Theoretical Study of Discrimination of Mechanisms of Thermal Inactivation of Urease by Monitoring its Oligomeric Composition*

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The possibility to discriminate the three-step mechanisms of thermal inactivation of jack bean urease was analyzed, the models being suggested in a previous study from the activity data. The simulations of the concentration courses of individual structural forms of urease incorporated in the mechanisms were performed. The results showed that all mechanisms could be discriminated if the dynamics of the evolution of oligomeric structural forms of urease was monitored. A careful choice of temperatures and combination of several analytical techniques is, however, required to achieve a reliable discrimination of the inactivation mechanism.

The quaternary structure, a three-dimensional arrangement, has some important biological functions in most enzymes such as forming active sites (directly or indirectly), providing stability or increasing solubility. It was reported recently that the lack of catalytic activity of oligomers is accompanied not only by the changes of the tertiary structure, but also by the dissociation of subunits [1]. Despite the fact that many dissociated subunits are inactive (possibly due to extreme conditions), some enzymes exist the subunits of which are more active than the oligomer [2]. For example, malate dehydrogenase dissociated in dependence on Mg^{2+} concentration and pH where tetrameric, dimeric as well as monomeric forms were active [3].

According to *Poltorak* and *Chukhray*, oligomeric enzymes are stabilized by a "conformational lock" due to the interaction of individual subunits [1]. In the first step of inactivation, the intersubunit contact is broken, rendering less stable but fully active enzyme subunits. Then, whatever enzyme may undergo, a number of distinct inactivation pathways (some of which may be irreversible) occur. These pathways may be linked through interconversion of the native states of the enzyme. It means that multisubunit proteins exhibit a complex inactivation pattern due to the changes in the quaternary structure of enzyme molecules.

Various physicochemical methods can be applied for the study of the above-mentioned changes in the quaternary structure. The most important ones are electrophoresis, chromatographic analysis, and light scattering. Size exclusion chromatography (SEC) was applied for the analysis of intermediates of lysozyme refolded by a different concentration of urea [4]. On the basis of comparison of the differences in hydrodynamic characteristics (Stokes radius and hydrodynamic volume), the authors proposed an appropriate refolding mechanism. Several publications have also dealt with the use of electrophoresis different nature for the detection of unfolding intermediates.

The native polyacrylamide electrophoresis (PAAG) was helpful to the identification of an intermediate active state and an inactive state of butyl cholinesterase at pressure and/or temperature inactivation. The existence of the intermediate accounted for the nonlinearity of the inactivation kinetics and the general expanded Lumry—Eyring model of inactivation could be proposed [5]. Another experimental method of great potential is light scattering that is used at the investigation of the homo- and heteroassociation of proteins and other biological macromolecules. The aggregation during the thermal inactivation of cutinase was investigated by dynamic light scattering (DLS) and detailed information on the changes in the aggregate size was used to propose an appropriate inactivation mechanism [6]. The fractionation of the polypeptide mixture immediately followed by a direct determination of the molecular mass of each fraction can be achieved by coupling the light scattering method with gel chromatography. This method is considerably more accurate than the molecular mass estimation based on the elution volume [7].

Several authors declared that the inactivation of

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Table 1. Three-Step Mechanisms of Inactivation of Urease

Model No.	Mechanism			Rate equations		
1	$N_6 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} 2D_3$	$2D_3 \xrightarrow{k_2} I_6$	$2N_6 \xrightarrow{k_3} I_{12}$	$r_1 = k_{+1}c_{N_6} - k_{-1}c_{D_3}^2$	$r_2 = k_2 c_{D_3}^2$	$r_3 = k_3 c_{N6}^2$
2	$N_6 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} 2D_3$	$D_3 \xrightarrow{k_2} I_3$	$2N_6 \xrightarrow{k_3} I_{12}$	$r_1 = k_{+1}c_{N_6} - k_{-1}c_{D_3}^2$	$r_2 = k_2 c_{D_3}$	$r_3 = k_3 c_{N6}^2$
3	$N_6 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} 2D_3$	$D_3 \xrightarrow{k_2} I_3$	$2D_3 \xrightarrow{k_3} I_6$	$r_1 = k_{+1}c_{N_6} - k_{-1}c_{D_3}^2$	$r_2 = k_2 c_{D_3}$	$r_3 = k_3 c_{D3}^2$

