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Transient model of thermal deactivation of enzymes

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Abstract

The kinetics of enzyme deactivation provide useful insights on processes that determine the level of biological function of any enzyme. *Photinus pyralis* (firefly) luciferase is a convenient enzyme system for studying mechanisms and kinetics of enzyme deactivation, refolding, and denaturation caused by various external factors, physical or chemical by nature. In this report we present a study of luciferase deactivation caused by increased temperature (i.e., thermal deactivation). We found that deactivation occurs through a reversible intermediate state and can be described by a Transient model that includes active and reversibly inactive states. The model can be used as a general framework for analysis of complex, multiexponential transient kinetics that can be observed for some enzymes by reaction progression assays. In this study the Transient model has been used to develop an analytical model for studying a time course of luciferase deactivation. The model might be applicable toward enzymes in general and can be used to determine if the enzyme exposed to external factors, physical or chemical by nature, undergoes structural transformation consistent with thermal mechanisms of deactivation.

Keywords

enzyme; deactivation; denaturation; heating; protein

1. INTRODUCTION

Mechanisms of enzyme deactivation by increasing temperature and various physical factors are complex and have not been completely understood. Traditionally a two-state model has been used to explain thermal deactivation of enzymes caused by denaturation [1–3]. The two-state model postulates an active enzyme form that irreversibly transforms into a denatured form upon heating. Yet, various experimental data show that the effect of temperature on enzymes cannot be adequately explained in terms of a two-state model that assigns the decrease of enzyme activity to irreversible denaturation [4–5]. The possibility for reversible enzyme denaturation has been discussed and recently an Equilibrium Model has been proposed to provide a quantitative explanation of the thermal behavior of enzymes [6–9].

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Transient model of thermal enzyme deactivation
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The Equilibrium Model introduces an inactive intermediate state which is in rapid equilibrium with the active state of the enzyme. This inactive intermediate simultaneously denatures irreversibly, albeit at a slower rate. It was hypothesized that the difference between the active and inactive states is due to small structural changes in the enzyme active site. The authors of the Equilibrium Model suggest that this model can represent a universal mechanism independent of the reaction or structure of the active site by which enzymes lose activity with an increase in temperature. In addition to two parameters of the “classical” model for enzyme activity, ΔG^*_{cat} and $\Delta G^*_{\text{inact}}$, the Equilibrium model introduces two new parameters ΔH_{eq} and T_{eq} , which are respectively the enthalpy of the equilibrium and temperature at the mid-point of the equilibrium.

Using a series of estimated thermodynamic parameters, the Equilibrium model has been successfully used for modeling enzyme activity as a function of temperature and exposure duration for a number of enzymes [9]. However, for some enzymes establishing a near-equilibrium condition between the active and inactive but not denatured enzyme states may not be an instant process. In such cases a complex, multiexponential decline of enzyme activity vs. time can be observed contrary to a simple single-exponential decline expected under the framework of Equilibrium model. Firefly luciferase, which is broadly used as a detection system in numerous biological assays based on ATP detection [10–11], is an important example of such an enzyme.

In this study we investigated time dependence of *Photinus pyralis* (firefly) luciferase activity at different temperatures as well as the recovery of activity following a period of heating. A transient model that does not depend upon an assumption of an initial equilibrium between the active and inactive states was developed. Firefly luciferase catalyzes the conversion of luciferin to oxyluciferin, with the concurrent emission of light. The emitted light is readily detectable with photon-counting luminometers having high sensitivity and broad dynamic range (e.g. 5 decades for the Orion II luminometer used in this study). Not only is luciferase activity readily measurable in a broad dynamic range with high sensitivity, luciferase activity can be measured continuously over time in response to various conditions making luciferase an excellent model enzyme to investigate deactivation. In addition to its broad use in gene reporter assays and in various bioanalytical assays (ATP detection down to attomole concentrations are possible), firefly luciferase was also used to study protein refolding as well as the effects of MHz electromagnetic radiation on biological systems [12–14]. Since luciferase is a fairly “typical” monomeric enzyme, detailed understanding of mechanisms of luciferase thermal stability will advance specific applications and can contribute to general understanding of mechanisms that affect stability of this and other enzymes under various conditions.

