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Thermal stability of proteins in intermolecular complexes

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Abstract

A general phenomenological model is proposed for the estimation of the influence of the formation of complexes with ligands on thermal stability of proteins. In this model the reversible processes of unfolding–refolding and of association–dissociation of protein–ligand complexes and of the irreversible chemical degradation of the unfolded protein were analyzed jointly. By using certain approximations, the analytical expressions for both the thermodynamic and kinetic stabilization are obtained. Two thermodynamic and four kinetic regimes of stabilization and destabilization can exist in such system. Each thermodynamic regime appears to be compatible with three different kinetic regimes. The effect of the formation of complexes on thermodynamic and kinetic stability of the protein is determined by the degrees of binding of the ligand to the folded and unfolded protein species and by the rates of irreversible degradation of free protein and protein in complex.

Keywords: Protein unfolding; Irreversible denaturation; Thermal stability; Ligand binding

1. Introduction

The stability of the native protein molecule in solution is now intensively investigated. The processes of reversible unfolding and refolding of simple monomeric proteins are usually described as a thermodynamic equilibrium between two states–folded (F) and unfolded (U) [1–3]. As a rule the unfolded in extreme conditions (at high temperature, high or low pH, etc.) protein molecule becomes a subject for subsequent chemical degradation, such as the deamidation and racemization of peptide groups, the destruction and β-elimination of disulfide bonds, and other processes [4–6]. Very often these irreversible reactions are proved to satisfy the first order kinetics with respect to protein concentration [6–8]. So the process of irreversible denaturation of proteins in many cases is well described by the following scheme [9,10]:

$$\mathrm{F} \underset{K_{\mathrm{U}}^{\circ}}{\rightleftharpoons} \mathrm{U} \xrightarrow[k_1]{} \mathrm{I}, \qquad (1)$$

where F, U and I denote the folded, unfolded and irreversibly (due to chemical degradation) unfolded states, respectively, of the protein molecule, $K_{\mathrm{U}}^{\circ} = [\mathrm{U}]/[\mathrm{F}]$ is an equilibrium constant, with [F] and [U] being the concentrations of the folded and unfolded species, and k_1 is the rate of irreversible degradation of the protein.

In the vast majority of the investigations the problem of the conformational stability of the protein is studied in a monomolecular approach

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as described above. Nevertheless, *in vivo*, many proteins exist in complexes with different ligands or other macromolecules, or are adsorbed in and on membranes. Moreover the interactions of proteins with adsorbents, polymeric gels and soluble synthetic polymers are often used in biotechnology as an empirical approach for the improvement of protein stability. However, there is no theoretical description of the conformational stability of proteins in complexes with other macromolecules in solution.

In this article a phenomenological model treatment of the reversible and irreversible denaturation of the protein is presented for the system in which a protein molecule can form complexes with other molecules in solution.

2. Denaturation model

Let the conformational state of the protein in solution is described by the scheme prestented in eq. (1) (Scheme 1). If this solution contains another solute P (it can be the synthetic or biological polymer or a low molecular weight ligand) the protein can interact with it, forming the soluble complexes of P with F, U and I:

$$\begin{array}{ccccc} F+P & \overset{K^{\circ}_U}{\rightleftharpoons} & U+P & \xrightarrow{k_1} & I+P \\ \alpha_F \Big\uparrow\Big\downarrow & & \alpha_U \Big\uparrow\Big\downarrow & & \Big\uparrow\Big\downarrow \\ FP & \rightleftharpoons & UP & \xrightarrow{k_2} & IP \end{array} \qquad (2)$$

Here $\alpha_F = [FP]/[F]$ and $\alpha_U = [UP/[U]$ are the ratios of the concentrations of the protein in the complex to those of the free protein in folded and unfolded state, respectively, and k_2 is the rate of irreversible degradation of the protein in complex. By using the scheme of eq. (2) (Scheme 2), the influence of the formation of complexes on the thermodynamic (in reversible processes of folding and unfolding) and kinetic (in irreversible processes) stability of the protein to the thermal denaturation will be analyzed in comparison with the scheme presented in eq. (1).

