Heat Denaturation of D-glyceraldehyde-3-phosphate Dehydrogenase Holoenzyme

(Short Communication)

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The conformational stability of a protein depends on the pH, temperature and composition of the medium and on the steric structure of the protein itself and it is reflected in the activation energy of denaturation. The aim of this work was to determine the rate constant and activation energy of heat denaturation of D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating) — EC 1.2.1.12 — containing the firmly bound NAD and the effect of pH and protein concentration on these parameters.

The heat denaturation of the enzyme, as determined by measuring the loss of enzymic activity in glycine buffer containing 0.03 M 2-mercaptoethanol, follows first order kinetics throughout the pH-range (pH 5.2 to 9.2), concentration range (0.030 to 5.0 mg/ml) and the temperature range (37 to 60 °C) examined even up to about 90% loss of initial activity. Some progress curves are presented in Fig. 1.

These results permit the assumption that the heat denaturation of glyceraldehyde-3-phosphate dehydrogenase holoenzyme follows the all-in-one mechanism (Keleti, 1971). However, in tris-HCl buffer, ionic strength 0.05, downward concave progress curves of inactivation were observed in the semilogarithmic plots after 70—80% loss of activity in agreement with the data obtained with the apoenzyme (Vas, Boross, 1972).

The first order rate constant of heat inactivation has a pH-minimum. This minimum is between pH 7.5 and 8.0 at 1 mg/ml protein concentration and 50 °C (Fig. 2).

The Arrhenius plots of heat inactivation at pH 7.5 to 8.0 in glycine buffer containing 0.03 M 2-mercaptoethanol, with protein solutions of 0.030 to 0.100 mg/ml (between 37 and 47°C), of 0.125 to 1.0 mg/ml (between 43 and 55 °C) and of 2.0 to 5.0 mg/ml (between 47 and 60 °C) gave straight lines and indicated an average value of activation energy of about 100 ± 15 kcal/mole. In tris-HCl buffer (0.05 ionic strength) the same activation energy was measured (5 mg/ml holoenzyme in the presence of 2-mercaptoethanol, between 50 and 57 °C). Even charcoal treated (NAD-free) apoenzyme gave the same activation energy at 1 mg/ml concentration (in 0.1 M glycine buffer, containing 0.1 M NaCl and 0.03 M 2-mercaptoethanol, between 43 and 55 °C).
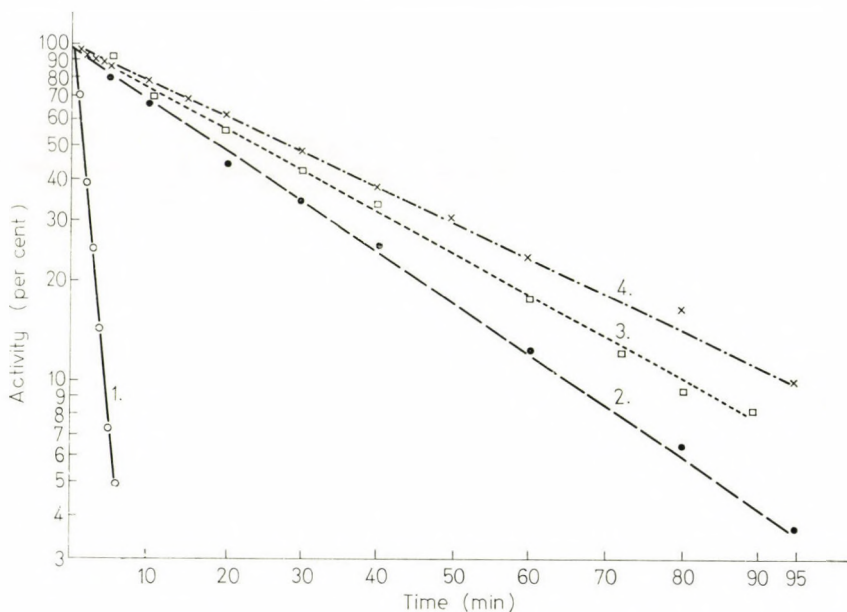
Fig. 1. Heat inactivation of D-glyceraldehyde-3-phosphate dehydrogenase under various conditions. Four times recrystallized swine muscle glyceraldehyde-3-phosphate dehydrogenase (Elődi, Szörényi, 1956) dissolved in 0.1 M glycine buffer, containing 0.1 M NaCl and 0.03 M 2-mercaptoethanol, pH 8.5 at 18 °C (which corresponds to pH 7.7 at 50 °C) was diluted with the same buffer heated to the temperature of the experiment. At the times indicated *aliquots* were cooled to 0 °C and diluted: a) with the same buffer to obtain the necessary concentration in the cuvette for enzymic activity assay, and b) with 0.1 M NaOH for the determination of protein concentration by using the molar extinction coefficient 145 000 for the holoenzyme at 280 nm. The enzymic activity was measured in a Hilger UVISPEK spectrophotometer at 340 nm in silica cells of 1 cm light path in the following assay mixture: NAD 2×10^{-3} M, phosphate 1×10^{-2} M, D-glyceraldehyde-3-phosphate 2×10^{-3} M, enzyme 7.15×10^{-9} M in 0.1 M glycine buffer, pH 8.5, at 18 °C. The figure shows the loss in per cent of specific enzyme activity in semilogarithmic presentation as a function of time. 1: 0.125 mg/ml protein, at 53 °C; 2: 1.0 mg/ml protein, at 55 °C; 3: 0.030 mg/ml protein, at 47 °C; 4: 5.0 mg/ml protein, at 57 °C