2. MATERIALS AND METHODS

2.1. Luciferase preparation and activity measurement

For all experiments, *Photinus pyralis* (firefly) luciferase was purchased from Sigma (Sigma-Aldrich, Cat. No. FLAA). The enzyme was diluted to 5 nM in 0.1 M Tris-acetate pH 7.7, 2 mM EDTA, 10 mM magnesium acetate, 0.1% BSA, 1 mM dithiothreitol, 0.4 mg/mL PVP, and 0.4 mM D-luciferin. Further dilution was used as required for maintaining luminescence signal within the dynamic range of luminometer. The luciferase concentration used in these studies was sufficiently low to minimize complicating aggregation reactions [13]. The luciferase solution was kept on ice until used. Luminescence detection was initiated by adding ATP to a final concentration of 40 μM (FLAA kit, Sigma-Aldrich) unless otherwise noted. Luminescence was recorded on an Orion II microplate luminometer (Berthold Detection Systems, GmbH, Pforzheim/Germany) equipped with a computer-controlled microplate temperature control unit.

2.2. Preliminary control experiments and results

Experimental conditions used in this study were typical for luciferase-based assays [15–16]. A number of preliminary tests were performed to ensure that observed effects are caused by enzyme deactivation and not due to exhaustion of ATP substrate, enzyme inhibition by reaction byproducts, or slow heating of samples. To check for ATP exhaustion and possible enzyme inhibition by reaction byproducts a fresh stock of luciferase was added to the sample in which enzyme activity had ceased after exposure to high temperature. After addition of fresh luciferase we observed signal restored to the original luminescence intensity indicating no exhaustion of ATP substrate and no substantial accumulation of reaction byproducts over the course of 15 min. To ensure that slow heating of samples cannot be responsible for the observed kinetics of luciferase deactivation, two samples were prepared in parallel on a pre-heated microplate. One sample was prepared by mixing 2 μL of enzyme solution at room temperature and 10 μL of reaction buffer pre-heated to the target temperature in the range of 25–45 $^{\circ}\text{C}$. In this preparation method the enzyme's temperature rises as quickly as the two solutions are mixed. Another sample was prepared by adding 12 μL of pre-mixed buffer and enzyme solution at room temperature to a well on the pre-heated microplate. Recording of luminescence started in approximately 5 s after loading the microplate into the luminometer. Luminescence was recorded as a function of time for each sample. Although it can be expected that the second sample would take longer to reach the target temperature, no statistically significant difference in kinetics was observed. This fact indicates that for small reaction volumes samples (<12 μL) the target temperature is reached before measurements can be started. In these studies, unless specified otherwise, low concentration of enzyme ($\leq 5 \text{ nM}$) and low concentration of ATP substrate ($\leq 40 \mu\text{M}$) was used to minimize effects of enzyme inhibition by reaction byproducts. Measurements were carried out for 15 min, during which time no ATP exhaustion was observed as shown by near-constant luminescence in control samples recorded at room temperature. The independence of the kinetics of enzyme deactivation with respect to the concentration of ATP substrate was verified by recording deactivation kinetics at ATP concentration below and above the K_m value for luciferase, as illustrated in Fig 1. The literature reports for the K_m value of *Photinus pyralis* varies widely possibly due to differences in buffers used and possible luciferase inhibition by reaction byproducts at high ATP concentration [17–21]. The $K_m = 19 \mu\text{M}$ at our experimental conditions was measured by detecting luminescence intensity at various ATP concentration. Subsequently, the $K_m = 19 \mu\text{M}$ was used as a criterion of high and low ATP substrate concentration. Luciferase deactivation kinetics at 40 $^{\circ}\text{C}$ were captured at ATP concentration of 5, 10, 20, 40, 80, and 160 μM and illustrated in Fig. 1. No dependence of the kinetics from ATP concentration supports the conclusion that under our experimental conditions the luminescence intensity is directly proportional to the number of active enzyme molecules.

2.3. Thermal deactivation and recovery

Enzyme deactivation was observed by heating luciferase solution to a given temperature and recording luminescence that represents the number of active enzyme molecules in solution over time. Before starting measurements, a microplate was incubated for 10 min until a desirable reaction temperature was reached in the range of 27 $^{\circ}\text{C}$ – 45 $^{\circ}\text{C}$. The luciferase solution was prepared as previously described and dispensed in three replicates into a 384-well pre-heated microplate. The plate was sealed by a transparent film to minimize sample evaporation. The preheating of the microplate to the target temperature before reagents were dispensed, combined with the small volume of reagents ensured that the reaction sample itself reached the desired temperature prior to recording of luminescence intensity. Luminescence signals were recorded as a function of time on the Orion II luminometer at approximately 1 s intervals.