N_6 – native hexamer, D_3 – reversibly denatured trimer, I_3, I_6, I_{12} – irreversibly inactivated trimer, hexamer, and dodecamer.

native hexamer of urease was accompanied by marked changes in the quaternary structure when the enzyme was inactivated by different chemical and physical denaturants [8—10]. This enzyme can undergo dissociation from hexamer to an active trimer and further into six monomers [9, 10]. *Hirai et al.* demonstrated that the dissociation of urease molecule into small subunits, induced by sodium dodecyl sulfate (SDS), was manifested by the decrease of urease maximum diameter detected by light scattering [9]. Gel electrophoresis and the analytical centrifugation were used for the identification of the dissociation product of urease in the presence of propandiol or glycerol [11]. Both the electrophoretic mobility and the sedimentation velocity of such products were consistent with the molecular mass of the trimer. A very sensitive silver activity staining of urease in PAAG gel can lead to another improvement of the differentiation of individual inactivation products [12, 13]. The occurrence of intermolecular interaction can be deduced from some studies dealing with the thermal and chemical denaturation of urease. *Contaxis* and *Reithel* have shown that active trimer species can also associate to form an inactive hexamer [14]. The conformational changes of the thermal unfolding of urease detected by fluorescence spectroscopy showed that at 80 °C urease visibly aggregated [15].

The main goal of this paper was to analyze the possibility of the discrimination of three-step mechanisms of the thermal inactivation of urease, which were suggested for the description of the experimental data published in our previous paper [16]. For this purpose, simulation calculations of concentrations of individual reaction forms were performed for all mentioned mechanisms at different temperatures. These simulation results were discussed in connection with different structural methods that allowed the information about undergoing structural changes during the inactivation of urease.

EXPERIMENTAL

The simulation of the course of concentration of individual reaction forms was based on the models in which the kinetic equations were derived directly from different three-step inactivation mechanisms presented in Table 1. A set of differential equations together with the enthalpy balance, describing the initial heating period during the thermal inactivation of urease, was used for the formulation of a mathematical model in the following general form

$$\frac{\mathrm{d}c_i}{\mathrm{d}t} = \sum_{j=1}^{3} \nu_{ij} r_j (c_{N6}, c_{D3}, c_{I3}, c_{I6}, c_{I12}, T) \qquad (1a)$$

$$\frac{\mathrm{d}T}{\mathrm{d}t} = K(T_B - T) \qquad (1b)$$

$$t = 0 \quad c_{N_6}/c_{N0} = 1 \quad c_{I6,I12} = 0$$
$$T = 293.15\ \mathrm{K} \quad T - T_B = 30\ \mathrm{K} \qquad (1c)$$

ν_{ij} is the stoichiometric coefficient of the i-th enzyme form in the j-th reaction step, r_j is the rate of the j-th reaction step, $c_{N6}, c_{D3}, c_{I3}, c_{I6}, c_{I12}$ are the molar concentrations of the native, reversibly denatured and irreversibly denatured enzyme forms, where the individual subscripts define the number of subunits. All concentrations were related to the initial molar concentration of the active enzyme form c_{N0}. T_B is the bath temperature and K ($K = 4.44 \times 10^{-2}\ \mathrm{s}^{-1}$) is a proportionality factor including the overall heat transfer coefficient estimated from the heat transfer experiments [16]. Eqn (*1a*) includes the concentration of hexamer N_6 and trimer D_3, two most frequently described levels of quaternary structure of urease [17]. The occurrence of products of intermolecular interactions (I_6, I_{12}) can be inferred from some studies dealing with the inactivation of oligomers [10]. The software Athena Visual Workbench (Stewart & Associates Engineering Software, Madison, Wisconsin, USA) was used for the calculation of the courses of individual reaction forms from the simulation models.

