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A Comparative Study of the Thermal Stability of Formate Dehydrogenases from Microorganisms and Plants

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Abstract—A comparative study of the thermostability of NAD^+-dependent formate dehydrogenases (FDHs; EC 1.2.1.2) from both methylotrophic bacteria *Pseudomonas* sp. 101 and *Moraxella* sp. C1, the methane-utilizing yeast *Candida boidinii*, and plants *Arabidopsis thaliana* and *Glycine max* (soybean) was performed. All the enzymes studied were produced by expression in *E. coli* cells. The enzymes were irreversibly inactivated in one stage according to first-order reaction kinetics. The FDH from *Pseudomonas* sp. 101 appeared as the most thermostable enzyme; its counterpart from *Glycine max* exhibited the lowest stability. The enzymes from *Moraxella* sp. C1, *C. boidinii*, and *Arabidopsis thaliana* showed similar thermostability profiles. The temperature dependence of the inactivation rate constant of *A. thaliana* FDH was studied. The data of differential scanning calorimetry was complied with the experimental results on the inactivation kinetics of these enzymes. Values of the melting heat were determined for all the enzymes studied.

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NAD^+-dependent formate dehydrogenase (FDH; EC 1.2.1.2) catalyzes the oxidation of a formate ion to carbon dioxide, coupled to NAD^+ reduction to NADH:

$$HCOO^- + NAD^+ \longrightarrow NADH + CO_2.$$

FDH is widely used for regeneration of NADH in dehydrogenase-assisted syntheses of optically active compounds. The practical irreversibility of the formate dehydrogenase reaction and its activity over a broad range of pH made this enzyme a universal biocatalyst capable of working efficiently at pH values optimal for a particular process. The company Degussa (Germany) recently started using commercial technology for the manufacture of *tert*-L-leucine, using FDH as a catalyst for NADH regeneration. The capacity of this process is one of the highest achieved by pharmaceutical chemistry for technologies involving enzymes. Another aspect underlying the industrial application of FDH is its high-thermal stability. However, the thermal stabilities of this enzyme differ considerably depending on the source from which it is isolated. Consequently, the selection of the most stable enzyme is a topical issue with regards to its industrial use.

Several types of FDH, the enzyme catalyzing the reaction in question, are met in the living organisms. These enzyme types differ from one another in (1) the number and type of their subunits and (2) the presence of metal ions and prosthetic groups [1]. The FDHs composed of two identical subunits and having neither metal ions nor prosthetic groups in the active center form a separated group. The FDHs of this type belong to the superfamily of D-specific 2-hydroxyacid dehydrogenases[2].

Until recently the FDH isolated from methylotrophic bacteria (*Pseudomonas* sp. 101, *Moraxella* sp. C1, *Mycobacterium* vaccae N10, etc.) and yeasts (*Candida boidinii, Pichia angusta* (*Hansenula polymorpha*), and *Pichia pastoris*) were the main objects of research in this field. The enzyme from *Pseudomonas* sp. 101 (P-FDH) is the best studied; crystal, X-ray-structure, analysis, data were obtained for the free enzyme and its various complexes [3–5]. The most studied yeast FDHs are the enzymes from *C. boidinii* (C-FDH) and *Saccharomyces cerevisiae.* Recently, an FDH of plant origin has attracted the increased attention of researchers. Unlike the bacterial and yeast enzymes localized to the cytoplasm, plant FDHs are transported to mitochondria after synthesis. Plant FDHs are involved in stress responses [6]. As a result from a stress response, the total amount of FDH may reach 9% of the total protein in mitochondria [6]. The genes encoding FDH are found in all plants [7], with some expressed in transgenic plants [8, 9]; attempts to produce a plant FDH in *E. coli* cells, however, were unsuccessful. Our laboratory was the first to express FDH genes from *Arabidopsis thaliana* (A-FDH) and *Glycine max* (G-FDH) in *E. coli* [10].