Luciferase recovery after exposure to elevated temperatures was studied by preparing two identical 20 μ L samples of luciferase solution. One was kept at 22 $^{\circ}$ C as a control and another was pre-heated to 55 $^{\circ}$ C for 30 s in a Cetus DNA cycler. Next, the heated luciferase sample was rapidly cooled to 22 $^{\circ}$ C by immersion in a water bath. Luminescence was initiated in both samples by adding ATP, and samples were subsequently dispensed in three replicates on a microplate. Luminescence was recorded on an Orion II luminometer over time at a constant plate temperature of 22 $^{\circ}$ C. Luciferase recovery was also examined for exposures to 45 $^{\circ}$ C for 90 s.

3. RESULTS

Luciferase thermal deactivation follows two-exponential decay kinetics

As previously mentioned, transient luminescent kinetics of the luciferase exposed to high temperature have nearly identical shapes at various ATP concentrations. The intensity of luminescence at $t = 0$ is directly proportional to the number of luciferase molecules added to the sample solution [21]. With no significant exhaustion of ATP and no enzyme inhibition by reaction byproducts under these experimental conditions, the luminescent intensity quantitatively represents the number of active luciferase molecule in solution.

Fig. 2 shows the luminescence signal as a function of time for various reaction temperatures. At reaction temperatures above 30 $^{\circ}$ C, the transient kinetics have fast and slow decline components that can be identified as two distinct linear segments on the logarithmic plot at higher reaction temperatures as illustrated in Fig. 2(h). The transient kinetics can be approximated by a two-exponential model, with the C's denoting constant terms and γ 's denoting the fast and slow decay constants:

$$I(t) = C_0 + C_{Fast} e^{-\gamma_{Fast} t} + C_{Slow} e^{-\gamma_{Slow} t} \quad (1)$$

Nonlinear least squares fitting was performed using Microsoft Excel's Solver add-in for finding parameters of Eq. 1 from experimental data.

The decrease in luciferase activity with increasing temperature that is observed shows that enzyme deactivation clearly is the dominating effect on reaction rate, rather than effects arising from an increase in the rate of effective collisions as predicted by the Arrhenius/Eyring equations. Contrary to experimental observations for luciferase, the Arrhenius/Eyring equations predict an *increase* in reaction rate with increased temperature. Indeed, by going from 27 $^{\circ}$ C to 45 $^{\circ}$ C, for an enzyme with typical activation energies of 25 kJ/mol the reaction rate would be increased by $k_{45\text{ }^{\circ}\text{C}}/k_{27\text{ }^{\circ}\text{C}} \approx 1.7$ -fold vs. the observed more than two orders of magnitude decrease of luminescence observed for luciferase as illustrated in Fig. 2(g).

After exposure to elevated temperature luciferase partially recovers its activity

We investigated the change of luciferase activity after it was briefly exposed to elevated temperature. Fig. 3 shows the changes in luminescence over time (a) for the control sample kept at room temperature and two samples exposed to (b) 45 $^{\circ}$ C for 90 sec and (c) 55 $^{\circ}$ C exposed for 30 sec. The flatness of the curve observed with the room temperature control indicate that the combined effects from possible substrate depletion and enzyme inhibition by reaction byproducts are negligible under these experimental conditions.

Due to thermal deactivation, the luminescence from the samples exposed to 45 $^{\circ}$ C and 55 $^{\circ}$ C is substantially lower than that of the control sample. Interestingly, after the exposed sample was brought to room temperature, its luminescence gradually increased consistent with the

idea of a partial recovery of previously deactivated luciferase molecules. We found the recovery was easier to observe in samples where luciferase was substantially deactivated (by 95% or more) exhibiting a lower residual luminescence and accumulating a larger number of enzyme molecules in the reversible inactive state that can revert to the active enzyme configuration.

A comparison of recovery curves (b) and (c) in Fig. 3 for two samples that were exposed to 55 °C for 30 s and 45 °C for 90 s, respectively reveals a common trend in the recovery of these two samples. Fitting the recovery curves by a single exponential model $A[1 - \exp(-Bt)] + C$, and comparing the constant parameters for each curve ($n = 3$) showed a very close rate of recovery and only a slightly different (i.e., 25%) number of molecules participating in recovery for two deactivating temperatures. Recovery data in Fig. 3 indicate that independently of the deactivation conditions enzyme molecules accumulating in the same reversible inactive state from which they can return back to the active state. This result suggests that the recovery is determined by the number of enzyme molecules that have been accumulated in the deactivated state rather than by how the molecules reached that state (i.e., the specific transition path and time frame). The similarity of the recovery curves at their outset suggests that similar number of molecules reached a reversibly inactive state, but the number that reached further irreversible deactivated states may have been different. The more than 4 min time course of the luminescence recovery implies that at least for luciferase, the reversibly inactive state and the active state do not form a rapid equilibrium; the equilibrium takes a substantial amount of time to become established.