3. Results

3.1 Thermodynamic stability

In the typical experiment on the protein stability to the reversible thermal denaturation, the dependence of K°_U on temperature should be studied. The assumption of the reversibility means that k_1, k_2 are sufficiently small and that the irreversible processes in Schemes 1 and 2 can be neglected. Scheme 2 then becomes a well known thermodynamic cycle from which the equation for the K_U—the equilibrium constant of unfolding—is readily obtained:

$$K_U = \frac{[U]+[UP]}{[F]+[FP]} = K^{\circ}_U \frac{1+\alpha_U}{1+\alpha_F}. \qquad (3)$$

If, in addition, the index of thermodynamic stabilization, s_T of the protein in complex will be determined as $s_T = K^{\circ}_U/K_U$, then it follows from eq. (3) that

$$s_T = \frac{1+\alpha_F}{1+\alpha_U}. \qquad (4)$$

This general formula can be applied to all the systems in which the thermodynamic state of the protein is well approximated by the model of two conformational states. The parameters α_F and α_U may depend on temperature, pH, ionic strength and (in case of co-operative binding) on the concentrations of protein and ligand. If $\alpha_F > \alpha_U$, then $s_T > 1$ and the protein is thermodynamically stabilized in complex form. This is realized in all complexes of proteins with specific ligands, enzyme–substrate and enzyme–inhibitor complexes, where α_F is large and α_U is small. If $\alpha_F < \alpha_U$, then $s_T < 1$ and the thermodynamic stability of the protein decreases due to the formation of complexes. This situation is often observed in non-specific complexes of proteins with synthetic polymers [11,12].

3.2 Kinetic stability

The stability of proteins against irreversible thermal denaturation is usually studied as fol-

lows. The protein is incubated at elevated temperature for different periods of time and from the time dependence of the irreversibly denatured product obtained the rate of the reaction is determined.

For the analytical solution of the system of differential equations which corresponds to Scheme 2, the assumption of equilibrium in the cycle of reversible reactions will be made. This assumption is equivalent to putting a restriction on the values of the rates k_1 and k_2, which must be much smaller than all the rates of partial equilibria in the cycle. The last restriction is realistic, because for thermal denaturation the values of k_1 were shown to be on the order of $(\text{hours})^{-1}$ and less [6,13]. Then the following equations are true at every moment of time, t:

$$[FP] = \alpha_F[F], \quad [U] = K_U^\circ[F],$$

$$[UP] = \alpha_U K_U^\circ[F]$$

Combining these restrictions with the mass laws:

$$[F] + [U] + [FP] + [UP] + [I] + [IP] = c_\Sigma,$$

and

$$\frac{d}{dt}([F] + [U] + [FP] + [UP] + [I] + [IP]) = 0,$$

and the initial conditions at the moment $t = 0$:

$$[F]_0 + [U]_0 + [FP]_0 + [UP]_0 = c_\Sigma,$$

the solution of the system can be obtained:

$$\frac{d([I] + [IP])}{dt} = -\frac{d([F] + [U] + [FP] + [UP])}{dt}$$

$$= -\frac{1 + \alpha_F + K_U^\circ(1 + \alpha_U)}{K_U^\circ}\frac{d([U])}{dt}$$

$$= [U](k_1 + \alpha_U k_2).$$

Then

$$[U] = [U]_0 \exp\left(-\frac{K_U^0(k_1 + \alpha_U k_2)}{[1 + \alpha_F + K_U^0(1 + \alpha_U)]}t\right),$$

and

$$([I] + [IP])$$

$$= c_\Sigma - ([F] + [U] + [FP] + [UP])$$

$$= c_\Sigma\left\{1 - \exp\left(-\frac{K_U^\circ(k_1 + \alpha_U k_2)}{[1 + \alpha_F + K_U^\circ(1 + \alpha_U)]}t\right)\right\}. \quad (5)$$

For the initial protein without ligand (Scheme 1) $\alpha_F = \alpha_U = 0$, and

$$[I] = c_\Sigma\left\{1 - \exp\left(-\frac{K_U^\circ k_1}{1 + K_U^\circ}t\right)\right\} \quad (6)$$

