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Inactivation of Formate Dehydrogenase at pH 8

N. S. Voinova, S. S. Savin*, A. A. Alekseeva, O. E. Skirgello, and V. I. Tishkov

Department of Chemical Enzymology

e-mail: vit@enz.chem.msu.ru

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Abstract—The first-order inactivation rate constant as a function of the phosphate buffer concentration has been studied for recombinant formate dehydrogenases from plants *Arabidopsis thaliana* and soybean and for mutant formate dehydrogenase from bacterium *Pseudomonas* sp. 101 (PseFDH GAV). Both stabilization and destabilization of the enzyme can be observed depending on the ionic strength of the buffer.

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NAD^+-dependent formate dehydrogenase (FDH, EC 1.2.1.2.) of different origin attracts much attention of researchers. In bacteria grown on methanol, this enzyme catalyzes the final stage of oxidation of methanol to carbon dioxide by means of the polyenzyme system (methanol dehydrogenase–formaldehyde dehydrogenase–FDH) and, thus, provides energy to the cell. In plants, FDH is localized in mitochondria and is among the most important enzymes synthesized under stress [1]. The interest in FDH is due to the fact that it is a model enzyme for study of the mechanism of action of D-specific $NAD(P)^+$-dependent 2-hydroxyacid dehydrogenases [2].

FDH is also of practical importance since it is widely used in biocatalytic synthesis of optically active compounds [3]. The efficiency of use of FDH in these processes is due to the broad pH optimum of activity; irreversibility of the catalyzed reaction; inexpensiveness of the second substrate, formate; and simplicity of removal of the bicarbonate ion during purification of the target product. For efficient use of an enzyme, comprehensive information on its stability under different conditions—temperature, pH, and ionic strength of a solution—is necessary. Unfortunately, such data are, as a rule, available only for one pH and buffer concentration value. There is only one work where the thermostability of recombinant FDHs from bacteria *Pseudomonas* sp. 101 (PseFDH) and *Mycobacterium vaccae* N10 was studied in a wide range of phosphate buffer concentrations at pH 7.0 [4]. In the present work, we studied how the first-order rate constant of thermoinactivation of recombinant formate dehydrogenases from plants *Arabidopsis thaliana* (AraFDH) and soybean (SoyFDH) and the mutant enzyme PseFDH GAV depends on the phosphate buffer concentration. The choice of these enzymes is caused by the fact that recombinant plant FDHs expressed in *E. coli* are virtually unstudied and PseFDH GAV is most widely used in practice.

EXPERIMENTAL

Plant formate dehydrogenases and PseFDH GAV were obtained as described in [3, 5]. According to analytical electrophoresis data, the purity of the preparations in a polyacrylamide gel in the presence of sodium dodecylsulfate was no less than 98%. The enzymes were kept in 0.1 M phosphate buffer at pH 7.0 in the presence of 20 mM EDTA. To study thermostability at pH 8.0 and different concentrations, on the day of the experiment, two 1-mL portions of each enzyme (activity, 10–20 units/mL) were sampled, and by means of gel filtration through Sephadex G-10 Superfine (1 × 10 cm column), one portion was transferred to 0.05 M phosphate buffer (pH 8.0) and the second portion was transferred to 1.5 M phosphate buffer (pH 8.0). The pHs of the buffers were controlled with an accuracy of 0.01 pH units on a pH meter (Ekoniks Ekspert, Russia) at the same temperature at which thermostability experiments were carried out.

The FDH activity was determined on a Shimadzu UV-1601PC spectrophotometer at 30°C by monitoring the NADH absorption at 340 nm ($\varepsilon_{340} = 6220\ M^{-1}\ cm^{-1}$).

The activity was measured in 0.1 M sodium phosphate buffer (pH 7.0) at concentrations of sodium formate and NAD^+ of 0.3 mol/L and 1.2 mg/mL, respectively. The exact concentrations of the initial NAD^+ solutions were determined spectrophotometrically at 260 nm ($\varepsilon_{260} = 18000\ M^{-1}\ cm^{-1}$). The exact concentration of sodium formate was determined under the same conditions as the FDH activity, namely, from the final concentration of NADH in the presence of excess NAD^+.

