0273-2289/96/6101-02—0067$07.00

Thermostability of Yeast Hexokinase and Yeast Glucose-6-Phosphate Dehydrogenase

E. A. ZAITZEVA,* E. S. CHUKRAI, AND O. M. POLTORAK

M. V. Lomonosov Moscow State University, Department of Chemistry, 119899, Moscow, Russia

ABSTRACT

Kinetic study of the mechanism of the temperature-induced loss of the catalytic activity by yeast hexokinase (HK) and yeast glucose-6-phosphate dehydrogenase (G-6-PDG) has shown the dissociative nature of the processes. In the temperature range 40–47°C, they are satisfactorily described in terms of consecutive reactions in which steps of irreversible denaturation of the monomeric units follow the reversible dissociation of inactive oligomeric forms into the active units, resulting in an increase in catalytic activity. The experimental data have been analyzed in the framework of the dissociative mechanism, and a semiquantitative method has been developed for calculating the individual rate constants.

Index Entries: Hexokinase; glucose-6-phosphate dehydrogenase; thermoinactivation; intermolecular interactions; kinetic mechanism.

INTRODUCTION

Thermal inactivation of enzymes with quaternary structures may have different mechanisms in which a significant role is played by association and dissociation of the protein globules. It is clear that the investigation of inactivation mechanisms in a number of cases provides valuable information on intermolecular interactions in such enzymatic systems.

Kinetic approaches are successfully used in analysis of thermostability of oligomeric proteins for which a reversible dissociation of oligomeric forms into subunits may precede their irreversible denaturation under certain conditions. The approach proposed earlier can be used for the

*Author to whom all correspondence and reprint requests should be addressed.

approximate treatment of second-order processes, and makes possible the calculation of the equilibrium dissociation constants and the rate constants for individual steps under different conditions (*1,2*). Until now, this approach was applied to the enzymatic systems in which a catalytically active associate dissociated into inactive monomeric units. There, however, exist a number of enzymes that have monomers or associates of an intermediate composition compared to the active units. The examples are yeast hexokinase (HK) and yeast glucose-6-phosphate dehydrogenase (G-6-PDG).

The purpose of this work was the investigation of the kinetic mechanisms of catalysis and inactivation of the above-mentioned enzymes, as well as elucidation of the role and evaluation of the physicochemical parameters of the intermolecular interactions of the proteins.

MATERIALS AND METHODS

HK (Fluka), G-6-PDG (Ferak Berlin), NADP as a monosodium salt, disodium salt of ATP, glucose-6-phosphate, α-D-glucose, ethylenediaminetetraacetic acid (EDTA), and magnesium chloride (all from Reanal) were used in this work.

Catalytic activities of the enzymes were determined by conventional techniques using α-D-glucose phosphorylation and oxidation in reactions with ATP and NADP, respectively. The initial reaction rate that defines the activity was calculated from the spectrophotometric data on the NADH formation.

The optical density at 340 nm was recorded in a differential regime using a thermostated cell compartment.

The HK activity was measured in 2.5 mL of the reaction mixture of the following composition: 1.4 mL of 0.2*M* borate buffer, pH 8.0, 0.2 mL of 0.4*M* glucose solution, 0.4 mL of HK solution of variable concentration, and 0.5 mL of the "operating mixture" in 0.2*M* borate buffer, pH 8.0, which had the following composition: 2.5 × 10 mg/mL G-6-PDG, 2.7 × $10^{-3}M$ ATP, 1.44 × $10^{-3}M$ NADP, and 7.5 × $10^{-3}M$ $MgCl_2$.

The enzymatic activity of G-6-PDG was determined in a similar way. The reaction mixture of 2 mL total volume contained 1.1 mL of the borate buffer, pH 8.0, 0.4 mL of the enzyme solution of the required concentration, and 0.5 mL of the "operating mixture" of the following composition: 9.4 × $10^{-4}M$ glucose-6-phosphate, 1.44 × $10^{-3}M$ NADP, and 7.5 × $10^{-3}M$ $MgCl_2$.

Thermoinactivation was carried out in a thermostated vessel in the temperature range 40–47°C. Aliquots of 0.4 mL were taken at certain time intervals and analyzed for enzymatic activity.

