STABILIZATION MECHANISM OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

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Yeast glucose-6-phosphate dehydrogenase (G6PDH) in solutions exists as the equilibrium mixture of dimers and tetramers. Only dimers are active. The equation responding to these conditions was used to determine the parameters: the specific activity of dimers and the dissociation equilibrium constant of tetramers. The stability of G6PDH dimers were shown to be maintained due to the conformational lock, a multiple-point intersubunit contact in the hydrophobic region of "cylindrical" protomers. The conformational lock is formed by two isolated contacts at least. In the consecutive mechanism of G6PDH thermal inactivation, the destruction of one of these contacts results in a labile dimer. The latter dissociates to inactive monomers. At 46°C, the equilibrium constant of this process $K_{\text{dis}}^{\text{II}} = (6.2 \pm 0.4)$ nM, whereas the specific activity dimers $a_2 = (13 \pm 0.2) \times 10^6$ mol NADPH/min.mol of active sites. The kinetic analysis showed that the inactivation process is preceded by tree steps: the dissociation of tetramers to active stable dimers, the emergence of labile dimers and their dissociation to inactive protomers. The final step of G6PDH thermal inactivation is an irreversible inactivation of protomers, the denaturation with the rate constant $k = 6 \times 10^{-3}$ min^{-1}.

G6PDH is glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate, oxidoreductase) belongs to one of the most important and vast class of enzymes NAD-dependent dehydrogenases. G6PDH catalyzes dehydration of glucose-6-phosphate (G6P) to 6-phosphategluconic lactone. As hydride-ion acceptor, the enzyme uses $NADP^+$. G6PDH amino acid composition was totally sequenced in 1969 [1]. A typical feature of G6PDH, like of other dehydrogenases, is its inactivation resulting from its dissociation [2, 3]. Not a single dehydrogenase was shown to have an active monomer. This is indicative of the importance of subunit contacts for catalytic properties to be pronounced. G6PDH from beer yeast have been most studied [2]. The method of sedimentation equilibrium was used to establish that G6PDH exists as monomer, dimer and tetramer entities. The molecular mass of the dimer is 102 kD. The second substrate of the reaction, $NADP^+$, affects the 2 dimers $\rightleftarrows$ 1 tetramer equilibrium in G6PDH solutions. The dimeric entities stable in a wide pH range and even in very diluted solutions, which is definitely defined by a fair reliability of the conformational lock stabilizing G6PDH dimer. The presence of $NADP^+$ and NADPH, unlike the second substrate G6P, causes the association of the active dimeric form of the enzyme to the tetrameric entity [3]. The work shows G6PDH dimeric form to contain two equivalent centers of $NADP^+$ binding with the dissociation constants $K_1 = 45$ μM and $K_2 = 13$ μM (pH 8.0, 27°C). Each dimer contains a hydrophobic region between the subunits-conformational lock, the distortion of which entails the dissociation of the active dimers to inactive monomers [3]. The same work reported that G6PDH monomers have a cylindrical shape 68×34 Å in size.

A detailed kinetic analysis for yeast G6PDH was reported [2, 4, 5]. The kinetics of the enzyme action obeys the Michaelis-Menten Law with an independent binding of substrates. At pH 8–9, $K_{\text{G6P}} = 3.5 \times 10^{-5}$ M and $K_{\text{NADP}} = 4 \times 10^{-6}$ M. $NADP^+$ analogs act on G6PDH thermal stability variously, which is explicable by various character of conformational changes upon binding of the analogs [6]. Hence, the analogs affect the reliability of the conformational lock and the capacity of the dimers to form tetramers. It was reported earlier [7] that at low temperatures, G6PDH is inactivated by the consecutive dissociation mechanism:

$$\underset{\text{inactive}}{E_4} \overset{k_2}{\underset{k_{-2}}{\rightleftarrows}} \underset{\text{active}}{2E_2} \overset{k_1}{\underset{k_{-1}}{\rightleftarrows}} \underset{\text{inactive}}{4E_1} \overset{k_d}{\rightarrow} 4E_d. \qquad (1)$$

Here $K_{\text{dis}}^{\text{I}} = \frac{k_2}{k_{-2}}$ and $K_{\text{dis}}^{\text{II}} = \frac{k_1}{k_{-1}}$.

The task of this research is to offer additional experimental results to confirm mechanism (1) and to kinetically analyze the stability of the conformational lock in G6PDH dimer.

Materials and Methods

Materials used: crystalline G6PDH extracted from yeast was a suspension in 3.2 M ammonium sulfate (Ferak, Berlin); $NADP^+$ monosodium salt and magnesium chloride (Russian production). The starting rate of the reaction was determined from kinetic curves for accumulation of NADPH (reaction product) at 340 nm on a spectrophotometer (Hitachi) in thermostatted cuvettes. The composition of the overall reaction mixture (2 ml) was 1.1 ml 0.2 M borate buffer, pH 8.0 + 0.4 ml enzyme of relevant concentration + 0.5 ml mixture of substrates containing

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9.4×10^{-4} M G6P, 1.44×10^{-3} M $NADP^+$ and 7.5×10^{-3} M $MgCl_2$.