Fast and slow rates of luciferase deactivation show the same dependence vs. temperature

Data in Fig. 2 were used to find the fast, γ_{Fast} , and slow, γ_{Slow} , rates of decay for thermal deactivation of luciferase according to Eq. 1. We used the Solver add-in utility of Microsoft Excel to perform nonlinear least-squares fitting of experimental data acquired at various temperatures. Both γ_{Fast} and γ_{Slow} increase rapidly with temperature from 30 °C to 45 °C (deactivation at 27 °C did not have a clear fit). Fig. 4 is a plot of γ_{Slow} vs. γ_{Fast} for various deactivation temperatures ($p = 0.000579$).

The plot of γ_{Slow} vs. γ_{Fast} fit well to a straight line as can be seen in Fig. 4 with $\gamma_{\text{Slow}} / \gamma_{\text{Fast}} = 0.153$ and remains constant over the entire range of temperatures used in this experiment. The constant $\gamma_{\text{Slow}} / \gamma_{\text{Fast}}$ ratio may indicate a synergy of the fast and slow processes that naturally occurs, for instance, in three state systems in which transitions occurs from the initial enzyme active state into one of two inactive states. The rate of transition can be due to involvement of the same enzyme state for both processes under the framework rather than participation of two unrelated or competing deactivation pathways. Indeed, involvement of two competing pathways for the fast and slow processes will likely result in different temperature dependencies for γ_{Slow} and γ_{Fast} and produce $\gamma_{\text{Slow}} / \gamma_{\text{Fast}} \neq \text{const}$ contrary to data in Fig. 4.

4. DISCUSSION

4.1. Model

By applying the Michaelis-Menten equation for calculating the rate of ATP consumption, the luminescence intensity, I_{Lum} , can be expressed as follows:

$$I_{\text{Lum}} \propto -\partial [\text{ATP}] / \partial t = k_2 S E / (K_m + S) \quad (2)$$

where K_m is the Michaelis-Menten constant, k_1 and k_2 is the rate of producing enzymatic product [22]. Eq. 2 can be rearranged by grouping terms with similar temperature (T) and time (t) dependence:

$$I_{lum}(T, t) = A(T) N_E(T, t) \quad (3)$$

$$A(T) = k_2 S / (K_m + S) \quad (4)$$

where $A(T)$ is the “Arrhenius” factor, which represents the increase of the rate of chemical reaction and which at a given temperature remains constant vs. time, and $N_E(T, t)$ is the number of enzyme molecules in an active state, which is a function of temperature and time. $A(T)$ can be excluded from Eq. 3 by normalizing luminescence kinetics with the luminescent intensity I_0 at $t = 0$:

$$I_{lum}(T, t) / I_0 = N_E(T, t) / N_0 \quad (5)$$

where $N_E(T, t) / N_0 = 1$ at $t = 0$. The normalized transient kinetic is directly proportional to the number of active enzyme molecules. Accordingly, the following analysis will be focused on modeling the number of enzyme molecules in an active state, $N_E(T, t)$.

Observed are two-exponential transient kinetics pointing to a possible two-step deactivation process which naturally can be accommodated by a three-state enzyme model shown in Fig. 5. In this model, state 1 represents enzyme in the active state, whereas states 2 and 3 represent partially unfolded, deactivated enzyme. Molecules in state 2 can refold and return to state 1, and molecules in state 3 can return to state 2. We assume that at $t = 0$ all enzyme molecules are in state 1 and subsequent transitions between states are driven by thermodynamic factors (temperature). Accordingly, we are not assuming that the system is in thermodynamic equilibrium at $t = 0$, but rather is in the process of reaching thermodynamic equilibrium. The model fulfills two requirements: first, to provide two-exponential decay of the number of enzyme molecules in active state 1; and second to exclude competing channels for decay in state 1 for consistency with experimental data shown in Fig. 4.

4.1.1. Rate equations for the number of enzyme molecules in different states

—Transitions of enzyme molecules between states 1, 2 and 3 are described by a set of conventional rate equations:

$$\partial N_1 / \partial t = -K_{12} N_1 + \gamma_{21} N_2 \quad (6)$$

$$\partial N_2 / \partial t = K_{12} N_1 - \gamma_{21} N_2 - K_{23} N_2 + \gamma_{32} N_3 \quad (7)$$

$$\partial N_3 / \partial t = K_{23} N_2 - \gamma_{32} N_3 \quad (8)$$

In addition:

$$N_0 = N_1 + N_2 + N_3 \quad (9)$$

and $\partial N_0 / \partial t = 0$ - the total number of enzyme molecules does not change.

