Dissociative mechanism for irreversible thermal denaturation of oligomeric proteins

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Abstract Protein stability is a fundamental characteristic essential for understanding conformational transformations of the proteins in the cell. When using protein preparations in biotechnology and biomedicine, the problem of protein stability is of great importance. The kinetics of denaturation of oligomeric proteins may have characteristic properties determined by the quaternary structure. The kinetic schemes of denaturation can include the multiple stages of conformational transitions in the protein oligomer and stages of reversible dissociation of the oligomer. In this case, the shape of the kinetic curve of denaturation or the shape of the melting curve registered by differential scanning calorimetry can vary with varying the protein concentration. The experimental data illustrating dissociative mechanism for irreversible thermal denaturation of oligomeric proteins have been summarized in the present review. The use of test systems based on thermal aggregation of oligomeric proteins for screening of agents possessing anti-aggregation activity is discussed.

Keywords Oligomeric proteins · Protein denaturation · Dissociative mechanism · Conformational lock

Introduction

One of the important characteristics of the proteins is their stability, which should be taken into account when studying biological functions of proteins and using protein preparations in biotechnology and medical practice (Joly 1965; Kurganov 2012; Sanchez-Ruiz 2010; Tanford 1968). Proteins lose their native structure under the action of physical and chemical factors resulting in the loss of biological activity. The denaturation processes, as a rule, are accompanied by aggregation of the unfolded protein molecules. In recent years, much attention has been given to the problem of protein aggregation due to the discovery of a great number of inherited diseases, known as “conformational diseases”. These diseases are originated when certain proteins undergo structural alterations favoring their aggregation (Hall and Edskes 2012; Murphy and Tsai 2006; Ramirez-Alvarado et al. 2010; Sanchez-Ruiz 2010; Uversky and Fink 2006, 2007). Therefore, investigations of the mechanisms of protein denaturation and aggregation and a search for the agents possessing the anti-aggregation activity acquire special importance.

The quaternary structure of a protein may be essential for its biological function. For example, the active site of the oligomeric enzyme may be located in the subunit interface of the oligomer with the participation of amino acid residues, which belong to the neighboring subunits (Harutyunyan et al. 1982; Kudou et al. 2007; Schnell et al. 2012). In addition, owing to their quaternary structure with certain symmetry elements, proteins can participate in the formation of multiprotein assemblies (Kurganov and Lyubarev 1988a, b; Kurganov et al. 1985; Levy et al. 2006; Lyubarev and Kurganov 1989, 1996). It is significant that assembling subunits into oligomeric structure provides enhancement of protein stability with respect to thermal stress, proteolysis and various harsh conditions (Steif et al. 1993).

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The denaturation process of an oligomeric protein may include the stages of reversible dissociation of the oligomer into subunits which reveal higher lability than the original oligomer. Owing to the existence of protein oligomer dissociation stages, the complex kinetics of thermal denaturation of oligomeric proteins cannot be described by simple kinetic schemes proposed for monomeric proteins. Special kinetic approaches should be elaborated for analyzing thermal denaturation of oligomeric proteins.

The data on thermal denaturation of oligomeric proteins are rather scattered. The goal of the present review is to summarize the experimental results demonstrating the fulfillment of the dissociative mechanism for irreversible thermal denaturation of oligomeric proteins.

Dissociative mechanism for irreversible thermal denaturation of oligomeric proteins

When analyzing the kinetics of thermal denaturation of oligomeric proteins, we should take into account that the denaturation process can include the stages of reversible dissociation of initial oligomeric forms into subunits. This circumstance results in the violation of direct proportionality between the initial rate of denaturation and the initial protein concentration. Since subunits are more labile than initial protein oligomers, the kinetics of denaturation becomes sensitive to variation of the initial protein concentration. If the heat-induced loss of the catalytic activity of the enzyme is studied, the increase in the protein concentration results in retardation of the inactivation process. As an example, Fig. 1 shows the kinetics of thermal inactivation of glycogen phosphorylase *b* (Phb) from rabbit skeletal muscles at 53 °C at enzyme concentrations equal to 1.6 and 5.2 mg/mL. The Phb molecule consists of two identical subunits with molecular masses of 97.4 kDa each. The data on analytical ultracentrifugation provide evidence of the dissociation of the dimer into monomers at elevated temperatures (Eronina et al. 2010; Kurganov et al. 2000; Meremyanin et al. 2008).

An important characteristic of the thermoinactivation kinetics is the order of inactivation with respect to the protein (n). To calculate the n value, the series of kinetic curves of inactivation at various initial protein concentrations should be obtained. The order of inactivation with respect to the protein is a power coefficient in the expression connecting the initial rate of inactivation (v_0) and the initial protein concentration C_0 (Borzova et al. 2015; Curto et al. 2012; Delahaije et al. 2015; Eronina et al. 2016; Kurganov 2013a, b, 2015, 2016; Le Bon et al. 1999; Roefs and De Kruif 1994; van Boekel 2009):

$$v_0 = \text{const } C_0^n. \quad (1)$$

Fig. 1 Kinetics of thermal inactivation of Phb from rabbit skeletal muscles at 53 °C (0.08 M Hepes–NaOH, pH 6.8). The relative enzymatic activity A/A_0 versus time plot was constructed on the basis of the data represented in the work by Kurganov et al. (2000). Enzyme concentration: 1 1.6 and 2 5.2 mg/mL

If v_0 is the initial rate of denaturation, n can be considered as the order of denaturation with respect to the protein. In the case of one-stage irreversible denaturation, the n value is equal to unity.