RESULTS AND DISCUSSION

Fig. 1 shows the selected inactivation profiles from our previous work where the thermal inactivation of jack bean urease in the temperature range from 55 °C to 85 °C was investigated [16]. It was shown that the

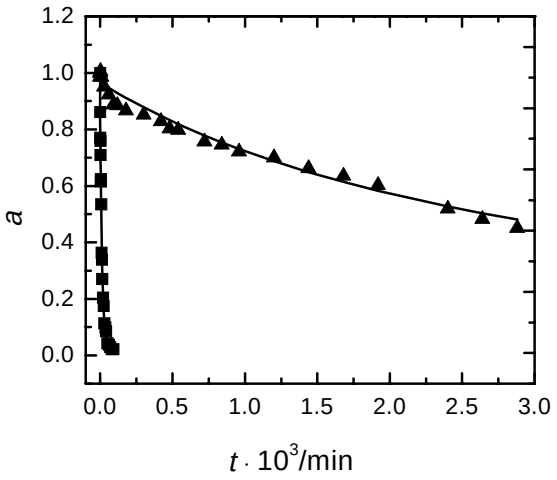


Fig. 1. The illustration of the urease inactivation courses and their fits using Model 1. Triangles represent the values of relative activity at 55 °C, the squares are the values of relative activity at 85 °C, and solid lines are the fitted curves.

inactivation at all investigated temperatures exhibited a biphasic pattern and the choice of all tested mechanisms was mainly influenced by the expected changes in the quaternary structure. The quality of the fit of experimental data by model 1 (Table 1) at 55 °C and 85 °C is illustrated in Fig. 1. Table 1 presents the three-step mechanisms selected in the final screening carried out in our previous work. The inactivation of urease could be identified as a three-step process consisting of the dissociation of the native form of enzyme in the first step, and two parallel reactions, where at least one of them was the association reaction of the native or denatured form. While mechanisms 1 and 2 are typical mechanisms with the association reaction of the native form, in mechanism 3, smaller oligomers produced by the denaturation reaction associate subsequently. The mean errors of the fitted activity using the above-discussed models were very similar (they varied from 0.036 for model 1 to 0.0425 for model 3). This fact confirmed that on the basis of the statistical evaluation used, it was not possible to discriminate the mentioned three-step mechanisms.

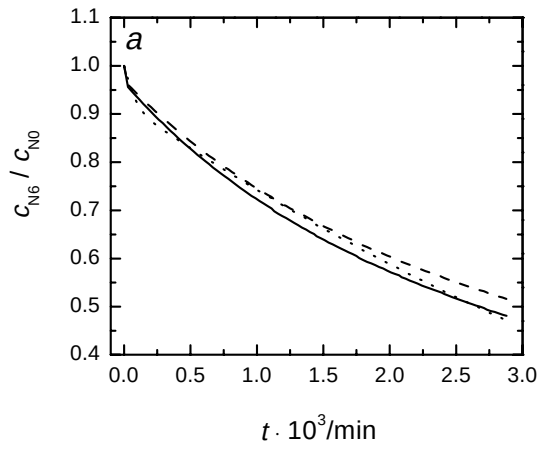
In order to improve the analysis of the inactivation mechanism of urease, the concentration courses of individual reaction forms were simulated for all three discussed mechanisms using previously calculated parameters (the rate constants and activation energies) (Table 2). Fig. 2 shows the simulation results for the boundary values of temperatures at which the urease inactivation was investigated. Figs. 2*a* and 2*b* present the concentration profiles of the native form N_6 at two temperatures for all three investigated mechanisms. It is evident that the mechanisms did not differ in the courses of the native form. Relatively small differences were also observed at the denatured form D_3 (Figs. 2*c* and 2*d*). Although, at 55 °C the relative concentration of denatured form D_3 at mechanism 3 was visibly higher than those at mechanisms 1 and 2 (Fig. 2*c*), this difference was not large in absolute concentrations. For that reason, the resolution of the mentioned mechanisms on the basis of denatured form by different structural methods would be difficult.