Let us determine the index of the kinetic stabilization s_k as $s_k = k_{free}/k_{compl}$, where k_{free} and k_{compl} are experimentally observed rates of irreversible degradation of the protein in Scheme 1 and in Scheme 2, respectively. Then, from (5) and (6)

$$k_{free} = \frac{K_U^\circ k_1}{1 + K_U^\circ}, \quad k_{compl} = \frac{K_U^\circ(k_1 + \alpha_U k_2)}{1 + \alpha_F + K_U^\circ(1 + \alpha_U)},$$

and

$$s_k = \frac{1 + \alpha_F + K_U^\circ(1 + \alpha_U)}{(1 + K_U^\circ)(1 + \alpha_U k_2/k_1)} \quad (7)$$

The value of the index s_k indicates the influence of the formation of complexes on the kinetic stability of the protein. When $s_k > 1$, the protein is kinetically stabilized in the complex, when $s_k < 1$, it is destabilized against irreversible denaturation.

The substitution of (7) into the inequality $s_k > 1$ gives:

$$\frac{1 + \alpha_F + K_U^\circ(1 + \alpha_U)}{(1 + K_U^\circ)(1 + \alpha_U k_2/k_1)} > 1,$$

$$\text{or } K_U^\circ(1 - k_2/k_1) > (k_2/k_1 - \alpha_F/\alpha_U).$$

Below two cases will be discussed, i.e. if $k_2/k_1 < 1$ and if $k_2/k_1 > 1$

3.2.1 Case 1: $k_2/k_1 < 1$

In this case the rate of irreversible denaturation of the protein in complex is less than that for

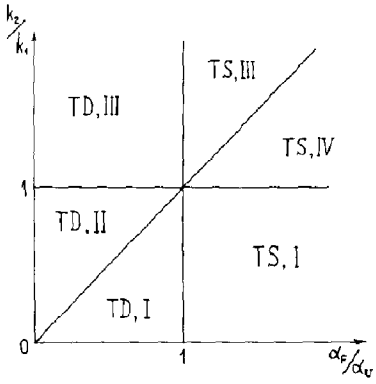

Fig. 1. Diagram of different regimes of stabilization and destabilization of the protein due to the formation of the complexes, calculated from Scheme 2. α_F and α_U–the ratios of the concentrations of the protein in the complex to those of the free protein in folded and unfolded state, respectively; k_2 and k_1–the rates of irreversible degradation of the unfolded protein molecules in the complex with ligand and in the free state. TS and TD–regimes of thermodynamic stabilization and destabilization of the protein. I and III–regimes of kinetic stabilization and destabilization at any temperature of irreversible denaturation; II and IV–regimes of kinetic low temperature destabilization, high temperature stabilization and kinetic high temperature destabilization, low temperature stabilization. The temperature is lower or higher than the temperature T_k (see the discussion in Section 3).

the initial protein. Then the formation of the complex has to stabilize the protein kinetically. But the redistribution between species in the cycle of reversible reactions (Scheme 2) also influences the experimentally observed rate k_{compl} and, as a result, two different regimes can be obtained:

$$K_U^\circ > \frac{k_2/k_1 - \alpha_F/\alpha_U}{1 - k_2/k_1}, \quad \text{and } k_2/k_1 < 1.$$

If $k_2/k_1 < \alpha_F/\alpha_U$, this is true for all the values of K_U° at any temperature, and the protein in complex is stabilized against irreversible denaturation (regime I in Fig. 1). If $k_2/k_1 > \alpha_F/\alpha_U$, this is true only at temperatures higher than some characteristic temperature T_k, which can be determined from the implicit equation (8):

$$K_U^\circ(T_k) = \frac{k_2/k_1 - \alpha_F/\alpha_U}{1 - k_2/k_1} \qquad (8)$$

Since $K_U^\circ(T)$ is a monotonically increasing function of temperature, the values of K_U°, experimentally obtained in the given conditions (pH, ionic strength, etc., but without ligands) can be used for the determination of the temperature scale in these inequalities. So the characteristic temperature T_k for the given values of α_F, α_U, k_1 and k_2 corresponds to the value of K_U° calculated from eq. (8). The explicit expression for T_k could be obtained provided the temperature dependences of quantities k_1, k_2 and α_F, α_U were known.

