MECHANISM OF THE THERMOINACTIVATION OF THE β-GALACTOSIDASE FROM *ESCHERICHIA COLI*

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The activity of oligomeric enzymes is sensitive to the formation of interprotein contact that make up the conformational lock. The mechanism for this is discussed in this article concerning the β-galactosidase from *Escherichia coli*. The thermoinactivation curve, obtained at 40°C, has induction period that may be ascribed to latent structural changes in conformational lock. The analysis of the kinetic curve has allowed us to calculate the minimum number of denaturation stages in the conformational lock ($n \cong 3$), i. e. tetramer becomes labile and capable of dissociation into two active dimers. These data support the kinetic scheme used to describe the dissociative inactivation of dimeric enzymes. The inactivation rate decreases in the presence of Mg^{2+}. As metal binding sequences are placed in domains 1 and 3, therefore, tetramer interglobular contacts stabilize each subunit. Formation of tetramer influences the activity of each subunit.

β-Galactosidase is the example of glycosidases. β-Galactosidase participates in lactose degradation. The enzyme catalyses lactose transformation into β-galactose and glucose. β-Galactosidase is metalloenzyme exhibiting broad substrate specificity [1]. The enzyme is a tetramer of four identical subunits. Within each subunit the 1023-amino acid polypeptide chain folds into five sequential domains plus an extended segment at the amino terminus. Each of the four active sites in the tetramer is formed by elements from two different subunits. The β-galactosidase from *Escherichia coli* is a large homotetrameric enzyme (MW = 465 412 Da). The active site of the molecule is possibly formed by the Mg ions therefore conformation changes in residues binding the ions would influence the enzyme activity.

The three types of interglobular tetramer contacts were studied according to procedure, proposed in [2]. The summary information concerning interactions between interglobular contacts and active site for each globule is presented in Table 1.

In interglobular interactions domains mainly involved domains 1, 3 and 5. As metal binding sequences are placed in domains 1 and 3, therefore, tetramer interglobular contacts stabilise each subunit and one way of activity regulation during some modification of interglobular regions is through domains' displacements. This way of regulation is characteristic for all three interglobular contact types. The other way, activity influence through displacement of adjacent to active site interglobular sequences is specific for AD-tetramer contact. Thus, formation of tetramer influences the activity of each subunit.

In accordance with this primary dissociation active tetramers on two active (and less stable) dimers must be connected with the relationship break-up AB and DC.

$$\begin{matrix} & \mathrm{A} & & & & & \\ \mathrm{B} & \times & \mathrm{D} & \rightleftarrows & \mathrm{AD} + \mathrm{BC} & \rightarrow & \mathrm{A} + \mathrm{B} + \mathrm{C} + \mathrm{D}. \\ & \mathrm{C} & & & & & \end{matrix}$$

catalytic active catalytic inactive

From kinetic data follows that dimers of β-galactosidase are catalytic active, but structural

Data speak of greater toughness of contacts AD and BC, near which are located important for the act of catalysis ions Mg^{2+}. The complex multipoint structure of contacts AB (and CD) allows multistage their destroying without the observable influence upon space removed active centres, situated near contacts AD and BC. This is according with the kinetic scheme (1), used for the thermodegradation description of β-galactosidase. It is less probable, but is principle possible the process of dissociation with primary break-up AD and BC contacts

Table 1

Active site and interglobular contact interactions in *Escherichia coli* β-galactosidase. A, B, C and D-globules [1].

Protein molecule site	Domain(s) involved	Overlap with metal binding sequence	Metal binding interdomain stabilisation
Mg 2002 binding	1, 3	—	—
Mg 2012 binding	1	—	—
Intg AB	3, 5	No	Yes
Intg AC	1, 2	No	Yes
Intg AD	1, (2), 3	Yes	Yes

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$$\begin{matrix} A \\ B \times D \\ C \end{matrix} \quad \rightleftarrows \quad AB + CD \quad \rightarrow \quad A + B + C + D.$$

catalytic active catalytic inactive

An example of such inactivation of β-galactosidase is given in [3].