To obtain FDH preparations in the phosphate buffer with pH 8.0 and a required concentration (c), the enzyme

* *OOO Innovations and High Technologies MSU Ltd.*

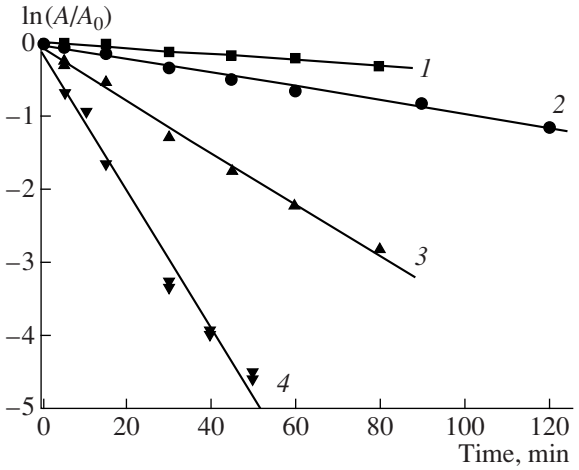

Fig. 1. Inactivation of formate dehydrogenase from *Arabidopsis thaliana* in 0.1 M phosphate buffer (pH 8.0) at different temperatures: (*1*) 56, (*2*) 58, (*3*) 60, and (*4*) 62°C.

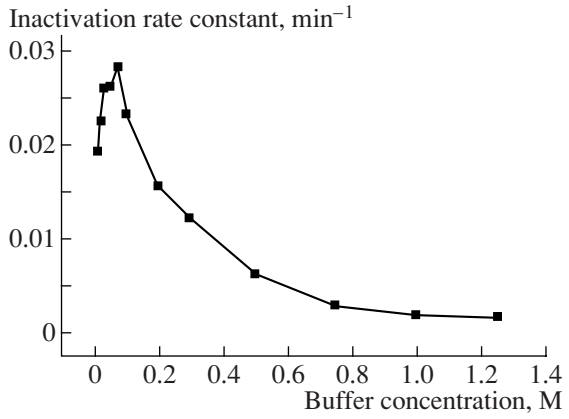

Fig. 2. Inactivation rate constant of recombinant formate dehydrogenase from soybean as a function of the concentration of the phosphate buffer (pH 8.0, 52°C).

preparations in 0.01 and 1.5 M phosphate buffers were mixed in a definite proportion, and a phosphate buffer of required concentration was added if necessary, so that the enzyme activity was 2–3 units/mL.

To study the thermoinactivation kinetics, a series of plastic tubes 1.5 mL in volume each containing 50 μL of an enzyme solution (0.2 mg/mL) in the required phosphate buffer were prepared. The tubes were placed in a water thermostat preliminarily heated to a necessary temperature (56–62°C, the accuracy of temperature control was ±0.1 K). At certain moments of time, one of the tubes was removed and placed in ice for 3 min; then, the tube was centrifuged for 3 min at 12000 rpm on an Eppendorf 5415D centrifuge to remove the enzyme precipitate. The residual FDH activity was measured as described above. The thermoinactivation rate constant k_i was determined as the slope of the plot of the natural logarithm of the residual activity versus time (semilogarithmic coordinates $\ln(A/A_0)$–t) by the linear regression method with the Origin 7.0 program.

RESULTS AND DISCUSSION

At temperatures above 45°C, inactivation of enzymes is mainly due to unfolding of the protein globule (thermodenaturation). The rate and depth of this process depends on different factors: temperature, pH of a medium, and the presence of substrates and inhibitors. The effect of a certain factor depends on what interactions in the protein molecule it influences and on the role of these interactions in stabilization of the protein globule. As is known, the entropy-driven assembly of a globular protein molecule is accompanied by packing of hydrophobic amino acid residues inside the protein molecule. As a result, the core of the globule is effectively stabilized due to hydrophobic interactions. However, other interactions, for example, electrostatic, can noticeably influence the stability of the protein globule. Electrostatic interactions are among the strongest noncovalent interactions. In different proteins, these two types of interactions make different contributions to the stabilization of their structure. Moreover, their ratio can depend on the concentration of the buffer solution. An increase in the buffer concentration leads to an increase in the ionic strength and electrostatic interactions in a protein molecule should become weaker. At the same time, an increase in the ionic strength ensures the enhancement of hydrophobic interactions. To elucidate the role of hydrophobic and electrostatic interactions in the thermostability of FDHs, we used the phosphate buffer. Phosphate ion is rather large. It does not inhibit the enzymatic activity and, according to X-ray crystallography, does not penetrate into the FDH globule. Thus, changing the concentration of the phosphate buffer, we can alter the electrostatic interactions at the surface of the protein molecule without disturbing the structure of the protein globule.

