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Comparison of Thermal Stability of New Formate Dehydrogenases by Differential Scanning Calorimetry

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Abstract—Formate dehydrogenases (FDHs) from different sources are systematically studied in our laboratory. Over the past few years, new genes of four FDHs from pathogenic bacterium *Staphylococcus aureus* (SauFDH), methylotrophic thermotolerant yeast *Ogataea parapolymorpha* (OpaFDH), yeast *Saccharomyces cerevisiae* (SceFDH), and moss *Physcomitrella patens* (PpaFDH) have been cloned and expressed in *E. coli* cells. By means of differential scanning calorimetry, a comparative study of thermal stability of new recombinant formate dehydrogenases and a number of FDHs from other sources has been performed. It was shown that two new enzymes, SauFDH and OpaFDH, are comparable to the FDH from *Pseudomonas* sp. 101 bacteria in their stability. SceFDH is the least stable FDH among the described formate dehydrogenases.

Keywords: thermal stability, formate dehydrogenase, differential scanning calorimetry

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INTRODUCTION

Formate dehydrogenases (FDH, EC 1.2.1.2.) from different sources have been studied in our laboratory for many years [1–3]. FDH is present in bacteria, yeast, and microscopic fungi, as well as in embryophytes and nonvascular plants. The importance of studying this enzyme is determined by the following factors: FDH is actively used in practice as a biocatalyst for cofactor regeneration [4], and also plays an important role in the vital functions of various organisms [1, 3]. In order to evaluate the possibility of using formate dehydrogenase as a biocatalyst, it is necessary to obtain information about the kinetic parameters and stability of this enzyme.

Genes encoding FDH in different organisms have been successfully cloned in *E. coli* in many laboratories all over the world. The creation of effective expression vectors made it possible to obtain recombinant FDHs in active and soluble forms. Our laboratory possesses the world's largest collection of cloned FDH genes. This collection includes genes from bacteria *Pseudomonas* sp. 101 (PseFDH), *Moraxella* sp. C-1, and *Mycobacterium vaccae* N10, methylotrophic yeast *Candida boidinii* (CboFDH), and plants *Arabidopsis thaliana* (AthFDH) and soya *Gycine max* (SoyFDH). In recent years, we have cloned and expressed active forms of new genes of FDH from pathogenic bacterium *Staphylococcus aureus* (SauFDH), methylotrophic thermotolerant yeasts *Ogataea parapolymorpha* (OpaFDH), baker's yeasts (SceFDH), and moss *Physcomitrella patens* (PpaFDH).

The study of the thermal stability of FDHs is an important task, since many biocatalytic processes are carried out for a long time at an elevated temperature. As a rule, the thermal stability of formate dehydrogenases is studied by the following two methods: through the inactivation kinetics and with differential scanning calorimetry (DSC). In the present work, comparative studies of the thermal stability of the four new and some of the previously obtained recombinant FDHs are performed by the DSC method.

Abbreviations: DSC, differential scanning calorimetry; SauFDH, PseFDH, OpaFDH, CboFDH, SceFDH, AthFDH, SoyFDH, and PpaFDH, recombinant formate dehydrogenases extracted from bacteria *Staphylococcus aureus* and *Pseudomonas* sp. 101, methylotrophic yeasts *Ogataea parapolymorpha* and *Candida boidinii*, baker's yeast, and plants *A. thaliana*, *Glycine max soya*, and *Physcomitrella patens*, respectively.