Analysis of the data published on FDHs demonstrates that their thermal stability has thus far received only minor attention from researchers. The relevant lit-

erature provides a vast variety of methods for the quantitative representation of thermal stability. In the case of FDH, many authors used the values of residual enzyme activity upon incubation at a set temperature and time period (15–30 min) [11–13] or reported the value of T_M, the temperature providing a 50% activity loss over 20 min [14]. The shortcoming of the first approach lies in the fact that the thermal inactivation of enzymes from different sources may follow distinct mechanisms; thus, inactivation kinetics have a complex time dependence. Therefore, the use of different time intervals may lead to opposite results. Moreover, the mechanism of thermal inactivation may change by an increased temperature. For example, the inactivation of *S. cerevisiae* FDH at a temperature below 42°C is reversible; however, at higher temperatures, inactivation follows a mechanism comprising of both reversible and irreversible stages [15].

A complex inactivation mechanism may also be the reason for the considerable difference between the T_M profiles within the same series of mutants, when using different periods of enzyme incubation. In addition, the T_M values fail to enable the quantitative assessment of thermal stability of an enzyme at other temperatures. Either by the studies of inactivation kinetics at various temperatures or by using differential scanning calorimetry (DSC) the most objective data on the thermal stability of enzymes is provided. The first approach gives quantitative characteristics of enzyme stability at different temperatures. The second, DSC technique, also allows the quantitative determination of the temperature transition between the native and denatured states of the protein globule.

A further note, yet another source of discrepancies, preventing an objective evaluation of FDS stability of diverse origin, is generated by the differences in the experimental conditions (first and foremost, the buffer solutions with different compositions, ionic strengths, and pH). As demonstrated with P-FDH, all these factors considerably affect the value of the thermal inactivation rate constant [16].

The goal of this work was to compare the thermal stabilities of FDHs isolated from the bacteria *Pseudomonas* sp. 101 and *Moraxella* sp. C1, the yeasts *Candida boidinii*, and the plants *Arabidopsis thaliana* and *Glycine max* under identical conditions, using an approach that involves the studies of thermal inactivation kinetics and differential scanning calorimetry.

MATERIALS AND METHODS

The genes coding for various FDHs were expressed in *E. coli* cells, and the recombinant enzymes were purified as described earlier [10, 16–18]. The purity of all enzymes was not less than 97% according to analytical SDS-PAGE.

FDH activity was determined spectrophotometrically by measuring NADH accumulation at a wavelength of 340 nm ($\varepsilon_{340} = 6220\ M^{-1}\ cm^{-1}$) in a Shimadzu UV 1601PC spectrophotometer (Japan) at 30° in 0.1 M of a potassium–phosphate buffer (pH 7.0). The concentrations of NAD^+ and sodium formate in the cuvette amounted to 1.5 mM and 0.3 M, respectively.

The thermal stability of FDH was measured in 0.1 M of potassium–phosphate buffer (pH 7.0), as described in [16, 18]. At which temperature incubation occurred depended on the thermal stability and varied from 62°C for P-FDH to 50°C for G-FDH. The thermal inactivation rate constant k_{in} was determined from the dependence of the residual activity A/A_0 on the time of the semilogarithmic coordinates ($\ln(A/A_0)$ against t, min) by linear regression, using the software Origin 7.5 (OriginLab, USA). Each value was the mean of at least three independent experiments.

The thermal stability of FDH was studied by DSC in a DASM-4M device (Specialized Design Office for Scientific Instrumentation, Pushchino, Russia) in a cell (480 μl) containing 0.1 M of potassium–phosphate buffer (pH 7.0). If not specified otherwise, the enzyme concentration in the cell amounted to 1 mg/ml and the heating rate was at 1 K/min. The experimental curves were processed and the values of denaturing heat were computed using Arina and Origin 1.16 programs.