Therefore the effect of the formation of complexes on the kinetic stability of the protein depends in the latter case on the temperature of the irreversible denaturation experiment. If for the given system the inequality $1 > k_2/k_1 > \alpha_F/\alpha_U$ is true, then the protein is kinetically stabilized in complex at the temperatures higher than T_k and destabilized at lower temperatures (regime II in Fig. 1).

3.2.2 Case 2: $k_2/k_1 > 1$

In this case, analogously to the previously considered, the protein will be kinetically stabilized in complex, if

$$K_U^\circ < \frac{k_2/k_1 - \alpha_F/\alpha_U}{1 - k_2/k_1}, \quad k_2/k_1 > 1.$$

This is false for any value of K_U° if $k_2/k_1 > \alpha_F/\alpha_U$ (since $K_U^\circ > 0$). It means that in this case the protein will be kinetically destabilized at any temperature (regime III in Fig. 1). If the inequality $\alpha_F/\alpha_U > k_2/k_1 > 1$ takes place, then the protein is kinetically stabilized at temperatures lower than T_k and destabilized at higher temperatures (regime IV in Fig. 1).

If the temperature of the experiment $T_e = T_k$, then the formation of the complex does not influence the kinetic stability of the protein.

4. Discussion

All these conclusions are summarized on the phase diagram shown in Fig. 1. It is clear that depending on the parameters of the system dif-

ferent regimes can be observed. The result of the formation of the complexes—kinetic stabilization or destabilization of the protein—depends on the dimensionless parameters α_F/α_U and k_2/k_1 and in certain cases (regimes II and IV) also on the temperature of the denaturation used in the experiment. Such regimes were observed for immobilized enzymes [14], and various hypotheses were proposed to explain these seeming contradictions. The general analysis above and Fig. 1 show that all these regimes can coexist in a given system.

The parameters α_F, α_U, k_1 and k_2 are the characteristics of the given pair protein–ligand and also depend on the conditions (pH, ionic strength, etc.). Thus for the certain pair protein–ligand the different regimes can be observed depending on the conditions of the experiment. To predict the result one has to know the values of α_F, α_U, k_1 and k_2 (or, more correctly, the values of α_F/α_U and k_2/k_1) in the given conditions of the experiment.

Moreover Fig. 1 illustrates that the thermodynamic stability and kinetic stability are the different and to certain extent the independent properties of the protein in such complexes. Most important is the fact that for the protein in complex three different regimes of kinetic stabilization or destabilization are compatible with any regime of thermodynamic stabilization or destabilization. This distinction must help to interpret the experimental data on the stability of immobilized proteins and enzymes to irreversible denaturation (inactivation).

Since k_1 and k_2 both depend on temperature, the ratio k_2/k_1 can either increase or decrease with temperature. In the former case, since at low temperatures the value of k_2/k_1 is less than at high temperatures, regime IV can be observed if at some temperature the inequality $\alpha_F/\alpha_U < k_2/k_1 < 1 (1 < k_2/k_1 < \alpha_F/\alpha_U)$ is fulfilled. Conversely, in the latter case, regime II can be observed again if only one of these inequalities is fulfilled. If at every temperature of experiment $k_2/k_1 > \max(\alpha_F/\alpha_U, 1)$, or $k_2/k_1 < \min(\alpha_F/\alpha_U, 1)$, then the regime of kinetic destabilization or kinetic stabilization, respectively, is observed.

The analytical solutions and the expression for the index of kinetic stabilization were obtained under the assumption that equilibrium is attained in the cycle of reversible reactions of folding–unfolding and association–dissociation of the protein–ligand complex. This assumption is valid if all the rates in this cycle are much higher than the rates or the irreversible reactions, k_1 and k_2. If this is not the case, the kinetics in the cycle must be taken into account. In the latter case the corresponding system of differential equations cannot be solved analytically and only a complicated numerical simulation with many unknown parameters is possible. But for the systems in which this approximation works all the regimes displayed on Fig. 1 can be predicted and the value of the index s_k can be calculated from the parameters of the system which can be obtained experimentally.

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