EXPERIMENTAL

Expression of Recombinant Formate Dehydrogenases

The expression of genes encoding the target formate dehydrogenase was performed in the *E. coli* BL21 (DE3) CodonPlus/pLysS cells. The producer

strains of recombinant FDHs were cultivated according to the following procedure. The museum collection cell culture was seeded in a 2YT medium (16 g/L of tryptone, 10 g/L of yeast extract, 1 g/L of sodium chloride, 1.5 g/L of sodium monophosphate, 1 g/L of disubstituted potassium phosphate, pH 7.0) and incubated overnight (37°C, 180 rpm) in the presence of antibiotics (150 μg/mL of Amp for PseFDH, SauFDH, CboFDH, SceFDH, AthFDH, and SoyFDH, and 30 μg/mL of Kan for PpaFDH and OpaFDH) and chloramphenicol (25 μg/mL). At the next stage, the night culture was transferred into a similar fresh medium with the same concentration of antibiotics and cultivated at 30°C in 250-mL or 1-L special flasks equipped with baffles. The seed volume was 10–15% of the total volume of the medium (20% of the volume of the flask). When the absorbance of a cell suspension at 600 nm (A_{600}) reached a value of 0.6–0.8, lactose as an inducer of the FDH biosynthesis was added to the medium until a final concentration of 20 mg/mL. Then the cells were cultured overnight at a maximum aeration at 20°C for SoyFDH, 25°C for CboFDH, SauFDH, and PpaFDH, and 30°C for the remaining enzymes. The cells were precipitated on a Beckman J-21 centrifuge (United States) at 6000 rpm for 20 min at 4°C.

Purification of Recombinant Enzymes

The enzymes expressed in *E. coli* cells were purified according to the procedure developed for recombinant FDHs from *Pseudomonas* sp. 101 [5]. The enzyme purification process included the destruction of cells (a suspension of 2 g of biomass in 10 mL of a 0.1 M sodium phosphate buffer solution, 0.01 M EDTA, and pH 8.0) at 0°C with a BraunSonic ultrasonic disintegrator (Germany), the heat treatment for 10 min (at 55°C for PseFDH and SauFDH, and at 50°C for OpaFDH), and the precipitation of ballast proteins with ammonium sulfate (35% of the saturation concentration). In the case of SoyFDH and PpaFDH, the additional precipitation was carried out at an ammonium sulfate concentration of 85% of the saturation concentration, and the precipitate was redissolved in a solution of ammonium sulfate (35% of saturation) in a 0.1 M phosphate buffer solution, pH 7.0 (solution A). Next, the hydrophobic chromatography on a Phenyl Sepharose Fast Flow (Amersham) under a descending ammonium sulfate concentration gradient (35–0% of the saturation concentration) and gel filtration on a column with Sephacryl S-200 were carried out. The purity of the FDH preparations was monitored by analytical electrophoresis in a 12% polyacrylamide gel in the presence of 0.1% of sodium dodecyl sulfate (BioRad electrophoresis apparatus). The resulting samples were at least of 98% purity.

Determination of Thermal Stability with Differential Scanning Calorimetry

The thermal stability was studied on a Nano DSC adiabatic differential scanning microcalorimeter. The working volume of the platinum capillary calorimetric cells was 300 μL. To prevent the formation of bubbles and the boiling of solutions upon an increase in the temperature, an excess pressure of 3 atm was maintained in the cells of the calorimeter. The instrumental baseline was determined before the experiment. During the measurements, a buffer solution was placed in the control cell and a solution of FDH under study in the same buffer solution in the working cell. The concentration of enzymes was 1–2 mg/mL, and the heating rate was 1°C/min.

RESULTS AND DISCUSSION

A comparative analysis of the primary structure of the enzymes of one family allows us, as a rule, to understand the relationship between the properties of particular enzymes and the presence/absence of certain amino acid residues in their sequences. The alignment of amino acid sequences for formate dehydrogenases from different sources for which the thermal stability was studied is shown in Fig. 1. The asterisks mark conservative amino acid residues. As is seen from Fig. 1, a specific sequence called a signal peptide (marked with italic in AthFDH and SoyFDH) is present at the N-terminus of plant enzymes. Practically all plant proFDHs having a signal peptide are inactive. It is known from the literature that this fragment is cleaved after the transport of FDH from the cytoplasm into the mitochondria. The enzyme from the moss (PpaFDH) has one of the longest signal peptides compared to other plant FDHs [3]. In addition, PpaFDH is the only exception known at the moment. This is the first example in the world among FDHs, when a full-length formate dehydrogenase proenzyme extracted from plants is active and has an uncleaved signal peptide. The partial deletion of the signal peptide exerts very little effect on the catalytic parameters (data to be published), but the yield of the active enzyme depends on the length of the cleaved sequence. It is shown in Fig. 1 that FDH from *S. aureus* has significant differences in the primary structure in comparison with other enzymes. The degree of homology of SauFDH with other bacterial FDHs (and with PseFDH) is less than 40%.