The condition (9) simplifies system (6)–(8) by reducing it to two linear differential equations.

$$\partial N_1 / \partial t = -K_{12}N_1 + \gamma_{21}N_2 \quad (10)$$

$$\partial N_2 / \partial t = K_{12}N_1 - \gamma_{21}N_2 - K_{23}N_2 + \gamma_{32}N_3 - \gamma_{32}N_1 - \gamma_{32}N_2 \quad (11)$$

A solution for this system is given by:

$$N_i = C_{O_i} + C_{F_i} \exp(-Y_F t) + C_{S_i} \exp(-Y_S t) \quad (12)$$

By substituting Eq. 12 into Eq. 10–12 and by balancing terms for $\exp(-Y_F t)$, $\exp(-Y_S t)$ and for no time dependence for states 1 and 2 we find γ_F , γ_S and C_{O_i} , C_{F_i} , C_{S_i} :

$$\gamma_{Fast} = (K_{12} + \gamma_{21} + K_{23} + \gamma_{32}) - (K_{12}K_{23} + \gamma_{32}(K_{12} + \gamma_{21})) / (K_{12} + \gamma_{21} + K_{23} + \gamma_{32}) \approx K_{12} + \gamma_{21} - \gamma_{Slow} \quad (13)$$

$$\gamma_{Slow} = (K_{12}K_{23} + \gamma_{32}(K_{12} + \gamma_{21})) / (K_{12} + \gamma_{21} + K_{23} + \gamma_{32}) \approx K_{23} + \gamma_{32} + \gamma_{32}\gamma_{21}/K_{12} \approx K_{23} + \gamma_{32} \quad (14)$$

where we assume that $K_{12}, \gamma_{21} \gg K_{23}, \gamma_{32}$. Next, for C_{O_i} , C_{F_i} , C_{S_i} :

$$(C_{S_1} + C_{O_1}) / C_{F_1} \approx \gamma_{21} / K_{12} = \exp(-\Delta H_{12} / kT) \quad (15)$$

$$C_{O_1} / C_{S_1} \approx \gamma_{32} / K_{21} = \exp(-\Delta H_{23} / kT) \quad (16)$$

here ΔH_{12} and ΔH_{23} are change of enthalpy for transition between corresponding states, k is Boltzmann's constant, and T is the absolute temperature.

Eq. 13–16 express model parameters K_{12} , γ_{21} , K_{23} , γ_{32} , ΔH_{12} , and ΔH_{23} through parameters γ_F , γ_S , C_{O_i} , C_{F_i} , and C_{S_i} that can be measured from experimental data.

4.1.2. Fast enzyme deactivation—By fitting Eq. 1 to experimental data shown in Fig. 2, we found γ_F , γ_S , C_{F_1} , C_{S_1} as a function of temperature. The fast component of the deactivation curves in Fig. 2 can be assigned to the transition between states 1 and 2. In the three-state model shown in Fig. 5, the deactivation process eventually leads to establishment of an equilibrium in which a small fraction of luciferase molecules, N_1^{Eq} remains in the active state (state 1):

$$N_1^{Eq} / N_0 = 1 / \{1 + \exp(\Delta H_{12} / kT) + \exp(\Delta H_{12} / kT + \Delta H_{23} / kT)\} \approx \exp(-\Delta H_{12} / kT - \Delta H_{23} / kT) \quad (17)$$

The molecules that remain in the active state contribute to a steady-state luminescence that is represented by C_{O1} . From data in Fig. 2: $C_{O1} \ll C_{F1}$, C_{S1} and we have for γ_{21}/K_{12}

$$\gamma_{21}/K_{12} \approx C_{S1}/C_{F1} \quad (18)$$

and for ΔH_{12}

$$\Delta H_{12} \approx -kT \ln(C_{S1}/C_{F1}) \quad (19)$$

Fig. 6 shows ΔH_{12} vs. temperature found from the experimental data in Fig. 2. On the energy scale, ΔH_{12} is changing from about +11 kJ/mol to -8 kJ/mol in the temperature range of 25 °C to 50 °C, which may correspond to the energy of a few hydrogen bonds or may correspond to the denaturation energy of a single protein residue. At low temperature, $T < 39$ °C, state 1 has lower energy than state 2, but rises above state 2 at $T > 39$ °C.