RESULTS AND DISCUSSION

Time dependences of residual FDH activities are shown in Fig. 1. When plotted in semilogarithmic coordinates these dependences are straight lines, thereby indicating that the thermal inactivation follows first-order reaction kinetics. The observed values of the inactivation rate constants k_{in} for all FDHs were independent of the enzyme concentration. Thus, these results demonstrate that the thermal inactivation of all five FDHs is a true monomolecular process and that kinetically significant intermediates do not appear during the transition from a native to denatured state (N and D, respectively):

$$N \xrightarrow{k_{in}} D. \quad (1)$$

As is evident from Fig. 1a, the FDH from *Pseudomonas* sp. 101 displayed the highest thermal stability among the five enzymes studied. In general, P-FDH was the most stable enzyme among the FDHs. Of the four left, the least stable was the soybean enzyme. The inactivation-rate constant for G-FDH at 50°C $(2.13 \pm 0.05) \times 10^{-4}\ s^{-1}$, is 1.5-fold higher than the corresponding constant for *Pseudomonas* sp. 101 FDH at 62°C. Accounting for the fact that the inactivation rate constant for P-FDH increased 10-fold as the temperature went up by 3°C [17], the difference between the thermal stabilities of these two enzymes at the same

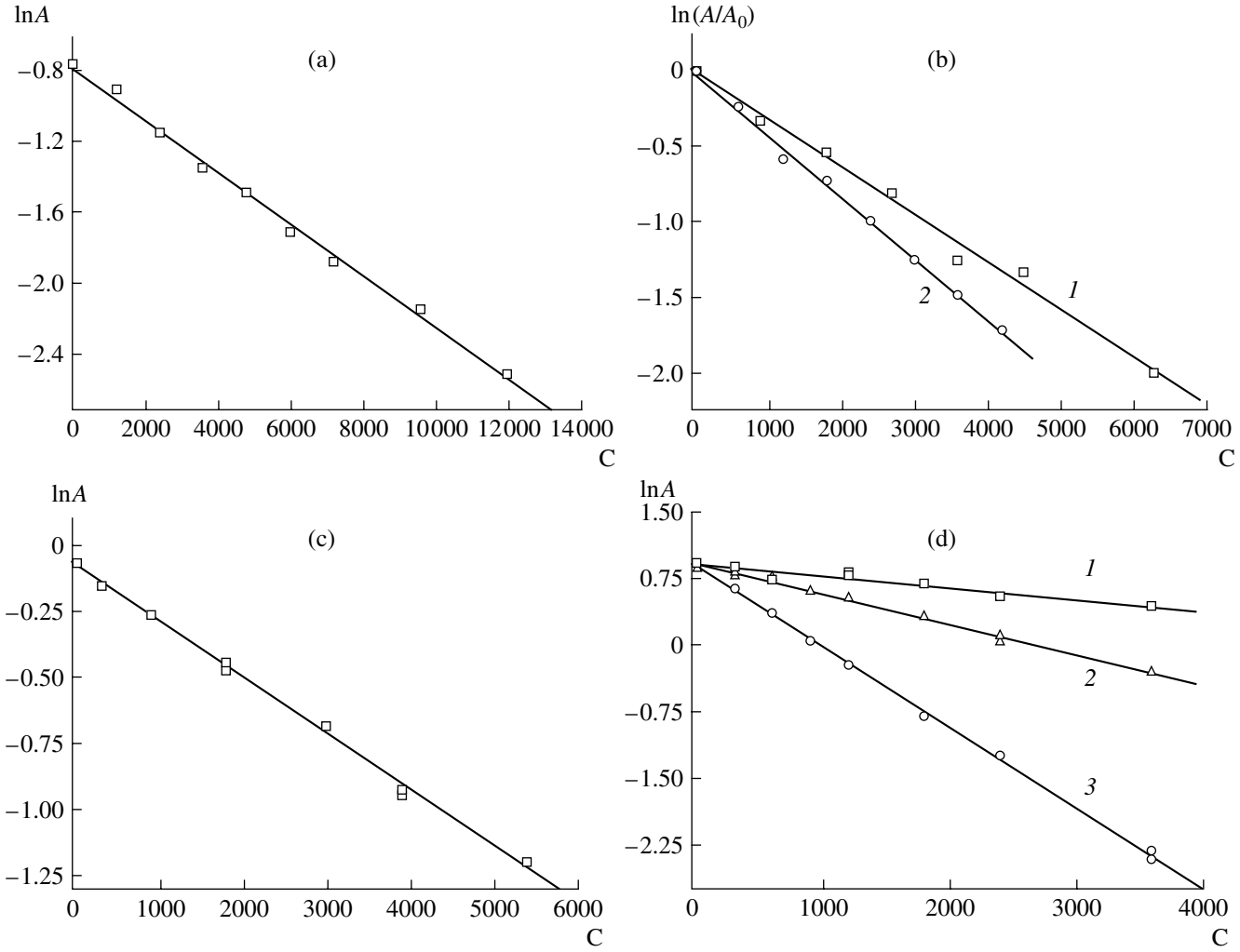

Fig. 1. Semilogarithmic plots of the time dependences of residual activity of FDHs of (a) *Pseudomonas* sp. 101 at 62°C; (b, *1*) *Moraxella* sp. C1 and (*2*) yeasts *C. boidinii* at 58°C; (c) plants soybean *Glycine max* at 50°C and (d) *A. thaliana* at (*1*) 56ºC, (*2*) 58ºC, and (*3*) 60ºC (0.1 M phosphate buffer pH 7.0; concentration of enzymes, 0.1 mg/ml).