Table 1
Kinetic Parameters of Associative-Dissociative Equilibrium of Oligomeric Forms of Yeast HK and G-6-PDG[a]

Enzyme	Equilibrium	Conditions	K_{ass}, M^{-1}	a, Specific activity, mol NADPH/min/ mol of act. centers	Equation used
HK (4,5)	$2E_1 \rightleftharpoons E_2$ active little active	20°C bidist. water, pH 6.2	$(2.9 \pm 0.3) \times 10^6$	$a_1 = (1.4 \pm 0.2) \times 10^4$ $a_2 = (9.2 \pm 0.7) \times 10^2$	(a)
HK (4,5)	$2E_1 \rightleftharpoons E_2$ active inactive	20°C glycine buffer, pH 8.6	$(1.4 \pm 0.1) \times 10^6$	$a_1 = (6.2 \pm 0.1) \times 10^3$ $a_2 = 0$	(b)
G-6-PDG (6,7)	$2E_2 \rightleftharpoons E_4$ active inactive	20°C borate buffer, pH 8.0	$(1.4 \pm 0.1) \times 10^9$	$a_2 = (9.5 \pm 1) \times 10^5$ $a_4 = 0$	(b)

[a]The parameters were calculated using Eqs. (a) and (b) (3):

$$a = a_2 + \sqrt{[(a_1 + a_2)/2K_{ass}]^{1/4} \times \sqrt{[(a_1 - a_2)/E_0]^{1/2}} \quad (a)$$

when $a_1 > a_2$, $a_2 > 0$,

$$1/a = 1/a_1 + (2K_{ass}/a_1^2) \times A \quad (b)$$

when $a_2 = 0$, where "a" stands for the specific catalytic activity; a_1 and a_2 are specific catalytic activities of the monomer and the dimer, E_0 is the initial protein concentration, A is the total enzyme activity, and K_{ass} is the association equilibrium constant.

RESULTS AND DISCUSSION

Effects of Associative–Dissociative Processes on the Enzymatic Activity of HK and G-6-PDG in Solution

Since we were interested in investigation of possible mechanisms of structural changes related to the catalytic function of the enzymes, it was necessary to determine the oligomeric composition of HK and G-6-PDG under the experimental conditions, and to compare the catalytic activity of different forms. From the dependence of the specific catalytic activity on the initial protein concentration using the calculation routine described in detail elsewhere (3), it was deduced that under the experimental conditions, G-6-PDG is a dimer that comprises catalytic unit while it is a monomer for HK. The equilibrium constants for association were calculated from these data as well and the results obtained are given in Table 1.

Determination of the Kinetic Parameters of G-6-PDG Thermoinactivation

Thermoinactivation of enzymes with the tertiary structures has complicated mechanisms comprising a few elementary steps. At relatively

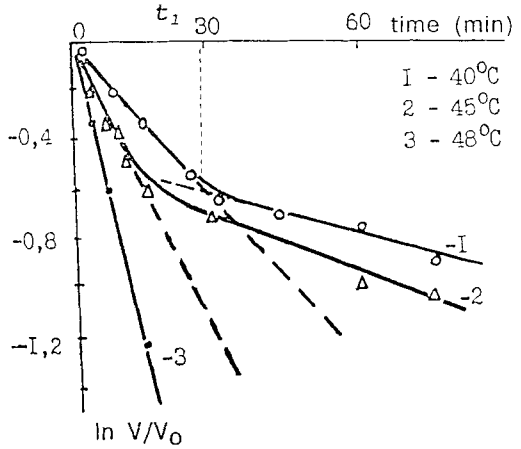

Fig. 1. Thermoinactivation kinetic curves for G-6-PDG at $[E_o] = 1.96 \times 10^{-9}M$. Activity was assayed at temperatures of inactivation at pH 8.0. V_o is the rate of the enzymatic reaction of time $t = 0$, and V_t is the rate at time t.

low temperatures, the inactivation of proteins with the quaternary structures may be usually described in terms of the dissociative mechanism. The thermoinactivation of G-6-PDG in the temperature range 40–46°C may be approximated by the following scheme:

$$E_4 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} 2E_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 4E_1 \xrightarrow{k_d} 4E_{den} \qquad (1)$$

where E_4 is the inactive tetrameric form, E_2 is the catalytically active dimer, E_1 is the inactive monomer, E_{den} is the denaturated monomeric form of the protein, and k_2, k_{-2}, k_1, k_{-1}, and k_d are the corresponding rate constants.

Scheme 1 includes the dissociation of the active dimer into the inactive monomer and inactive denaturated product E_{den}:

$$E_2 \underset{}{\overset{k_1}{\rightleftharpoons}} 2E_1 \xrightarrow{k_d} 2E_{den} \qquad (2)$$

Kinetic curves of the thermoinactivation in a given concentration range have two linear regions (Fig. 1). The position of the intersection point of these linear regions changes regularly, depending on the temperature and the initial concentration of the protein. The first region corresponds to nonsteady-state kinetics of dissociation of the active dimer into the inactive monomers. The second region reflects the slow kinetics of irreversible changes of the monomers.