The enzyme in solution forms an equilibrium mixture of tetrameric, dimeric and monomeric species (Hoagland, Teller, 1969; Lakatos et al., 1972; Ovádi et al., 1971), the firmly bound NAD's have different dissociation constants (Koshland, Neet, 1968; De Vijlder, Slater, 1968) and the apparent first order rate constant of heat inactivation of the apoenzyme changes with protein concentration (Vas, Boross, 1972). Considering the aforementioned data one would expect the activation energy to change with protein concentration and a deviation from linearity of the kinetics of denaturation and of the Arrhenius plots. However, we were unable to demonstrate any of the above phenomena in the concentration and temperature ranges used.
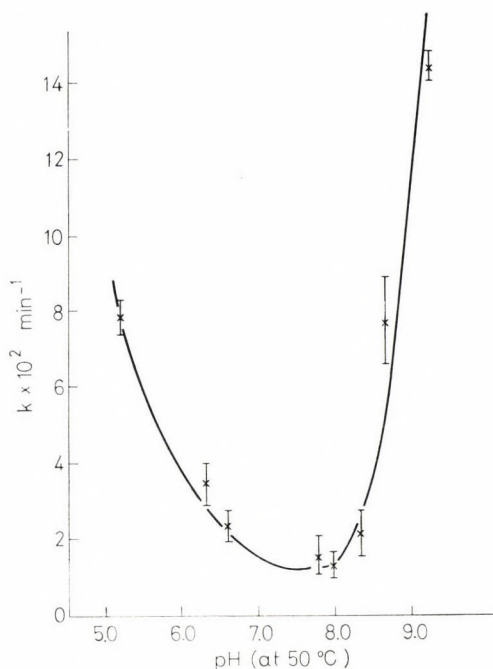
Fig. 2. pH-dependence of first order rate constant of heat inactivation of glyceraldehyde-3-phosphate dehydrogenase. The first order rate constants were determined as in Fig. 1, at 1 mg/ml protein concentration and 50 °C. The pH was measured with a Radelkisz blood pH-meter at 50 °C at the beginning and at the end of heat exposure and only those experiments were accepted where the difference in pH did not exceed ± 0.2 units

The rate constants of heat inactivation of the holoenzyme were only slightly influenced by protein concentration. This change may partially be due to the change in the saturation by NAD, since it is known that the binding of NAD alters the steric structure of the protein and it becomes more stable (Elődi, Szabolcsi, 1959; Szabolcsi 1958; Listowsky et al., 1965; Závodszky et al., 1966). Indeed, we have shown that the saturation of the enzyme in dilute solution by excess NAD decreases the first order rate constant of inactivation by a factor of about 0.7, as compared to the "holoenzyme", which in dilute solution (0.030 to 0.125 mg/ml) contains about 2 moles of firmly bound NAD per mole of protein. A similar protective effect was found by increasing the SO_4^{2-} concentration of dilute enzyme solutions.

The dimeric form of the enzyme is probably less stable than the tetramer as it was shown in experiments with enzyme-ATP complex (Ovádi et al., 1971). However, at higher temperatures higher average molecular weights can be determined (Závodszky, 1965) and the dissociation of the enzyme into subunits caused by ATP or salts is promoted by low but reversed by high temperature (Constantinides, Deal 1969, 1970; Nagradova, Gùseva, 1971). These data suggest

that higher temperatures favour the association of the subunits. Further data suggest that NAD may also displace the equilibrium towards the associated form (unpublished results).

If the assumption is true that under the conditions used the equilibrium is greatly shifted towards the tetrameric form, the observed denaturation is that of the tetrameric form of glyceraldehyde-3-phosphate dehydrogenase. This notion is supported by the higher activation energy of heat inactivation of the holoenzyme at any protein concentration and of the apoenzyme at high concentration and high temperature (about 100 kcal/mole) as compared to that of the apoenzyme dissociated into monomers (about 30 kcal/mole, see Vas, Boross, 1972).

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