Biochemistry (Moscow), Vol. 70, No. 7, 2005, pp. 804-808. Translated from Biokhimiya, Vol. 70, No. 7, 2005, pp. 974-979.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-188, December 5, 2004.

Use of Ramachandran Plot for Increasing Thermal Stability of Bacterial Formate Dehydrogenase

A. E. Serov, E. R. Odintzeva, I. V. Uporov, and V. I. Tishkov*

Department of Chemical Enzymology, Faculty of Chemistry, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (7-095) 939-2742; E-mail: vit@enz.chem.msu.ru

Received July 13, 2004
Revision received August 17, 2004

Abstract—From analysis of Ramachandran plot for NAD^+-dependent formate dehydrogenase from the methylotrophic bacterium *Pseudomonas* sp. 101 (FDH, EC 1.2.1.2), five amino acid residues with non-optimal values ϕ and ψ have been located in β- and π-turns of the FDH polypeptide chain, e.g., Asn136, Ala191, Tyr144, Asn234, and His263. To clarify their role in the enzyme stability, the residues were replaced with Gly by means of site-directed mutagenesis. The His263Gly mutation caused FDH destabilization and a 1.3-fold increase in the monomolecular inactivation rate constant. The replacements Ala191Gly and Asn234Gly had no significant effect on the stability. The mutations Asn136Gly and Tyr144Gly resulted in higher thermal stability and decreased the inactivation rate by 1.2- and 1.4-fold, respectively. The stabilizing effect of the Tyr144Gly mutation was shown to be additive when introduced into the previously obtained mutant FDH with enhanced thermal stability.

Key words: formate dehydrogenase, *Pseudomonas* sp. 101, stabilization, site-directed mutagenesis, conformational tension, optimization of conformation, β-turn, π-turn

Polypeptide chain conformation is determined by the dihedral angles ϕ_i and ψ_i for each amino acid residue in the sequence [1]. Protein structures usually exhibit values for ϕ and ψ angles that provide minimal repulsion between the amino acid side chains. Figure 1 shows the Ramachandran plot for L-Ala inside a polypeptide chain. The plot demonstrates two deep minima for the energy of the residues in the β-structure and right-hand α-helix. Amino acid residues with the values of the dihedral angles outside these regions increase the energy for the protein globule and make it less stable toward thermal treatment. Therefore, a possible approach to increasing the protein thermal stability is to free the polypeptide chain from the conformational tension by replacing these residues with Gly. The glycine residue has no β-carbon atom and, therefore, has a much wider range of "permitted" values for ϕ and ψ compared to other amino acid residues [2].

In accordance with protein structure statistics, a large number of residues are located in non-optimal regions of the Ramachandran plot, far from deep energy minima, and the conformation of about 1% of non-glycine residues is considered as "restricted" [3]. The most promising searching regions for the replacements for Gly are the turns of the polypeptide chain. The structural organization of some types of turns requires amino acid residues with positive values of the dihedral angle ϕ [4, 5]. These values are non-optimal for all amino acid residues except glycine. The Ramachandran plot for L-amino acids shows one energy minimum corresponding to the left-hand α-helix [2] (Fig. 1).

Systematic studies on the structure–function relationships for NAD^+-dependent formate dehydrogenase from the methylotrophic bacterium *Pseudomonas* sp. 101 (FDH, EC 1.2.1.2), with special emphasis on the enzyme stability and kinetic properties, have been carried out in this laboratory for a number of years [6, 7]. This enzyme exhibits higher activity and stability compared to other FDHs (from bacteria, yeast, fungi, and plants). The thermal stability of FDH was improved earlier using an approach based on the hydrophobization of α-helices (Ser residues replaced with Ala) [8]. The high resolution crystal structures for apo- and holo enzyme forms [9] allowed us to perform analysis of the ϕ and ψ angles in the FDH polypeptide chain and locate a number of non-conserved non-glycine amino acid residues in non-optimal conformation in β- and π-turns. The aim of this work was to improve the enzyme thermal stability by optimizing the conformation of the peptide chain in the region of these turns.

* To whom correspondence should be addressed.