Thermal inactivation was conducted in thermostatted vessels at 45–46 °C. In certain time intervals, aliquots (0.4 ml) of this mixture were taken to measure the enzymatic activity.

Results and Discussion

It was reported earlier [3] that G6PDH is an equilibrium mixture $E_4 \rightleftarrows 2E_2$, tetramers being inactive. The specific catalytic activity as a function of protein concentration in solution is a declining curve in the concentration range $[E_0]$ 0.5–18 nM (Fig. 1). It is notably simpler to define the association constant from the concentration dependence of the specific activity when one of the entities is inactive. At $a_4 = 0$ (with inactive tetramers), expression

$$\frac{1}{a} = \frac{1}{a_2} + \frac{2K_{\text{ass}}}{a_2^2 A} \tag{2}$$

holds. Here A and a are the overall and specific activities, respectively; a_2 is the specific activity of dimers. The experimental results should be linearized in the coordinates $1/a, A$. Using relationship (2), the obtained results were treated to derive the necessary parameters tabulated below.

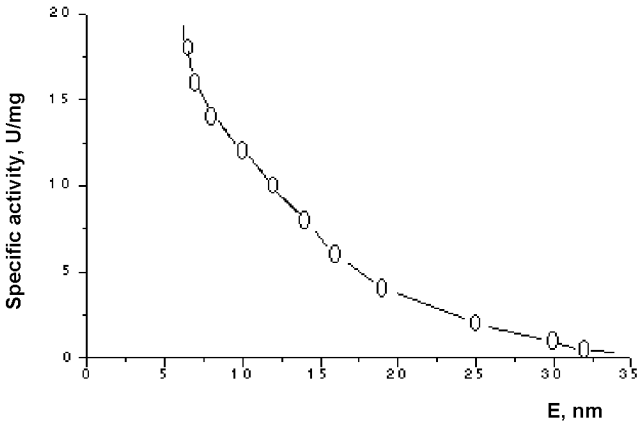

Fig. 1. Dynamics of G6PD relative activity after its thermal inactivation in 0.2 M borate buffer, pH 8.0, at 46 °C. $[E]_0 = 23$ nM.

Table 1

Kinetic parameters of G6PDH dissociative processes at various temperatures in 0.2M borate buffer, pH 8

t, °C	$K^{\text{I}}_{\text{dis}}$, nM	$K^{\text{II}}_{\text{dis}}$, nM	a_2, mol NADPH/min.mol G6PDH
20	0.71 ± 0.95	—	$(9.5 \pm 1) \times 10^{-5}$
46	24 ± 3	6.2 ± 0.4	$(13 \pm 0.1) \times 10^{-6}$

The kinetic curves of thermal inactivation in the coordinates "relative activity (b)—time (t)" have a maximum each. The physical sense of (b) value is simple: this is the ratio of the observed enzymatic activity for the initial protein concentration E_0 to the enzymatic activity for the same concentration provided all protein is in the active dimeric form. From this, at any $[E]_0$, $b \geq 1$. This curve has a maximum and describes process

$$\underset{\text{inactive}}{E_4} \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} \underset{\text{active}}{2E_2} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \underset{\text{inactive}}{4E_1}. \tag{3}$$

According to scheme (3), the intermediate active entity is dimer. At the first step of the process, the dissociation of tetramers increases the activity but emergence of inactive monomers entails its decrease. This fact hinders to calculate $K^{\text{I}}_{\text{dis}}$ at 46 °C. In this case, another procedure turns out useful: to represent the experimental results obtained at various initial protein concentrations in the coordinates of linear equation

$$\frac{1-b}{2b_{\max}} = \frac{1}{\gamma} + \frac{1}{K^2_{\text{dis}}}[E]_0 b_{\max}, \tag{4}$$

where b_{max} is the value of b in the maximum point; γ is the ratio of the constants k_2/k_1 (scheme (3)). Thus derived $K^{\text{I}}_{\text{dis}}$ value is given in the table. The method of approximated calculations supposes to define the ratio of the constants from the length of the intercept cut off on Y-axis. It was reported [7] that the dimeric entity of the protein in solution dissociates further. For this reason, it is possible to define the k_2/k_1 value for test system, where k_2 is the rate constant for decomposition of inactive tetramers to active dimers whereas k_1 is the rate constant for decomposition of active dimers to inactive monomers. According to the kinetic analysis thus conducted, $\gamma = k_2/k_1 = 14.3$. The value of k_1 was derived in the same conditions at $[E]_0 \ll K^{\text{I}}_{\text{dis}}$, i. e., in absence of tetramers. In this case, the kinetics of thermal inactivation is described by a declining curve which has a knee in semilogarithmic coordinates. The starting region of the curve is linearized in the coordinates of equation (5).