The melting curves of the studied formate dehydrogenases are shown in Fig. 2. In addition to new FDHs, the data are given for a number of enzymes that are considered as a model for formate dehydrogenases from various sources. The main parameter characterizing the stability of FDH in the case of DSC is the maximum temperature value in these curves. As is seen from Fig. 2, the bacterial formate dehydrogenases SauFDH and PseFDH show the highest stability.

Fig. 1. Alignment of amino acid sequences of formate dehydrogenases from different sources: OpaFDH from *O. parapolymorpha*; CboFDH from *Candida boidinii*; SceFDH from *Saccharomyces cerevisiae*; SauFDH from *S. aureus*; SauFDH and PseFDH from *Pseudomonas* sp. 101; SoyFDH from *Glycine max* (soya); AthFDH from *Arabidopsis thaliana*; PpaFDH from *Physcomitrella patens* (moss). Conservative amino acid residues are marked with asterisks. Confirmed sequences of signal peptides in AthFDH and SoyFDH are shown in italics. Conservative regions in FDH coenzyme-binding and catalytic domains are highlighted by light and dark gray colors, respectively.

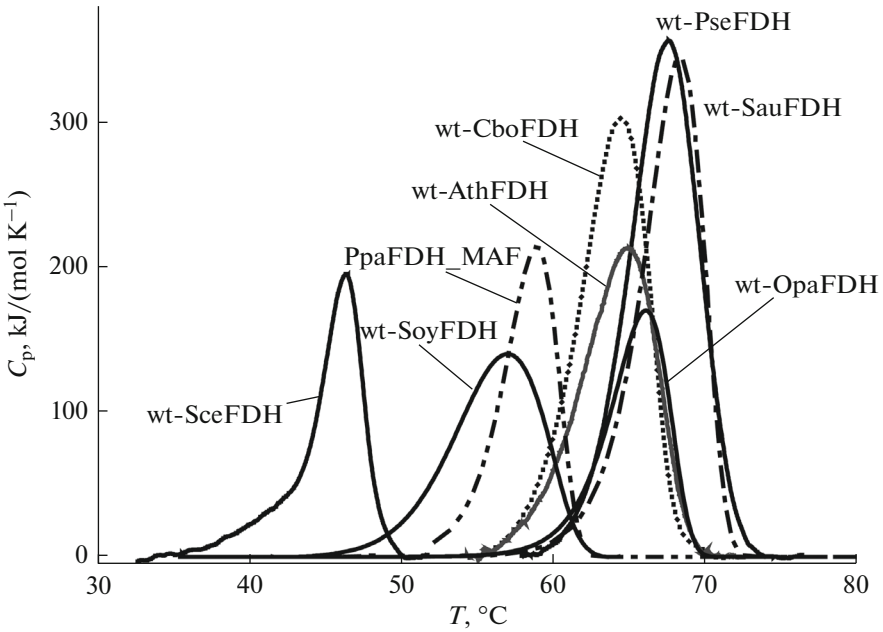

Fig. 2. Melting curves for wild-type formate dehydrogenases from different sources. SauFDH, PseFDH, OpaFDH, CboFDH, SceFDH, AthFDH, SoyFDH, and PpaFDH are recombinant formate dehydrogenases from bacteria *Staphylococcus aureus* and *Pseudomonas* sp. 101, methylotrophic yeasts *Ogataea parapolymorpha*, and *Candida boidinii*, baker's yeasts, plant *A. thaliana*, soya *G. max*, and moss *Physcomitrella patens*, respectively (a 0.1 M sodium phosphate buffer, pH 7.0, an enzyme concentration of 1–2 mg/mL, and scanning rate of 1 grad/min).