temperature should be approximately 10000-fold. The thermal stabilities of the FDHs from the bacterium *Moraxella* sp. C1, the yeast *C. boidinii*, and the plant *A. thaliana* are comparable, being considerably lower than that of P-FDH.

In addition to the earlier experiments with P-FDH [16, 17], we studied the temperature dependence of A-FDH inactivation kinetics (Fig. 1d). It appeared that the enzyme inactivation in the temperature range studied (53–60°C) followed the first-order kinetics; i.e., the mechanism of thermal denaturing does not change during the process. As demonstrated repeatedly in experiments with wild- and mutant-type variants of P-FDH [16–18], the theory of an activated complex is applicable to a description of thermal inactivation. According to this theory, the temperature dependence of a rate constant on a first-order reaction (in our case the inactivation rate constant is k_{in}) is described by the following equation:

$$k_{in} = \frac{k_B T}{h} e^{-\left(\frac{\Delta H^{\neq}}{RT} - \frac{\Delta S^{\neq}}{R}\right)}, \qquad (2)$$

where T is in Kelvin degrees; k_B and h, the Boltzmann and Plank constants, respectively; R, the universal gas constant; and $\Delta H^{\neq}$ and $\Delta S^{\neq}$, activation enthalpy and entropy, respectively. This dependence can be linearized by plotting $\ln(k_{in}/T)$ against $1/T$. As evident from Fig. 2, this theory is applicable to the inactivation of A-FDH, as the temperature dependence of the inactivation rate constant is linear in the coordinates used. The value $\Delta H^{\neq}$ determined from the slope of the linearized dependence amounted to (408 ± 7) kJ/mol, which is slightly lower compared to the analogous value for P-FDH, (540 ± 15) kJ/mol. However, both values fall

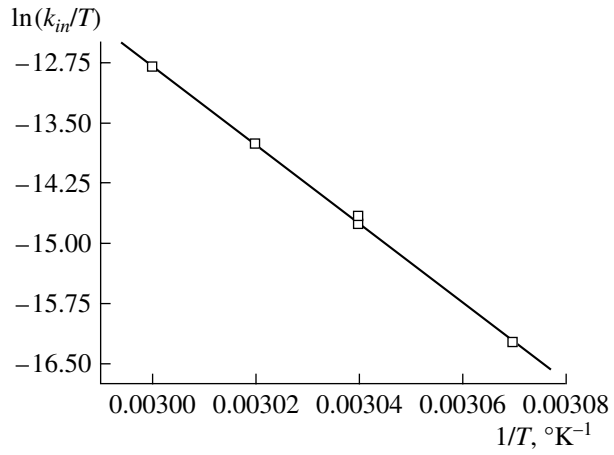

Fig. 2. Temperature dependence of the inactivation rate constant for *A. thaliana* FDH on in the ln(k_{in}/T) versus 1/T coordinates.