0006-2979/05/7007-0804

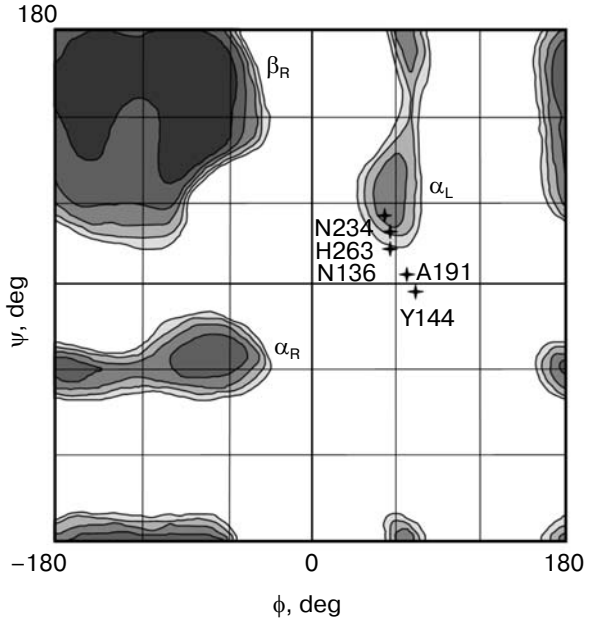

Fig. 1. Ramachandran plot for L-alanine residue [2]. Gradation of gray color corresponds to about 1 kcal/mole. Asterisks denote positions of residues Asn136, Ala191, Tyr144, Asn234, and His263 in formate dehydrogenase from *Pseudomonas* sp. 101.

MATERIALS AND METHODS

Reagents. DNA-polymerase, DNA-ligase, T4 polynucleotide kinase, and restriction endonuclease *Stu*I were from New England Biolabs (USA). All other reagents used for genetic engineering were of "molecular biology grade" from Sigma (USA). Kinetic experiments were run with NAD^+ (grade V, 99% purity) from Sigma and sodium formate (analytical grade) from Reakhim (Russia).

Site-directed mutagenesis of bacterial FDH was carried out in accordance with the Kunkel method [8] using the single strand form of the pFDH6 plasmid [10] with uracil replacing thymidine as a matrix. Primary screening of plasmids for the Asn234Gly mutation was performed using the additionally introduced restriction site for *Stu*I endonuclease. To confirm the unique mutation sites, the *FDH* gene-containing plasmids were sequenced in both directions using an automated DNA sequencer from Applied Biosystems (USA), model 370A, and an ABI PRISM DNA Sequencing Kit based on fluorescent labeled terminators (Applied Biosystems). Mutant FDH forms expressed in *E. coli* cells were isolated and purified according to the protocol developed for the recombinant wild-type FDH [11]. The enzyme preparations obtained were of at least 93-95% purity as judged by analytical SDS-PAGE (12% gel).

FDH activity was assayed spectrophotometrically by NADH accumulation at 340 nm ($\varepsilon_{340} = 6220\ M^{-1}\cdot cm^{-1}$) in 0.1 M K-phosphate buffer, pH 7.0, using a Shimadzu UV 1601PC spectrophotometer at 30°C. Concentrations of NAD^+ and sodium formate in the cell were 1.5 mM and 0.3 M, respectively.

Thermal stability of mutant FDHs and the wild-type recombinant enzyme was analyzed in 0.1 M K-phosphate buffer, pH 7.0. A series of 1.5-ml plastic tubes containing 100-μl aliquot of the enzyme solution (0.2 mg/ml) was prepared for each mutant FDH. The tubes were placed into a water thermostat (precision ±0.1°C) kept at 62°C. At selected time intervals, tubes were taken one by one and placed into a cooling mixture (ice water) for 5 min. The tubes were centrifuged for 2 min at 13,000 rpm on a Beckman 5415D microcentrifuge, and then the residual enzyme activity was measured. The first order rate constant of thermal inactivation, k_{in}, for the mutant and wild-type FDHs was determined from a semi-logarithmic plot for the dependence of the residual activity, $\ln(A/A_0)$, on time, t, using the linear regression method of SigmaPlot 2000 program (SPSS Inc., USA).