Study of processes of thermoinactivation under more low temperatures (55–61 °C) is more informative. In this interval of temperature thermal inactivation may occur via dissociative inactivation according to the kinetic scheme:

$$E_4 \rightarrow E'_4 \rightarrow \ldots \rightarrow 2E_2 \rightarrow 4E_1 \rightarrow 4E_d, \qquad (1)$$

where E_4 is stable tetramer, E'_4 and E_2 are active intermediate forms (labile tetramer and active dimer), E_1 is inactive monomer, and E_d is a denatured monomer. Presence of dimer and labile tetramers in the process of thermoinactivation of β-galactosidase is discovered in work [3]. When raising a temperature to separate stages of scheme (1) become kinetic indistinguishable, but kinetic process is described by first-order equation. Kinetic study of scheme (1) enables to define from experimental data efficient constants of velocity of inactivation. It is clear, that the real mechanism of dissosiative inactivation have turned out to be complex. The inactivation curves shown in Figs. 1 and 2 are observed for oligomeric enzyme—β-galactosidase. For this enzyme at 40 °C some induction period is observed during thermal inactivation. This is illustrated in Fig. 1 by kinetic curve *1*. It is important that in this case only one quantity is to be additionally determined from experiment—the induction period (latency period) and in scheme (1) there is unknown number of conversion steps with unknown number of rate constants for reciprocal transitions. Thus, this problem has no conclusive solution, and it cannot be solved only by the methods of chemical kinetics. In this case, other information must be added to kinetic analysis of inactivation, for example the X-ray data on the spatial structure of dimeric enzymes, which give additional information on the state of protein before its dissociation.

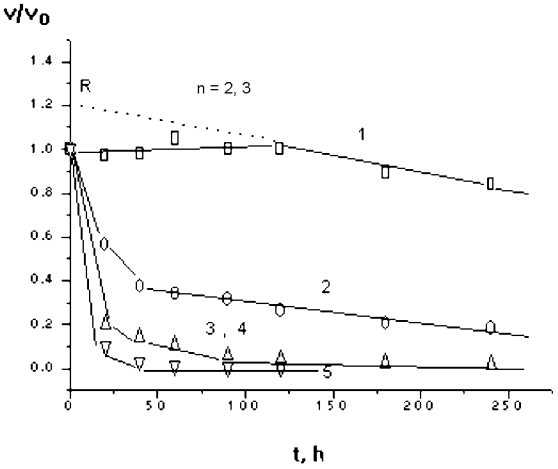

Fig. 1. Kinetics of thermal inactivation of β-galactosidase for different temperatures at pH 7.5: *1*, 40 °C; *2*, 45 °C; *3*, 48 °C; *4*, 50 °C, and *5*, 52 °C.

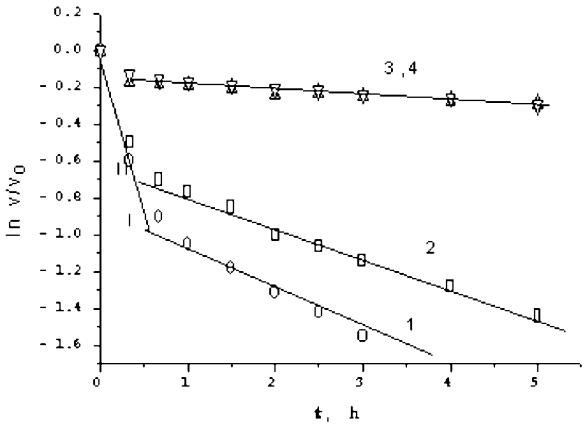

Fig. 2. Kinetic of thermoinactivation of β-galactosidase at pH 7.5 and 48 °C and various concentrations of enzyme: (*1, 3*) 36.55 mg/l and (*2, 4*) 7.3 mg/l; (*1, 2*) in absence of Mg^{2+} ions and (*3, 4*) in the presence 0.01 M of Mg^{2+}.