It is impossible to solve the problem related to the second-order associative–dissociative processes in the most general case. Therefore, several approximations were used in determination of the kinetic parameters in Scheme 2. The basic assumptions are the following. (1) The process $E_2 \rightarrow E_{2,den}$ was excluded from the consideration, and (2) on estimating the

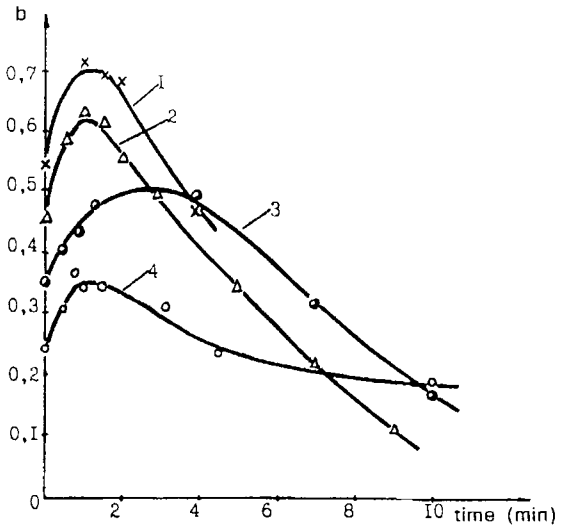

Fig. 2. Changes of the relative activity b in the course of thermoinactivation of G-6-PDG at = 46°C at different [E_o]. The enzyme activity was measured at 46°C. 1, [E_o] = 5.83 × $10^{-9}M$; 2, [E_o] = 1.17 × $10^{-8}M$; 3, [E_o] = 2.33 × $10^{-8}M$; 4, [E_o] = 4.66 × $10^{-8}M$; pH 8.0.

kinetic parameters, it was suggested that up to the moment of time corresponding to the break-point on the kinetic curve, the concentration of the denaturated form should be negligible. This makes possible the calculation of k_1, the individual rate constant for the decay of the dimer, in accordance with Eq. (3) (*1*):

$$F(V) = 2(V/V_o) - 1/2/(V/V_o)^2 = 3/2 - k_1 t \quad (3)$$

where V_o is the enzyme activity at time $t = 0$; V is the current activity at time t. For temperatures 40 and 45°C, the following values were obtained: $k_1^{40} = (2.0 \pm 0.2) \times 10^{-2}$/min and $k_1^{45} = (3.5 \pm 0.3) \times 10^{-2}$/min.

The estimate of the dissociation equilibrium constant was derived from the position of the kinetic curves break-points (t_1 and V_t) (Fig. 1) (*1*):

$$K^{40} = k_1/k_{-1} = 4E_o \times (V_o - V_t)^2/(V_o \times V_t) = 1.7 \pm 1 \times 10^{-9}M \quad (4)$$

At higher G-6-PDG concentrations, it was found that initial portions of the kinetic curves displayed an increased enzymatic activity that could be brought about by the dissociation of the inactive tetrameric form into the active dimeric units. The kinetic curves obtained in this case are shown in Fig. 2.

The relative activity "*b*", defined as the ratio of the observed total enzymatic activity A at the initial concentration E_o to the enzymatic activity at the same concentration when all the protein is in its active form, was used as a kinetic parameter: $b = A/a_2E_o$, where a_2 is the specific catalytic activity of the dimer.

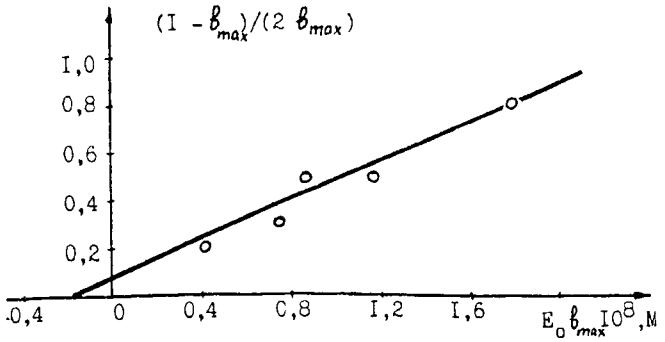

Fig. 3. Linear anamorphosis of experimental data (*see* Fig. 2 and Eq. [5]).