Computer analysis of FDH structure surrounding the mutated residues was performed on a Silicon Graphics Indy workstation with the software package the InsightII program (Accelrys, USA).

RESULTS AND DISCUSSION

Glycine residues play a key role in the appropriate folding of regular elements of secondary structure by providing the necessary turns of the polypeptide chain [1, 12]. Non-structured regions in proteins contain 55% of the total number of glycine residues [12]; 23% are located in the turns, among which 80% exhibit positive values for the ϕ angle. Glycine is a preferred residue in the third position of type II β-turns [13] and in the fifth position of π-turns [4, 5].

The refined crystal structure of *Pseudomonas* sp. 101 dimeric FDH demonstrates seven type II β-turns and seven π-turns for each subunit [9]. Almost half of the positions with positive values of the ϕ angle are occupied by glycine residues. Nevertheless, three type II β-turns contain Asn136, Asn164, and Asn234 in the third position, and four π-turns contain Ala191, Tyr144, His263, and Tyr326 in the fifth position (table) [9]. These seven residues are potential targets for replacement with Gly. However, mutagenesis of conserved residues usually has a negative effect on the catalysis and substrate or cofactor binding. The alignment of the amino acid sequences of FDHs from different sources visualizes the conserved residues. Currently, about 30 complete sequences for FDH from bacteria, yeast, fungi, and higher plants are known [14], some of which are shown in Fig. 2. The alignment demonstrates that only Asn164 is conserved among all the resides discussed above. The rest of the residues, except for Tyr326, are replaced by Gly in the majority of the non-bacterial FDHs (Fig. 2).

Non-conservative amino acid residues in β- and π-turns of formate dehydrogenase polypeptide chain with non-optimal values of ϕ and ψ and first order inactivation constants for wild type and mutant FDHs (62°C, 0.1 M potassium phosphate buffer, pH 7.0)

Residue	Secondary structure element	ϕ (degree)	ψ (degree)	Mutation	$k_{in} \times 10^4$, sec^{-1}
His263	β-turn type II 261-264	57.2	36.8	H263G	3.3 ± 0.3
Ala191	β-turn type II 189-192	69.3	4.2	A191G	2.7 ± 0.2
Asn234	π-turn 230-235	54.5	51.5	N234G	2.7 ± 0.2
Asn136	π-turn 132-137	59.1	32.4	N136G	2.1 ± 0.1
Tyr144	β-turn type II 142-145	73.9	–4.9	Y144G	1.8 ± 0.1
Tyr144	same	73.9	–4.9	T4/Y144G	1.1 ± 0.1
Wild type FDH					2.5 ± 0.1

It should be noted that the replacement of amino acid residues with non-optimal values of ϕ and ψ angles with glycine residues is not guaranteed to improve thermal stability because of energy losses that could take place once the interaction between the side chains is disrupted. To reliably predict the results of the conformational optimization one has to thoroughly analyze the effect of the planned mutations on the interactions in the enzyme tertiary structure. We have analyzed such interactions in the surrounding of Asn136, Ala191, Tyr144, Asn234, and His263 residues. The analysis revealed the surface location of the side chain of the His263 residue, which can