They are followed by FDH from the thermotolerant yeast *O. parapolymorpha* (OpaFDH). It strongly surpasses by this parameter the highly homologous FDH from methylotrophic yeast *Candida boidinii* (CboFDH), which is now widely used in practice. Taking into account the fact that OpaFDH also outperforms CboFDH in terms of the catalytic properties, it can be assumed that OpaFDH will also find application in practice in the near future. The lowest stability is exhibited by the FDH from the *S. cerevisiae* yeast (SceFDH), which proved to be even one of the most unstable enzymes among the described formate dehydrogenases. Two plant FDHs (SoyFDH and the new enzyme PpaFDH) also show a low level of thermal stability, but FDH from *A. thaliana* is much more stable and even overcomes the yeast CboFDH in stability.

The analysis of the alignment of amino acid sequences makes it possible to reveal a number of trends which determine the differences in the thermal stability of the studied formate dehydrogenases. The high thermal stability in the case of bacterial formate dehydrogenases is partly attributed to the presence of an additional sequence at the N-terminus (Fig. 1). This additional sequence contains up to seven proline residues and, as was shown earlier [6], represents an unstructured rigid loop that is responsible for providing stability in this region of the protein globule. As noted above, FDH from the golden staphylococcus strongly differs in its amino acid sequence from all the other formate dehydrogenases. Differences are also observed in the Gly(Ala)XGlyXXGly characteristic sequence (X is any residue) for the adenine-binding domain (the so-called finger print, highlighted in gray in Fig. 1); furthermore, the Gly residue in the first position provides the optimal φ and ψ angles in the Ramachandran map. The substitution of A198G in PseFDH (the first amino acid residue in the characteristic sequence) gives rise to an increase in the thermal stability [7].

It is also interesting to analyze the effect of the composition of the XP(A/K)QP semi-conservative sequence (it includes the catalytically important Gln residue [5]) on the thermal stability of the studied enzymes. In most of the formate dehydrogenases, this sequence is XPQP, where X is either the Phe or Tyr hydrophobic residues. It was shown that the hydrophilization of this position leads to an increase in the thermal stability. For example, the replacement of the Phe190 residue in SoyFDH with the negatively charged residues Asp and Glu gives rise to an increase in the thermal stability by more than 50 times [8, 9]. Let us note that the Asp residue in AthFDH of the wild type is already present in this position, which is probably the reason for the high level of thermal stability of the natural enzyme. In PpaFDH, this position contains the asparagine residue, which does not provide, according to the directed mutagenesis of SoyFDH, a high stability level as in the case of FDH of the wild-type soya.

The XP(A/K)QP sequence in SceFDH also has a nonstandard composition, specifically, FKQP (Fig. 1); i.e., the Lys residue is used instead of the Pro residue in addition to the hydrophobic Phe residue, which explains the low thermal stability level of this enzyme. The thermal stability data for the bacterial and yeast enzymes are also consistent with an increase in the thermal stability data upon replacing the phenylalanine residue at the X position with the tyrosine residue [10]. Previously, it was shown for SoyFDH [9] and for PseFDH [10] that the F/Y replacement in this position leads to an increase in the thermal stability of PseFDH, CboFDH, and SoyFDH. In OpaFDH and SauFDH, the tyrosine residue is initially located in this position, which explains the higher stability of these enzymes compared to CboFDH and PseFDH, respectively, which have a phenylalanine residue in this position.

CONCLUSIONS

In summary, note that the DSC data on the thermal stability of the new enzymes are in good agreement with previously published results on the stability of other FDHs [8–12], as well as with the data on the thermal stability obtained through the kinetics of thermal inactivation at different temperatures.

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