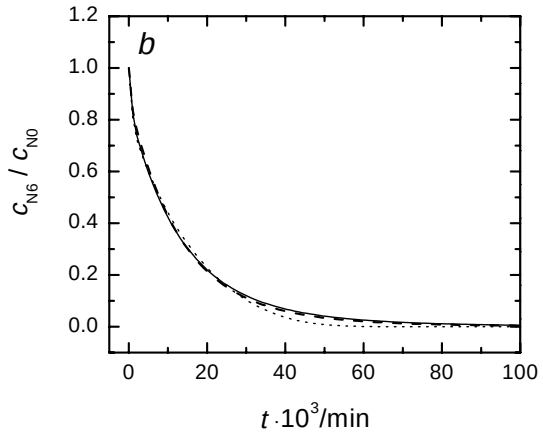
On the contrary, the differences in the courses of inactivated dodecamers for mechanisms 1 and 2 were significantly larger (Fig. 2*j*) and they could eventually be distinguished using the structural methods mentioned in the introduction. The difference of 16 % in the relative concentrations of the dodecamer at mechanisms 1 and 2 would be observable as a visible discrepancy in the intensity of light scattering when the methods of static and dynamic light scattering are applied. The best discrimination of mechanisms 1 and 2 could, however, be achieved if the concentration dependences of irreversibly inactivated forms I_6 and I_3 were compared. In the case of mechanism 1, the irreversibly inactivated hexamer (oligomer with the comparable number of subunits as the native form) could be a dominant form at higher temperatures (Fig. 2*f*).

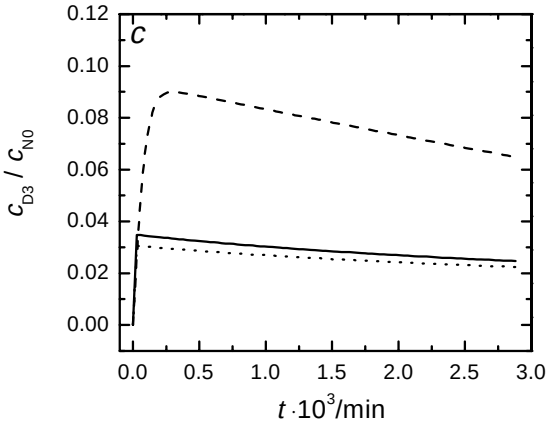
Table 2. The Values of the Estimated Parameters of all Three-Step Models together with their 95 % Confidence Intervals