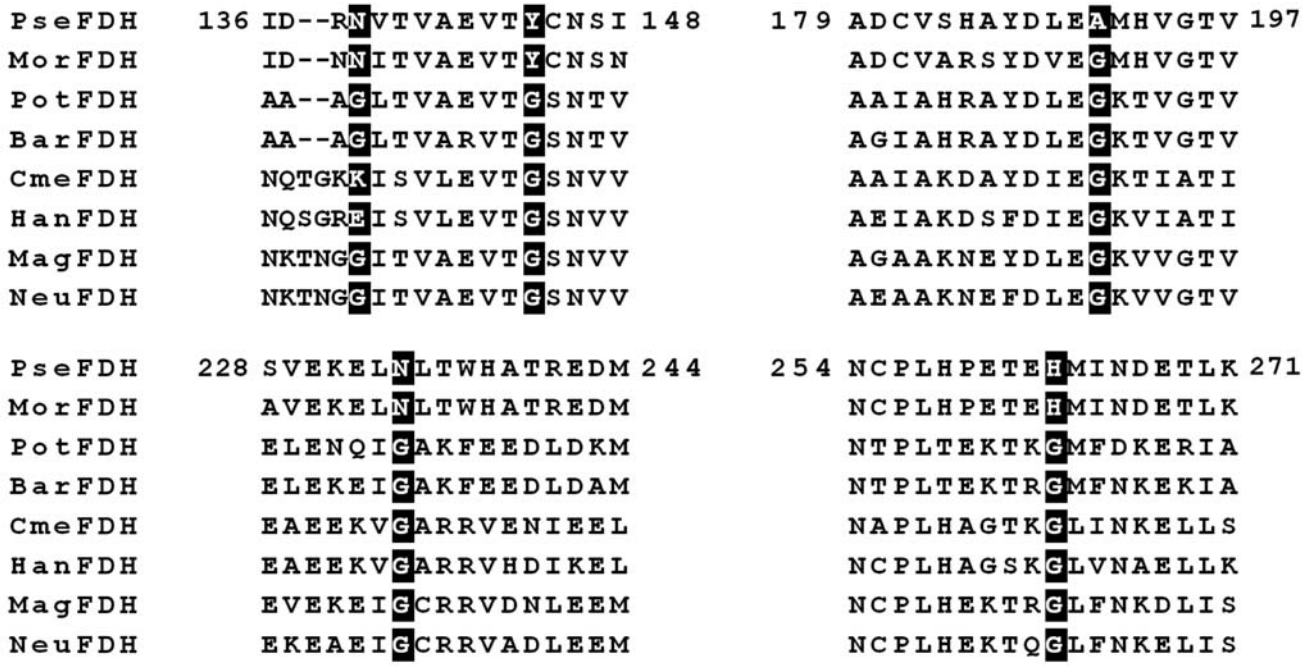

Fig. 2. Partial alignment of formate dehydrogenase amino acid sequences from bacteria *Pseudomonas* sp. 101 (PseFDH, SWISS-PROT:FDH_PSESR) and *Moraxella* sp. C-1 (MorFDH, EMBL Accession Y13245); plants: potato (PotFDH, EMBL Z21493) and barley (BarFDH, EMBL D88272); yeasts: *Candida methylica* (CmeFDH, EMBL X81129) and *Pichia angusta* (former *Hansenula polymorpha*, HanFDH, EMBL P33677); and fungi *Neurospora crassa* (NeuFDH, EMBL L13964) and *Magnaporthe grisea* (MagFDH, EMBL AA415108). Numbering of amino acid residues is show according to numbering for formate dehydrogenase from *Pseudomonas* sp. 101.

form hydrogen bonds with carbonyl groups of the Lys286 and Ser288 residues. In the case of Ala191, its methyl group participates in hydrophobic interaction with the second enzyme subunit. Therefore, the replacement of these residues with Gly may have either stabilizing or destabilizing effect on the structure. The side chains of the other three amino acid residues do not participate in ionic, hydrogen, or hydrophobic interactions.

To test the effects of the non-conserved amino acid residues with the non-optimal values of ϕ and ψ on the thermal stability of FDH, Asn136, Ala191, Tyr144, Asn234, and His263 residues have been replaced with Gly. The table presents the results of the thermal stability measurements at 62°C for the obtained mutant FDHs. FDH inactivation at elevated temperatures occurs as an irreversible unfolding of the protein globule without dissociating into the individual subunits, in accordance with the first-order kinetics [8]:

$$N \xrightarrow{k_{in}} D,$$

where N and D are active and denatured forms of the enzyme, respectively. The rate constant for inactivation k_{in} is truly first order, being independent of the enzyme concentration. The values for each mutant FDH obtained in this work were also identical for different enzyme concentrations. This justifies the use of the obtained k_{in} values for the quantitative comparison of the stabilization effects upon introducing the above described mutations.