The kinetics of inactivation of all three FDHs, from plants *Arabidopsis thaliana* (AraFDH) and soybean (SoyFDH) and mutant bacterial FDH from *Pseudomonas* sp. 101 PseFDH GAV, was studied at pH 8.0 in a wide range of temperatures. We showed that, at any concentration of the phosphate buffer, the activity loss in all cases was described by one exponential curve and did not depend on the enzyme concentration. This is evidence that thermodenaturation of these three FDHs is a monomolecular process and that the first-order inactivation rate constant directly characterizes the thermostability of the protein globule under given conditions. As an example, Fig. 1 shows the plots of residual activity versus time for AraFDH.

Inasmuch as the FDHs under consideration differ in thermostability from each other (this is especially true for SoyFDH, for which the thermostability is at least 1000-fold lower than that of AraFDH and, especially

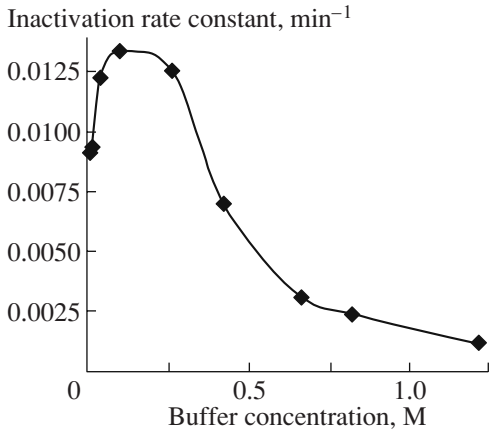

Fig. 3. Inactivation rate constant of recombinant formate dehydrogenase from *Arabidopsis thaliana* as a function of the concentration of the phosphate buffer (pH 8.0, 58°C).

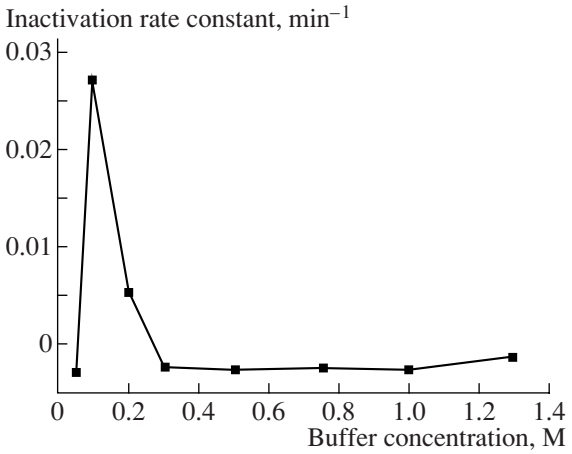

Fig. 4. Inactivation rate constant of mutant formate dehydrogenase PseFDH GAV as a function of the concentration of the phosphate buffer (pH 8.0, 63°C).

PseFDH GAV), the dependence of the thermoinactivation rate constant on the phosphate buffer concentration was studied at different temperatures: at 52°C for SoyFDH, at 58°C for AraFDH, and at 63°C for PseFDH GAV. The results are shown in Figs. 2–4. As is seen, the plots of the inactivation rate constant versus the buffer concentration differ significantly from one another not only in shape but also in value. For the plant enzymes, electrostatic interactions do not play a leading role in the stability of a protein globule: as the phosphate buffer concentration increases to 0.1 M, the inactivation rate constant increases by a factor of no more than 1.4–1.5, whereas a further increase in the phosphate concentration causes the enzyme to become ten times more stable due to the enhancement of hydrophobic interactions. For AraFDH (Fig. 3), the range of buffer concentrations at which the enzyme has the lowest stability is larger than in the case of SoyFDH (Fig. 2). The narrowest "instability range" is observed for mutant PseFDH GAV. Actually, the stability of this enzyme is independent of the phosphate buffer concentration in a very wide range, 0.3–1.3 M (Fig. 4). These data differ strongly from the data on the wild-type enzyme, for which the maximal instability range is 0.1–0.35 M [4]. This is likely associated with the fact that the thermostability of PseFDH GAV was increased by means of mutations enhancing the hydrophobicity of the protein globule.

Thus, our findings demonstrate a more hydrophobic character of the protein globules of plant FDHs as compared with the FDH from bacterium *Pseudomonas* sp. 101. Stabilizing the bacterial enzyme by increasing the hydrophobicity of its protein globule made it possible to obtain a biocatalyst with a constant stability in a wide range of ionic strength.

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