According to the data in Fig. 6, ΔH_{12} has a linear dependence vs. temperature and crosses zero (i.e. $\Delta H_{12} = 0$) at 39.6 °C. This result is consistent with the assumption used in the Equilibrium Model [7]. Accordingly, the data in Fig. 6 can be used to find parameters of T_{eq} and H_{eq} of the Equilibrium Model: $T_{eq} = 39.6$ °C when $H_{eq} = 0$.

4.1.3. Slow enzyme deactivation—The energy of state 3 is lower than the energy of state 1 and 2 at all the temperatures used in our experiments. Therefore $\gamma_{32} \ll K_{23}$, and from Eq. 10 $K_{23} = \gamma_{Slow}$. Unfortunately, no reliable data are available for C_{O1} to find ΔH_{23} and we have to use an alternative approach to estimate ΔH_{23} . Here we use data for recovery of luciferase activity after exposure to elevated temperature (see Fig. 3). In view of the fact that the number of recovered luciferase molecules is given by the total number of deactivated molecules and we can expect $N_2 \ll N_3$, the fraction of recovered luciferase molecules is given by N_1^{Eq} in Eq. 13 and Eq. 13 can be used to estimate ΔH_{23} . From Fig. 3 $N_{eq}/N_0 = 7.23 \times 10^{-3}$ and extrapolating data in Fig. 7 to $T = 22$ °C we find $\Delta H_{23} = -25.06$ kJ/mol. The ΔH_{23} energy is substantially smaller than the energy of complete denaturation of a 100 kDa protein, that typically can be expected to be approximately 500 kJ/mol. Accordingly, states 2 and 3 likely represent luciferase molecules at early phases of denaturation, when just a few hydrogen bonds or links between a few neighboring protein residues have been altered. There is a high probability that changes occur at the enzyme catalytic site, but changes outside the catalytic pocket also can lead to loss of enzymatic activity as is known from the studies of allosteric inhibitors.

4.2. Energy diagram

Based on the analysis of fast and slow deactivation of luciferase we designed the energy diagram shown in Fig. 7. We hypothesize that the relative position of states 2 and 3 does not change with time for consistency with data in Fig. 4, i.e., the fact that the ratio $\gamma_{Fast}/\gamma_{Slow}$ remains nearly constant with respect to temperature under the actual experimental conditions. Accordingly, the dependence of ΔH_{12} vs. temperature in Fig. 6 can be explained by the change of position of state 1 on the energy diagram as the temperature increases and the enzyme structure undergoes small reversible conformational changes.

5. Conclusions

The results of our study, as well as previous literature reports, indicate that the conventional two-state model of enzyme deactivation needs to be revised. Here we presented a three-state model for describing thermal deactivation of *Photinus pyralis* that shares some common elements with the previously proposed three-state Equilibrium model [9]. However, there

are also important differences in assumptions used, and features of thermal enzyme deactivation captured by each model. The Equilibrium model exercises a phenomenological approach for quantitative description of enzymatic activity. In this model two parameters have been introduced to describe transitions between active and inactive enzyme states: the enthalpy of equilibrium ΔH_{Eq} , and equilibrium temperature T_{Eq} , which can be defined as the temperature at which an equal number of enzyme molecules are found in the active and inactive states. The Equilibrium model assumes that equilibrium in the number of enzyme molecules in active and inactive state can be reached instantly. An additional assumption of the Equilibrium model is that the equilibrium constant K_{Eq} for enzyme in the active and inactive states can be expressed vs. temperature as following:

$$\ln(K_{Eq}) = (\Delta H_{Eq}/R)(1/T_{Eq} - 1/T) \quad (20)$$

It can be shown that the last equation can be derived by postulating a linear temperature dependence of the enthalpy change ΔH for transition between active and inactive enzyme states: $(\partial \Delta H / \partial T) = \text{const.}$ Under this assumption we have for the equilibrium enthalpy ΔH_{Eq} and temperature:

$$\Delta H_{Eq} = \Delta H_o (1 - T/T_{Eq}) \quad (21)$$

Substituting Eq. 21 into the Arrhenius equation leads to Eq. 20 which is implicitly assumed in the Equilibrium model. By neglecting the process of establishing equilibrium, the Equilibrium model can accommodate only a single-exponential decline of enzyme activity vs., time, contrary to experimental observation for *Photinus pyralis* luciferase.