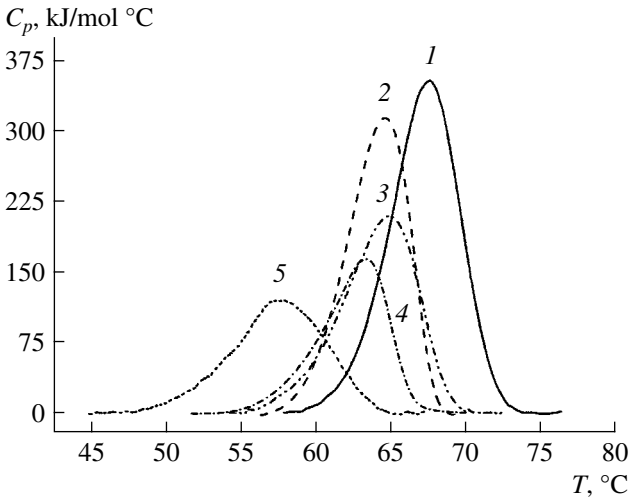

Fig. 3. Normalized melting curves of the FDH from (*1*) bacteria *Pseudomonas* sp. 101, T_m = 67.6°C and (*4*) *Moraxella* sp. C1, T_m = 63.4°C; (*2*) yeasts *C. boidinii*, T_m = 64.5°C, and (*3*) plants *A. thaliana*, T_m = 64.9°C and (*5*) soybean, T_m = 57.9°C (concentration of enzymes, 1 mg/ml; heating rate, 1 K/min; 0.1 M phosphate buffer pH 7.0).

within the range characteristic of processes related to protein globule unfolding.

DSC data on the thermal inactivation of FDHs (Fig. 3) agree with the results of kinetic experiments. The enzyme was irreversibly inactivated in all the cases studied. The value of the melting heat of the protein globule, ΔH_m, was independent of the protein concentration and heating rate. The half-width of the peaks for all enzymes except G-FDH amounted to 4.9–5.9 degrees, indicating a high cooperation in the unfolding of the protein globule. As the most stable enzyme P-FDH has the highest values of T_m (67.6°C) and ΔH_m (calculated from the peak area). Characteristic of the G-FDH denaturing was a low cooperation (the half-width of peak, 7.3 degrees) and the lowest values of T_m (57.9°C) and ΔH_m, which is 2.2-fold lower compared with the corresponding values for P-FDH. M-FDH, C-FDH, and A-FDH displayed close T_m values; however, the enzymes differed considerably in their ΔH_m values (Fig. 3). Presumably, this is accounted for by different contributions of enthalpy and entropy factors to thermal inactivation. In order to obtain deeper insight into the role of these components in the thermal inactivation of the enzymes, we plan to study the effect of ionic strength on the thermal stability of FDHs. A comparison of the data on the effect of ionic strength on P-FDH thermal inactivation [16] with the results of the preliminary experiments involving M-FDH demonstrates that the entropy factor contributes greater to the stability of the latter enzyme.

Thus, the experiments performed allowed us to demonstrate that the thermal inactivation of these five FDHs is an irreversible monomolecular process. The study of the inactivation kinetics under identical conditions gave comparative quantitative characteristics of their thermal stabilities. The thermal inactivation of FDHs was for the first time examined by differential scanning calorimetry. The results obtained comply with the data of kinetic experiments.

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