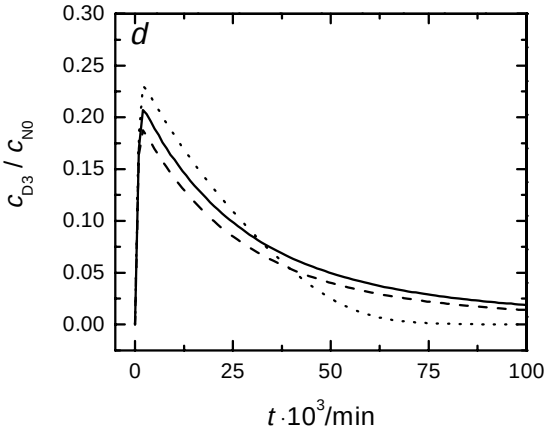
Parameter	95 % Confidence interval		
	Mechanism 1	Mechanism 2	Mechanism 3
$k_{+10}/(\mathrm{min}^{-1})$	$(8.51 \pm 0.29) \times 10^{-1}$	$(3.04 \pm 1.52) \times 10^{1}$	$(0.47 \pm 0.19) \times 10^{-2}$
$k'_{-10}/(\mathrm{min}^{-1})$	$(8.36 \pm 0.28) \times 10^{1}$	$(3.58 \pm 0.12) \times 10^{3}$	$(7.61 \pm 4.81) \times 10^{-2}$
$k'_{20}/(\mathrm{min}^{-1})$	$(1.74 \pm 0.79) \times 10^{-2}$	$(0.93 \pm 0.43) \times 10^{-3}$	$(1.88 \pm 0.43) \times 10^{-3}$
$k'_{30}/(\mathrm{min}^{-1})$	$(0.24 \pm 0.04) \times 10^{-3}$	$(0.35 \pm 0.03) \times 10^{-3}$	$(3.25 \pm 1.16) \times 10^{-3}$
$E_{\mathrm{a},+1}/(\mathrm{kJ\ mol}^{-1})$	373 ± 21	281 ± 23	278 ± 51
$E_{\mathrm{a},-1}/(\mathrm{kJ\ mol}^{-1})$	247 ± 4	138 ± 12	233 ± 62
$E_{\mathrm{a}2}/(\mathrm{kJ\ mol}^{-1})$	116 ± 23	200 ± 20	13 ± 2
$E_{\mathrm{a}3}/(\mathrm{kJ\ mol}^{-1})$	93 ± 28	153 ± 12	194 ± 21

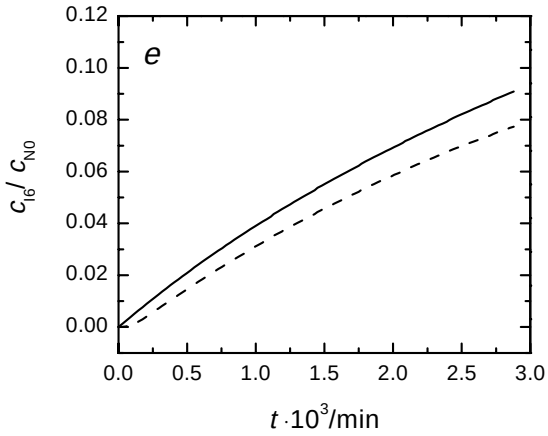
k_{i0}, k'_{i0} – rate constants at the reference temperature of 70 °C, $E_{\mathrm{a}i}$ – activation energies. The true rate constants k_{-1}, k_2, and k_3 contained the dimension of reciprocal concentration, so they could be estimated from the dimensionless form of the material balances. For that reason, the model parameters were the modified rate constants k'_{-1}, k'_2, and k'_3. The relationship between the true and modified rate constants was $k'_i = k_i c_{\mathrm{N}0}$.