From the analysis of the experimental and simulated kinetic curves, it was concluded that the process may be described satisfactorily by Scheme 5:

$$\underset{\text{inact.}}{E_4} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{act}}{2E_2} \xrightarrow{k_1} \underset{\text{inact.}}{2E_1} \tag{5}$$

With the help of the above-mentioned approximations, it turned out to be possible to represent the experimental data as a linear anamorphosis (Fig. 3).

$$(1 - b_{max})/2b_{max} = 1/g + (1/K) \times (E_o \times b_{max}) \tag{6}$$

where b_{max} is the maximal value of b, K is the dissociation equilibrium constant at the temperature used in the experiment, and g is the ratio k_2/k_1. The determined parameters of Scheme 6 were the following:

$$K^{46} = (2.4 \pm 0.3) \times 10^{-8}M, k_2/k_1 = 14.3 \tag{7}$$

Using the value of k_1 found previously, it was possible to estimate the value of $k_2 = (5 \pm 2) \times 10^{-1}$/min. The estimated enthalpy of dissociation of tetrameric units into the dimer ΔH^o equals 25 ± 1 kcal/mol.

The Kinetic Mechanism of the Yeast HK Thermoinactivation

The yeast HK belongs to the most thermolabile enzymes. Up to now, its thermoinactivation mechanism was not clear. Main difficulties were associated with the oxidation of the sulfhydryl groups during the thermoinactivation of the native enzyme, and with contamination of commercially available enzyme preparations with a thermolabile proteolytic form that is unable to form the associates. These factors excluded by addition of EDTA (0.001*M*), and using fresh preparations of enzyme allowed us to describe the inactivation of HK by a two-step scheme in the temperature range 40–47°C:

$$\underset{\text{inact.}}{E_2} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{act}}{2E_1} \xrightarrow{k_d} \underset{\text{inact.}}{2E_{den}} \tag{8}$$

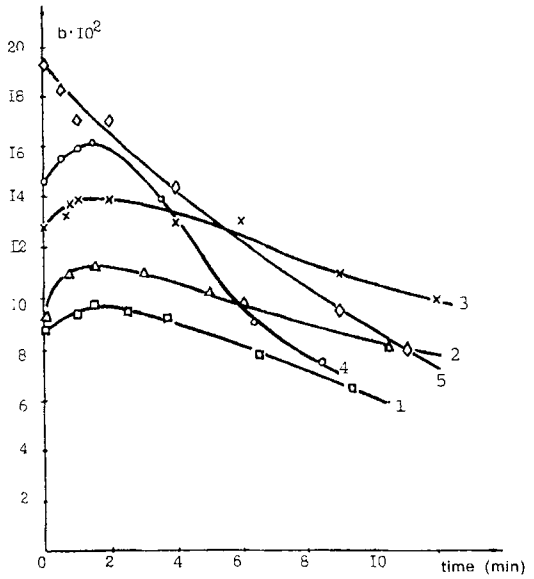

Fig. 4. Thermoinactivation kinetic curves for yeast HK in aqueous solution; pH 6.2; 0.001*M* EDTA, 47°C; b = $V/a_1[E_o]$; a_1 is the specific activity of the monomer; 1, $[E_o]$ = 0.41 mg/mL; 2, $[E_o]$ = 0.35 mg/mL; 3, $[E_o]$ = 0.28 mg/mL; 4, $[E_o]$ = 0.15 mg/mL.

where k_1, k_{-1}, and k_d are the rate constants, E_2 is the inactive dimer, E_1 is the catalytically active monomer, and E_{den} is the inactive denaturated form of the protein.

The kinetic curves of thermoinactivation of HK for the conditions described above are shown in Fig. 4. From these data and using Eq. (6), the following parameters were determined graphically:

$$K^{47} = k_1/k_{-1} = (2.7 \pm 0.2) \times 10^{-6}M, k_1/k_d = 0.67 \quad (9)$$

with ΔH^o = 14 ± 1 kcal/mol.

CONCLUSION

Thermoinactivation of soluble forms of yeast HK in the temperature range 40–47°C accompanied by an increase in the catalytic activity in a certain concentration range may be satisfactorily described by a kinetic scheme with consecutive reactions in which steps of irreversible denaturation follow a dissociative decay of inactive oligomers into active catalytic units.

A semiquantative routine was developed allowing, on the basis of experimental data, the calculation of physicochemical parameters of the process, such as the rate constants of elementary steps and equilibrium dissociation constants.

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