The data show that the His263Gly replacement resulted in 1.3-fold increase in the inactivation rate constant (table). The replacements Ala191Gly and Asn234Gly had no significant effect on the stability. Mutation Asn136Gly improved FDH thermal stability by 1.2-fold. The maximum stabilization was achieved with the Tyr144Gly replacement, i.e., this mutation caused a 1.4-fold decrease in the inactivation rate constant.

Replacements His263Gly, Ala191Gly, Asn234Gly, and Asn136Gly did not change the FDH kinetics properties, in contrast to the Tyr144Gly mutation, which increased K_m for formate by 75% compared to the wild-type enzyme. This increase in K_m is most likely due to the location of Tyr144 adjacent to the highly conserved Asn146 (Fig. 2) that participates in the catalysis and formate binding in the active center [7].

The experimental data confirm the theoretically predicted effects of the His263Gly, Ala191Gly, Asn234Gly, Asn136Gly, and Tyr144Gly mutations on FDH thermal stability. In the case of the His263Gly and Ala191Gly replacements, the energy gain from the conformational optimization was lesser than the energy losses from the disruption of the stabilizing interactions. However, we note that the decreased stability of the His263Gly mutant (k_{in} increased by 30%) shows just modest destabilization compared to those that could be expected from the loss of hydrogen bonding. For instance, the loss of hydrogen bond upon introducing the Tyr165Phe mutation results in an 18-fold increase in the inactivation rate constant [15]. The difference in the stabilizing effects of the Tyr144Gly and Asn136Gly replacements can be seen from the topology of the Ramachandran plot. According to the classification of the regions in the Ramachandran plot for L-amino acids [3], the conformations of His263, Ala191, Asn234, and Asn136 are "partially permitted", while the region of values, where the Tyr144 ϕ and ψ angles belong, is "restricted" for all non-glycine amino acids (Fig. 1). Overall, the maximum efficiency of a single replacement was about 40% among all amino acid residues with non-optimal values of dihedral angles in β- and π-turns of the bacterial FDH.

As a rule, an increase in thermal stability upon introducing single-point mutations is not significant [16]. A significant stabilization of a mutant protein can be achieved with a number of single-point mutations because of their stabilization effects are very often additive. For example, the positive effects of mutations Ser131Ala, Ser160Ala, Ser184Ala, and Ser228Ala, aimed to stabilize bacterial FDH by hydrophobization of α-helices, were 1.2, 1.2, 1.1, and 1.1-fold, whereas the four combined mutations Ser→Ala (T4 FDH mutant) results in a 1.6-fold decrease in k_{in} compared to the wild-type recombinant FDH [8]. To test whether the stabilizing effect of Tyr144Gly mutation is additive, the mutation has been introduced in T4 FDH previously produced in our laboratory. The new mutation resulted in the additional stabilization of the enzyme (Fig. 3). The inactivation rate constant ratio for the mutant T4/Tyr144Gly FDH and the wild-type enzyme was about 2.3 (table). Within the experimental error, this value coincides with the result of multiplication of the stabilization effects for

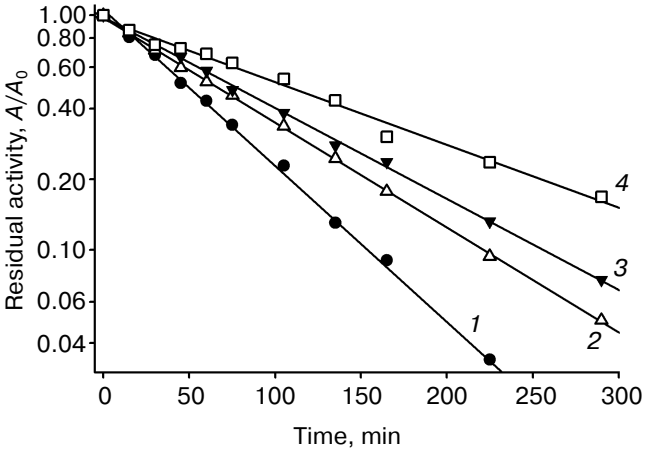

Fig. 3. Dependence of the enzyme residual activity on time at 62°C for wild-type (*1*) and mutant formate dehydrogenases with replacements Tyr144Gly (*2*), T4(Ser/131,160,184,228/Ala) (*3*), and T4/Tyr144Gly (*4*). Conditions: 0.1 M potassium phosphate buffer, pH 7.0.

the mutations in T4 FDH (Ser131Ala, Ser160Ala, Ser184Ala, and Ser228Ala) and Tyr144Gly mutation ($1.6 \times 1.4 = 2.24$). These data show the additive character of the introduced mutations. Thus, the Tyr144Gly mutation can be used further to create a "super stable" FDH form.