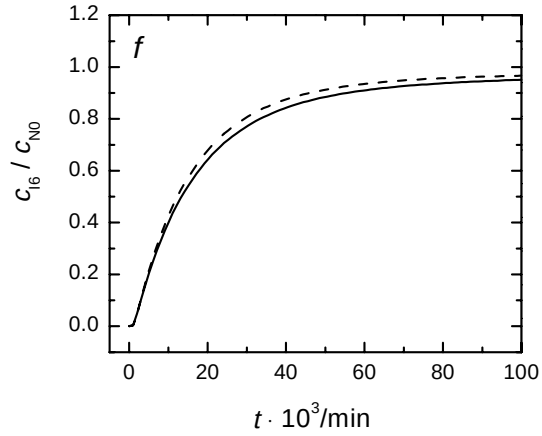

a
c_{N6} / c_{N0}
$t \cdot 10^3$/min

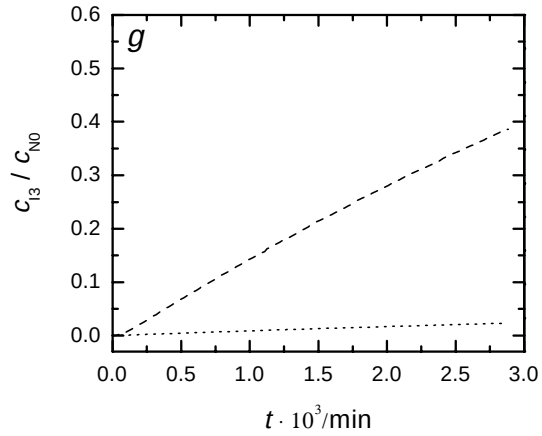

b
c_{N6} / c_{N0}
$t \cdot 10^3$/min

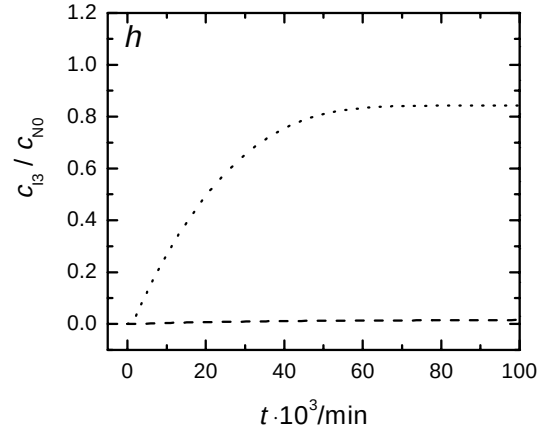

c
c_{D3} / c_{N0}
$t \cdot 10^3$/min


d
c_{D3} / c_{N0}
$t \cdot 10^3$/min


e
c_{I6} / c_{N0}
$t \cdot 10^3$/min


f
c_{I6} / c_{N0}
$t \cdot 10^3$/min


g
c_{I3} / c_{N0}
$t \cdot 10^3$/min


h
c_{I3} / c_{N0}
$t \cdot 10^3$/min

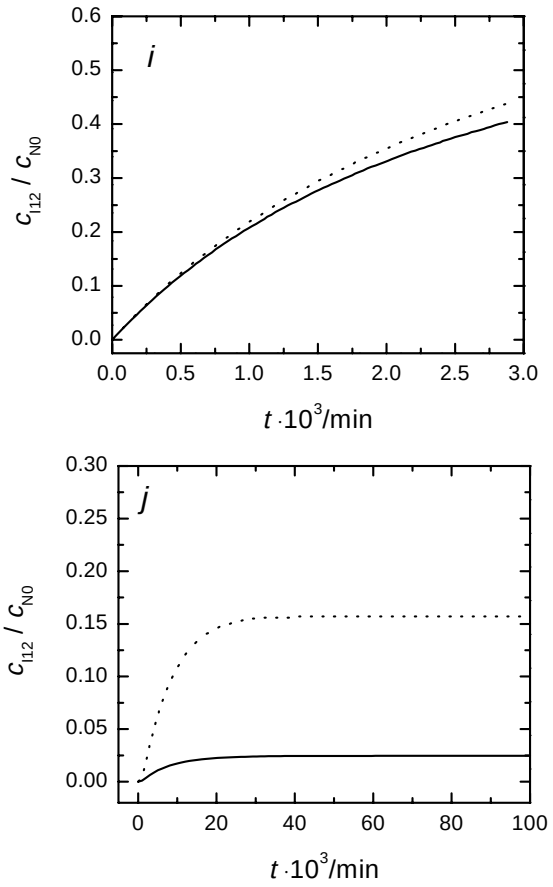


Fig. 2. The simulated concentration (relative to the initial value) courses of individual enzyme forms at 55 °C and 85 °C for all three-step models described in Tables 1 and 2. Native enzyme form N_6 at 55 °C (*a*) and 85 °C (*b*), denatured intermediate D_3 at 55 °C (*c*) and 85 °C (*d*), product of association reaction of the denatured form I_6 at 55 °C (*e*) and 85 °C (*f*), inactivated intermediate I_3 at 55 °C (*g*) and 85 °C (*h*), product of association reaction of the native form I_{12} at 55 °C (*i*) and 85 °C (*j*). The lines represent the simulation profiles for the following mechanisms: mechanism 1 (solid line), mechanism 2 (dotted line), mechanism 3 (dashed line).