The Transient model is based on a microscopic approach. Similar to Equilibrium model, the Transient model postulates that each enzyme molecule can exist in one of three states: active, and two reversibly inactive states. Transitions between these states are driven by thermodynamic factors. In this study we are using additional simplifying assumptions that on an energy scale the inactive state 3 is lower than state 2 and transitions from active state 1 to state 3 occur mostly through the intermediate state 2 as illustrated in Fig. 5. We believe under such assumptions the Transient model has quite broad applicability and encompasses the Equilibrium model as a special case in the limit of fast transitions between active and irreversibly inactive state 1 and 2. Under the Transient model Eq. 20 can be used to find T_{Eq} of the Equilibrium Model from experimental data for *Photinus pyralis* luciferase as shown in Fig. 6. Here T_{Eq} corresponds to the temperature at which $\Delta H_{12} = 0$ and from data in Fig. 6: $T_{Eq} = 39.6^\circ\text{C}$. Unlike the Equilibrium Model, the Transient model does not assume a linear dependence of enthalpy vs. temperature. Accordingly, the Transient model can accommodate more complex temperature dependences and can be used to test the validity of that assumption of the Equilibrium model. Interestingly, in the case of *Photinus pyralis* (firefly) luciferase, the experimental data indicate that the enthalpy vs. temperature for the active state fits well to a linear dependence (see Fig. 6). This fact can be viewed as experimental justification for the key assumption of the Equilibrium Model in the case of *Photinus pyralis* luciferase. Two special features that naturally arrive from the Transient model are two-exponential decline of luminescence activity vs. time and the gradual partial recovery of enzyme activity after short exposure to a high temperature observed for luciferase. An additional advantage of the Transient model is that the same uniform set of parameters is used for all states of the Transient model. This fact can facilitate expansion of this model by including additional states for describing enzymes with large numbers of conformations with similar enthalpies. According to the Transient model, irreversible deactivation of an enzyme can be viewed as a set of consecutive transitions, each of which is

reversible, and which leads to the enzyme state with lowest enthalpy (i.e., the state representing a complete denaturation). In such expanded model the well-developed formalism and techniques for analysis of multi-level systems can be used to analyze transient kinetics and measure model parameters.

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REFERENCES

- [1]. Copeland, RA. *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*. Wiley-VCH; New York: 2000.
- [2]. Dixon, M.; Webb, EC. *Enzymes*. Longman Group Ltd; London: 1979.
- [3]. Garrett, RH.; Grisham, CM. *Biochemistry*. Brooks/Cole – Cengage; Boston: 2010.
- [4]. Thomas TM, Scopes RK. The effects of temperature on the kinetics and stability of mesophilic and thermophilic 3-phosphoglycerate kinases. *Biochem. J.* 1998; 330:1087–1095. [PubMed: 9494072]
- [5]. Gerike U, Danson MJ, Russell NJ, Hough DW. Sequencing and Expression of the Gene Encoding a Cold-Active Citrate Synthase from an Antarctic Bacterium, Strain DS2-3R. *Eur. J. Biochem.* 1997; 248:49–57. [PubMed: 9310359]
- [6]. Daniel RM, Danson MJ, Eysenbach R. The temperature optima of enzymes: a new perspective on an old phenomenon. *Trends Biochem. Sci.* 2001; 26:223–225. [PubMed: 11295553]
- [7]. Daniel RM, Danson MJ, Eysenbach R, Lee CK, Peterson ME. New parameters controlling the effect of temperature on enzyme activity. *Biochem. Soc. Trans.* 2007; 35:1543–1546. [PubMed: 18031263]
- [8]. Daniel RM, Danson MJ, Eysenbach R, Lee CK, Peterson ME. The effect of temperature on enzyme activity: new insights and their implications. *Extremophiles.* 2008; 12:51–59. [PubMed: 17849082]
- [9]. Daniel RM, Peterson ME, Danson MJ, Price NC, Kelly SM, Monk CR, Weinberg CS, Oudshoorn ML, Lee CK. The molecular basis of the effect of temperature on enzyme activity. *Biochem. J.* 2010; 425:353–360. [PubMed: 19849667]
- [10]. Gould SJ, Subramani S. Firefly luciferase as a tool in molecular and cell biology. *Anal. Biochem.* 1988; 175:5–13. [PubMed: 3072883]
- [11]. Marques SM, Esteves da Silva JCG. Firefly Bioluminescence: A Mechanistic Approach of Luciferase Catalyzed Reactions. *IUBMB Life.* 2009; 61:6–17. [PubMed: 18949818]
- [12]. Binkowski B, Fan F, Wood K. Engineered luciferases for molecular sensing in living cells. *Curr. Opin. Biotechnol.* 2009; 20:14–18. [PubMed: 19299118]
- [13]. Herbst R, Schäfer U, Seckler R. Equilibrium intermediates in the reversible unfolding of firefly (*Photinus pyralis*) luciferase. *J. Biol. Chem.* 1997; 272:7099–7105. [PubMed: 9054403]
- [14]. Younousse, S.; Enrica, A.; Pierre, Z.; Farhad, R.; Pierre, G.; Jean-Pierre, Z. High frequency electromagnetic radiations induce a heat shock-like response in *Physcomitrella patens*. The Annual International Meeting for Moss Experimental Research; Laboratory of Plant Cell Genetics, Institute of Ecology, CH 1015 Lausanne, Switzerland. 2003.
- [15]. Agah A, Aghajan M, Mashayekhi F, Amini S, Davis RW, Plummer JD, Ronaghi M, Griffin PB. A multi-enzyme model for pyrosequencing. *Nucleic Acids Res.* 2004; 32(21):e166. [PubMed: 15576673]
- [16]. Sun Y, Jacobson KB, Golovlev V. A multienzyme bioluminescent time-resolved pyrophosphate assay. *Anal Biochem.* 2007; 367(2):201–9. [PubMed: 17540325]
- [17]. Yousefi-Nejad M, Hosseinkhani S, Khajeh K, Ranjbar B. Expression, purification, and immobilization of firefly luciferase on alkyl-substituted Sepharose 4B. *Enzyme Microb. Technol.* 2007; 40:740–746.