Minimization of conformational tensions cannot be considered as an established method for protein stabilization because of the lack of experimental confirmation. The possibility in principal to use the approach was earlier shown for *Staphylococcus* nuclease [17]. Our work gives another confirmation of the successful application of this method. However, the overall stabilization depends on the relative contribution of the opposite effects, i.e., conformational optimization and disruption of the side group interactions. That is why we observed improvement in thermal stability for two cases only. The 20-40% stabilization effects described in this work are enough high for single-point mutations in the enzyme superior over all known analogs with respect to thermal denaturation [8].

The work was supported by grants from the Russian Foundation for Basic Research (projects No. 02-04-49415 and 05-04-49073).

REFERENCES

1. Cantor, C., and Schimmel, P. (1984) *Biophysical Chemistry* [Russian translation], Mir, Moscow, pp. 248-256.
2. Brant, D. A., and Schimmel, P. R. (1967) *Proc. Natl. Acad. Sci. USA*, **58**, 428-435.
3. Gunasekaran, K., Ramakrishnan, C., and Balaram, P. (1996) *J. Mol. Biol.*, **264**, 191-198.
4. Wilmot, C. M., and Thornton, J. M. (1990) *Protein Eng.*, **3**, 479-493.
5. Rajashankar, K. R., and Ramakumar, S. (1996) *Protein Sci.*, **5**, 932-946.
6. Tishkov, V. I., Galkin, A. G., Fedorchuk, V. V., Savitsky, P. A., Rojkova, A. M., Gieren, H., and Kula, M. R. (1999) *Biotechnol. Bioeng.*, **64**, 187-193.
7. Popov, V. O., and Tishkov, V. I. (2003) in *Protein Structures: Kaleidoscope of Structural Properties and Functions* (Uversky, V. N., ed.) Research Signpost, Kerala, pp. 441-473.
8. Rojkova, A. M., Galkin, A. G., Kulakova, L. B., Serov, A. E., Savitsky, P. A., Fedorchuk, V. V., and Tishkov, V. I. (1999) *FEBS Lett.*, **445**, 183-188.
9. Lamzin, V. S., Dauter, Z., Popov, V. O., Harutyunyan, E. H., and Wilson, K. S. (1994) *J. Mol. Biol.*, **236**, 759-785.
10. Tishkov, V. I., Matorin, A. D., Rojkova, A. M., Fedorchuk, V. V., Savitsky, P. A., Dementieva, L. A., Lamzin, V. S., Mezentzev, A. V., and Popov, V. O. (1996) *FEBS Lett.*, **390**, 104-108.
11. Tishkov, V. I., Galkin, A. G., Marchenko, G. N., Tsygankov, Y. D., and Egorov, A. M. (1993) *Biotechnol. Appl. Biochem.*, **18**, 201-207.
12. Ramakrishnan, C., and Srinivasan, N. (1990) *Curr. Sci.*, **59**, 851-861.
13. Hutchinson, E. G., and Thornton, J. M. (1994) *Protein Sci.*, **3**, 2207-2216.
14. Tishkov, V. I., and Popov, V. O. (2004) *Biochemistry (Moscow)*, **69**, 1252-1267.
15. Serov, A. E., Rojkova, A. M., and Tishkov, V. I. (2000) *Byul. Mosk. Univ., Ser. 2, Khim.*, **41**, 358-361.
16. Lee, B., and Vasmatzis, G. (1997) *Curr. Opin. Biotechnol.*, **8**, 423-428.
17. Stites, W. E., Meeker, A. K., and Shortle, D. (1994) *J. Mol. Biol.*, **235**, 27-32.