Model 2, on the contrary, predicted the dominance of the irreversibly inactivated dissociated forms that could be trimer or monomer eventually (Fig. 2*h*). The products of the second reaction step in mechanism 1 and mechanism 2 (inactivated hexamer or trimer) could be distinguished by PAAG or SEC methods where inactivated hexamer would reach a lower electrophoretic mobility or lower elution volume, respectively, in comparison with the inactivated trimer.

Unlike in the previous case, the mechanisms 1 and 3 can be distinguished only at a low temperature. While the product of association reaction I_{12} became significant for mechanism 1 (Fig. 2*i*), in the case of mechanism 3, the inactivated trimer was the dominant enzyme form (Fig. 2*g*). At a temperature of 85 °C, the prevailing form was the irreversibly inactivated hexamer, but no visible discrepancies in its courses at mechanisms 1 and 3 were observed. Mechanisms 2 and 3 could be distinguished at low as well as at high temperatures. The associated inactivated form (Fig. 2*i*) and inactivated trimer (Fig. 2*g*) at mechanisms 2 and 3, respectively, were prevailing at 55 °C. At the temperature of 85 °C, it was predicted at mechanism 2 that urease lost more than 80 % of the total activity due to the denaturation reaction and the inactivated trimer I_3 could be a dominant enzyme form (Fig. 2*h*). Model 3, on the contrary, predicted the dominance of the association reaction of the denatured form where the content of I_6 could be as high as 95 %.

It seems from the simulation experiments that the observed differences in the dynamics of the individual structural forms could be used for the discrimination of inactivation mechanisms. These simulation results together with the knowledge of undergoing structural changes associated with the inactivation process can be useful for the determination of an appropriate mechanism. However, it would be difficult to improve the analysis of an unknown inactivation system by the application of a sole method. The employment of several analytical techniques significantly enhances the quality and reliability of the verified mechanism.

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SYMBOLS

a	relative activity	
c_{N6}	molar concentration of the native enzyme form	mol m^{-3}
c_{D3}	molar concentration of the denatured enzyme form	mol m^{-3}
c_{I3}, c_{I6}, c_{I12}	molar concentrations of irreversibly denatured enzyme forms	mol m^{-3}
c_{N0}	initial molar concentration of active enzyme form	mol m^{-3}
D_3	reversibly denatured trimer	
E_{ai}	activation energy of the i-th reaction	kJ mol^{-1}
I_3	irreversibly inactivated trimer	
I_6	irreversibly inactivated hexamer	
I_{12}	irreversibly inactivated dodecamer	
i	total number of enzyme forms	
j	total number of reaction steps	
K	proportionality factor including the overall heat transfer coefficient	s^{-1}
k_{+1}	rate constant of forward reaction in the first reaction step	min^{-1}
k_{-1}	rate constant of backward reaction in the first reaction step	m^3 mol^{-1} min^{-1}
k_2	rate constant of the second reaction step (in mechanism 1)	m^3 mol^{-1} min^{-1}
k_2	rate constant of the second reaction step (in mechanisms 2 and 3)	min^{-1}

k_3	rate constant of the third reaction step	$m^3\ mol^{-1}\ min^{-1}$
k_{i0}	rate constants of the i-th reaction at $T_0 = 343.15$ K	min^{-1}
k'_{-1}, k'_2, k'_3	modified rate constants, $k'_i = k_i c_{N0}$	min^{-1}
N_6	native hexamer	
r_j	the rate of the j-th reaction step	$mol\ m^{-3}\ min^{-1}$
r_1, r_2, r_3	the rates of the individual reaction steps	$mol\ m^{-3}\ min^{-1}$
T_B	bath temperature	K
T	temperature	K
ν_{ij}	the stoichiometric coefficient of the i-th enzyme form in the j-th reaction step	

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