- [18]. Lee RT, Denburg JL, McElroy WD. Substrate-binding properties of firefly luciferase. II. ATP-binding site. Arch. Biochem. Biophys. 1970; 141:38–52. [PubMed: 5480123]
- [19]. Ataulakhanov F, Vitvitskiĭ VM, Zhabotinskiĭ AM, Pichugin AV, Sinauridze E. The recording of ATP in the erythrocytes using luciferase injected into the cells. Izv Akad Nauk SSSR Biol. 1989; 6:813–21. [PubMed: 2621278]
- [20]. Zako T, Ayabe K, Aburatani T, Kamiya N, Kitayama A, Ueda H, Nagamune T. Luminescent and substrate binding activities of firefly luciferase N-terminal domain. Biochim. Biophys. Acta. 2003; 1649:183–189. [PubMed: 12878037]
- [21]. Brovko, L. Bioluminescence for food and environmental microbiological safety. SPIE Press; Bellingham, Washington, USA: 2007.
- [22]. Berg, JM.; Tymoczko, JL.; Stryer, L. Biochemistry. W.H. Freeman and Company; New York: 2007.

Research Highlights

- Enzyme deactivation upon heating proceeds in multiple steps
- Deactivation is rapid, when compared to denaturation
- Transient model to describe sequential changes – different from Equilibrium model

Fig. 1.

a) Michaelis-Menten kinetics for luciferase in Tris-acetate buffer (pH= 7.7). b) Independence of transient luminescent kinetics with changing ATP concentration measured at 40 °C and ATP concentration of 5, 10, 20, 40, 80, and 160 μM .

Fig. 2. Thermal deactivation of luciferase. Luminescence in relative light units per second (RLU/s) over time at various reaction temperatures (a–g): 27 °C, 30 °C, 33 °C, 36 °C, 39 °C, 42 °C, and, 45 °C; (h) illustrates two linear segments of transient kinetics consistent with two-exponential deactivation process.

Fig. 3.

Recovery of luciferase activity after exposure to high temperature: (a) control sample at 23 °C; (b) sample exposed to 45 °C for 90 s; (c) sample exposed to 55 °C for 30 s. Luminescence intensity for samples (b) and (c) have been multiplied by 10 to be on comparable scale with the negative control (a). The recovery effect is similar (details in section 3.2). Any differences can be ascribed to the longer treatment duration at 45 °C that allowed more enzyme molecules to reach the irreversibly inactive state or states.

Fig. 4. Relation of decay constants. γ_{Slow} vs. γ_{Fast} (in s^{-1}) from Eq. 1 found for various deactivation temperatures. The data points can be approximated by a linear dependence shown by the line.

Fig. 5. Model of transition between different enzyme states. 1 is enzyme in active state; 2 and 3 represent partially denatured deactivated enzyme states.

Fig. 6. Transition enthalpy, ΔH_{12} (in kJ/mol) vs. temperature ($^{\circ}\text{C}$) calculated from data in Fig. 2.

Fig. 7. Energy diagram vs. temperature for active (1) and (2, 3) reversibly inactive states of *Photinus pyralis* (firefly) luciferase. T_{Eq} illustrates the position of the active state at the equilibrium temperature of 39 °C for *Photinus pyralis* luciferase.