The kinetic problem has also turned out to be more definitive. It has been found that a limiting value of the number of possible intermediate steps of protein conversion can be determined. A simple approximate relation for practical calculations has the form [4]:

$$n = (0.13 + \delta)/(0.13 - 0.05\delta),$$

where n is a certain quantity that describes the irreversible transitions of n enzyme oligomeric forms with similar rate constants (equal to k_1), $R = (1 + \delta)$ is the ordinate intercept produced by a tangent to the inflection point of the kinetic curve in Fig. 1, and δ is a dimensionless parameter used to calculate a minimal number of active intermediate enzyme forms. This makes it possible to estimate the minimal number of step protein conversion for dissociation into catalytically inactive monomers. It has been found $n = 2.9$, as illustrated in Fig. 1. Within the error of calculation it means, that it may be three active forms in scheme (1)

$$E_4 \rightarrow E'_4 \rightarrow E_2 \quad \text{or} \quad E_4 \rightarrow E_2 \rightarrow E'_2.$$

Here E_4 is stable tetramer, E'$_4$ is labile tetramer ABCD and E_2 and E'_2 is stable and labile any active dimers AD or BC. Based on kinetic data, it is impossible to judge either the real number of intermediate active forms of an enzyme or of the nature of organisation of the conformational lock. Here, an alternative arises that cannot be understood without appealing to the data on a real spatial structure of β-galactosidase. This alternative is the following: whether the conformational lock opens through two steps within one spatial site of interprotein contact AD–BC, or there are two such sites within interprotein contact A–D (or B–C).

Thermal inactivation of β-galactosidase at 45 °C, 48 °C, 50 °C, and 52 °Cas have shown in Fig. 1 curves *2–5* does not proceed through an induction period. A specific feature of these kinetic curves plotted in co-ordinates of first-order equation is the presence of inflection points. As seen from Fig. 2, in fact these inflection points I and II are the intercepts of asymptotes to a continuous straight line. It is not difficult to determine the inflection points in experiments. Position of inflection points and the slopes

of curves in coordinates of first-order kinetic curve allow us to determine three elementary constants of kinetic scheme (2)—degradation of dimeric protein E_2,

$$E_2 \Leftarrow K_{dis} \Rightarrow 2E_1 — k_d \rightarrow 2E_d. \quad (2)$$

Here E_1 is a monomeric protein capable of a reversible restoration of initial structure and E_d is the product of an irreversible changing of E_1. Kinetic scheme (2) has no explicit analytical solution, because in addition to two monomolecular processes, it involves a bimolecular stage (k_{-1}). However, chemical kinetics has available approximation methods develop to describe complex reactions. For a zero approximation, the description of one process is used. At the initial moment of time ($t = 0$) the single occurring process is dissociation with the rate constant k_1. It is described by first-order equation. The slope of the straight line plotted in linear co-ordinates of first-order equation ($\ln[E_2]$ against t) gives value of constant k_1: 3.6×10^{-5} s^{-1} (45 °C); 1.3×10^{-4} s^{-1} (48 °C and 50 °C) and 3.3×10^{-4} s^{-1} (52 °C).

An approximate determination of k'_d, the constant for inactivation of native monomer capable of reversible association may be carried out for quasi-equilibrium concentrations of intermediate form E_1. The rate constant determined by the slope of the straight line at $t > \tau$, in the point $t = \tau$ (inflection point) is related to the elementary rate constant k_d that characterises of native monomeric protein as follows:

$$k_d = k'_d(v_0 + v\tau)/2(v_0 - v\tau),$$

where k'_d is the effective rate constant determined by slope of the straight line at $t > \tau$: (Fig. 2) 1.3×10^{-4} s^{-1} (36.5 mg/l protein) and 1.5×10^{-4} s^{-1} (7.3 mg/l protein). In the presence of Mg^{2+} ions k'_d is 3.8×10^{-5} M and does not depend on the protein concentration (Fig. 2, curves *3* and *4*). The inactivation rate decreases in the presence of Mg^{2+}. In interglobular interactions domains mainly involved domain 1, 3 and 5. As metal binding sequences are placed in domains 1 and 3, therefore, tetramer interglobular contacts stabilise each subunit. Formation of tetramer influences the activity of each subunit.

References

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