$$F(\nu) = 2\frac{\nu}{\nu_0} - \frac{1}{2}\left(\frac{\nu}{\nu_0}\right)^2 = \frac{3}{2} - k_1 t. \tag{5}$$

Neglecting the temperature difference 1 °C at the known value of $k_1(45\,^\circ\text{C}) = 0.035$ min^{-1}, it is easy to derive $k_2(46\,^\circ\text{C}) = 0.5$ min^{-1}. The computed analysis of scheme (3) showed that at $K^{\text{I}}_{\text{dis}} = 10K^{\text{II}}_{\text{dis}}$ and at the selected values $\gamma = 20$, 10, 5, and 1, respectively; the maximum appears only at $\gamma > 5$ whereas at $\gamma \leq 1$ the maxima were not observed on the kinetic curves. This analysis shows that when the constants are equal, it is impossible to kinetically discriminate two consecutive processes in scheme (3). Yet, the number of steps preceding the inactivation step is definable from the kinetic curves $\nu/\nu_0 \div 1$ (Fig. 2) on having drawn a tangent at the knee to its cross with Y-axis at the point $R = \delta + 1$. The value δ is related to the number of steps n through empirical expression as follows [8],

$$n = \frac{0.13 + \delta}{0.13 - 0.05\delta}. \tag{6}$$

Figure 2 shows that all tangents cross in one point $R = 1.22$. From this, derive $\delta = 0.22$, which corresponds to the minimal number of the ulterior steps $n = 3$. That is in scheme (1), there is an additional step of conversion of

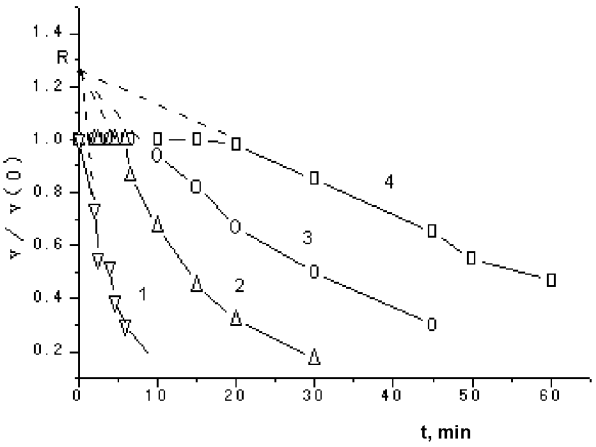

Fig. 2. Determination of the dimensionless relative lag period $\delta = R - 1$ from kinetic curves of G6PD thermal inactivation in 0.2 M borate buffer, pH 8.0, at 46 °C. $[E]_0 = 1$ nM (*1*); 6 nM (*2*); 9 nM (*3*), and 19 nM (*4*).

the active stable dimer to the active labile dimer capable of dissociation.

$$E_4 \overset{\text{I}}{\longleftrightarrow} 2E_{2\text{stab}} \overset{\text{II}}{\longleftrightarrow} 2E_{2\text{lab}} \overset{\text{III}}{\longleftrightarrow} 4E_1. \qquad (7)$$

From this, it follows that G6PDH dimer is fixed with two multiple-point contacts. Destruction of one of them entails the emergence of less stable isomer E_2. The conformational lock is located in the hydrophobic region of the cylindrical molecule of G6PDH protomer [3]. Evidently, the dimer is stabilized due to the presence of the conformational lock and the formation of tetramers which can be considered as a reserve for the native enzyme. The amount of this reserve is regulated by the second substrate of NADPH and its analogs [6]. Thus, this work has successfully showed the procedure for G6PDH stabilization and kinetically described the mechanism of the low-temperature inactivation of the enzyme, i. e., derived all kinetic parameters in scheme (1): $K^{\text{I}}_{\text{dis}}$, $K^{\text{II}}_{\text{dis}}$, k_1, k_2, and k_d. At 46 °C, $K^{\text{I}}_{\text{dis}} = (24 \pm 3)$ nM whereas $K^{\text{II}}_{\text{dis}} = (6.2 \pm 0.4)$ nM. This implies that at operative concentrations of the enzyme 1–19 nM and at this temperature, the concentration of tetramers can be neglected. The rate constant for dissociation of tetramers $k_2 = 0.5$ min^{-1}, which is higher by a factor than the rate constants of labile dimers $k_1 = 0.035$ min^{-1} whereas the rate constant of the irreversible inactivation (denaturation $k_d = 6 \times 10^{-3}$ min^{-1}. The stability of G6PDH dimers is provided by the conformational lock formed, at least, by two independent intersubunit contacts in the hydrophobic region of protomers. A similar structure of the conformational lock was disclosed kinetically and proved by structural studies for the dimer of alkaline phosphatase from *E. coli* [9].

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