak points' in the protein globule. That is why the Ser-168-Asn-164 hydrogen bond can fix the three-dimensional structure of FDH.

3.3. Double and four-point mutants S(131,160)A, S(184,228)A, S(131,160,184,228)A

Positive mutations were combined to form two double mutants S(131,160)A, S(184,228)A and a four-point mutant S(131,160,184,228)A. Steady-state kinetic experiments have shown that double and four-point mutants as well as single FDH mutants, have the same kinetic parameters (k_{cat} and K_m with formate and NAD^+) as the recombinant wild-type enzyme. Thermal inactivation of multi-point mutants (Fig. 2) proves that the combination of mutations led to the enhanced stabilization effects, and the four-point mutant overcame the single and double point mutants in terms of the enzyme thermal stability. To evaluate the degree of additivity, we used the theory of the activated complex [26]. According to the theory of the activated complex the dependence of the first-order constant of enzyme inactivation k_{in} on T is described by the equation:

$$k_{in} = \frac{kT}{h}\cdot e^{-\left(\frac{\Delta G}{RT}\right)} = \frac{kT}{h}\cdot e^{-\left(\frac{\Delta H}{RT}-\frac{\Delta S}{R}\right)} \quad (1)$$

where T is the absolute temperature in K, k and h are the constants of Bolzmann and Plank, respectively, R is the universal thermodynamic constant, ΔG, ΔH and ΔS are the activating parameters of changes in free energy, enthalpy and entropy for the process of enzyme thermal inactivation. This dependence can be linearized using $[\ln(k_{in}/T)]-1/T$ plot. The linearity of the above dependencies for the wild-type FDH and its four-point mutant (Fig. 4) confirmed the applicability of the activated complex theory to describe the process of FDH thermal inactivation. The similar slope of the lines in Fig. 4 ($\Delta H/R$ according to Eq. 1) shows that the decrease in the rate of the four-point mutant FDH thermal inactivation is comparable with the wild-type enzyme due to the increase in the ΔS component only. The change in the ΔG_{mut} value for the mutant FDH, compared to that for the wild-type enzyme (ΔG_{wt}), can be presented as a sum of two parameters:

$$\Delta G_{mut} = \Delta G_{wt} + \Delta\Delta G_{mut} \quad (2)$$

where ΔG_{wt} corresponds to the change in free energy for the

Table 1
First-order inactivation constants of recombinant wild-type and mutant formate dehydrogenases from *Pseudomonas* sp.101 at 60°C (0.1 M potassium phosphate buffer, pH 7.0)

Mutant	Inactivation constant, $s^{-1}\times10^5$	Ratio of inactivation constants for mutant and wild-type FDH, α
Recombinant wild-type FDH	8.8 ± 0.3	1.0
Single mutations		
Ser131Ala	7.3 ± 0.2	0.83
Ser131Leu	21.6 ± 0.5	2.45
Ser160Ala	7.1 ± 0.2	0.807
Ser160Val	63 ± 8	7.16
Ser160Leu	405 ± 23	46.0
Ser168Ala	14.7 ± 0.2	1.67
Ser184Ala	7.8 ± 0.3	0.886
Ser228Ala	8.1 ± 0.2	0.92
Double mutants		
Ser(131,160)Ala	6.3 ± 0.1	0.716
Ser(184,228)Ala	6.9 ± 0.2	0.784
Four-point mutant		
Ser(131,160,184,228)Ala	5.8 ± 0.3	0.659

wild-type FDH and $\Delta\Delta G_{mut}$ is an additional change in free energy due to mutation. In this case Eq. 1 can be transformed to:

$$k_{in}^{mut} = \frac{kT}{h}\cdot e^{-\left(\frac{\Delta G_{mut}}{RT}\right)} = \frac{kT}{h}\cdot e^{-\left(\frac{\Delta G_{wt}}{RT}+\frac{\Delta\Delta G_{mut}}{RT}\right)}$$

$$= k_{in}^{wt}\cdot e^{-\left(\frac{\Delta\Delta G_{mut}}{RT}\right)} = \alpha\cdot k_{in}^{wt} \quad (3)$$

where the coefficient α is the k_{in}^{mut}/k_{in}^{wt} ratio. The α value is <0 or >0 when mutation results in the increase or decrease in the enzyme thermal stability, respectively. In the case of full additive effect of mutations the $\Delta\Delta G_{mut}$ value for the double mutant is the sum $(\Delta\Delta G_{mut1} + \Delta\Delta G_{mut2})$ and $\alpha = \alpha_{mut1} \times \alpha_{mut2}$. The comparison of α coefficients for the single, double and four-point FDH mutants shows that the additivity is not accurate. For example, the theoretical and experimental α values for the double mutant S(131,160)A are 0.670 and 0.716 (Table 1), respectively. Comparison of the same α values for the four-point mutant FDH (theoretical $\alpha = 0.83 \times 0.807 \times 0.886 \times 0.92 = 0.543$ and experimental 0.659) shows that the additivity of mutations in this mutant is $(0.543/0.659) \times 100\% = 82\%$.

Thus, the general approach based on hydrophobization of α-helices allowed us to yet more improve the stability of the most stable enzyme among the NAD^+-dependent FDHs. The highest stabilization effect for *Pseudomonas* sp.101 FDH obtained using hydrophobization of α-helices was 1.5-fold. A higher stabilization effect can be obtained using the approaches based on optimization of electrostatic interactions or water elimination from a hydrophobic core of the protein globule. The results of application of these approaches to improve the FDH stability will be presented in forthcoming publications.

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