The first attempt to characterize the kinetics of thermal denaturation of dissociable enzymes was made by Kurganov (1966), who analyzed the order of denaturation with respect to the protein for the dissociable enzyme system of the type dimer (D) $\rightleftharpoons$ 2 monomer (M):

(U and U' are the unfolded states of the monomer and dimer, respectively). It is assumed that there is a rapidly established equilibrium between the monomeric and dimeric forms. The association constant for this equilibrium is expressed as follows:

$$K_{\text{ass}} = \frac{[\text{D}]}{[\text{M}]^2} \quad (3)$$

([M] and [D] are equilibrium molar concentrations of the monomeric and dimeric forms, respectively). For the denaturation process (2), the unfolding of the monomeric and dimeric forms proceeds as a one-stage irreversible process with the rate constants k_1 and k_2 , respectively. Let ε be the k_1/k_2 ratio. The expression for the order of denaturation with respect to protein n as a

function of protein concentration has the following form (Kurganov 1966):

$$n = 1 - \frac{(\varepsilon - 1)(\sqrt{1 + 8K_{\text{ass}}C_0} - 1)}{\sqrt{1 + 8K_{\text{ass}}C_0}(2\varepsilon - 1 + \sqrt{1 + 8K_{\text{ass}}C_0})} \quad (4)$$

In this expression, C_0 is the total molar concentration of the protein calculated on the monomer.

Consider the case when only the monomeric form undergoes irreversible denaturation ($k_2 = 0$). The dependence of n on the initial protein concentration acquires the following form:

$$n = \frac{1}{2} \left(1 + \frac{1}{\sqrt{1 + 8K_{\text{ass}}C_0}} \right) \quad (5)$$

The limiting value of n at $C_0 \rightarrow 0$ is equal to unity. When $C_0 \rightarrow \infty$, the n value approaches 0.5. Thus, for the system stable dimer $\rightleftharpoons$ labile monomer, the order of denaturation with respect to protein n is less than unity ($0.5 \leq n \leq 1$).

The lag period, which appears on the kinetic curves of thermal inactivation of oligomeric enzymes, has been interpreted by Poltorak and colleagues (Atyaksheva et al. 2008, 2009; Chukhrai and Atyaksheva 2010; Poltorak and Chukhrai 1986; Poltorak et al. 1998) in terms of the concept of the conformational lock. The works by Moosavi-Movahedi and colleagues (Amani et al. 2005; Moosavi-Movahedi et al. 2013; Moosavi-Nejad et al. 2003) has contributed significantly to the development of this concept. It is assumed that the destruction of the intersubunit contacts proceeds in a stepwise manner, the dissociation stage being accompanied by the loss of the enzymatic activity:

The unfolding pathway involves m intermediates being formed during the conformational lock destruction. The minimum number of such intermediates can be determined by experiment as follows. The linear part is selected on the dependence of the relative enzymatic activity A/A_0 on time after passing the lag period (see, e.g., Fig. 2; A and A_0 are the current and initial values of the relative enzymatic activity). The length cut-off on the ordinate axis by the linear dependence of A/A_0 on time is designated as δ . The minimum value of intermediates of conformational lock destruction is calculated by the expression (Poltorak et al. 1998):

$$m \geq \frac{0.13 + \delta}{0.13 - 0.05\delta} \quad (7)$$

Fig. 2 The kinetics of thermoinactivation of Phb. The dependence of the relative enzymatic activity A/A_0 on time is plotted for the data represented in Eronina et al. (2009). Conditions: 0.08 M Hepes buffer, pH 6.8, containing 0.1 M NaCl; 48 °C

For the kinetic curve of Phb thermoinactivation represented in Fig. 2, the value of m was found to be 3.7 ± 0.1 . Thus, in the case of the thermoinactivation of Phb, $m \geq 4$.

Figure 3 demonstrates the contact regions between the subunits in Phb dimer. The subunit interface of Phb dimer consists of two main contact regions which are located on the opposite sides of the enzyme molecule (Barford and Johnson 1989). One of these contacts (contact region I in Fig. 3a) is formed by the antiparallel association of the $\alpha 7$ helices. Two hydrogen bonds between Arg 269 of the subunit A and Asn 270 of the subunit A' (Fig. 3b) and nonpolar interactions between interface residues underlie this association. The other contact region (contact region II in Fig. 3a) involves about 30 amino acid residues from four secondary structure elements of each subunit (Fig. 3c, d): N-terminal domain, $\alpha 1$ and $\alpha 2$ helices, and the cap region (a loop, which connects $\alpha 1$ and $\alpha 2$ helices). The residues belonging to the $\alpha 1$ helix make hydrogen bonds and hydrophobic contacts with the residues belonging to the N-terminal domain, $\alpha 1$ and $\alpha 2$ helices of the opposite subunit, while the residues of the cap region of one subunit make hydrogen bonds with the residues of the $\beta 7$ strand of the other subunit and hydrophobic contacts with the residues belonging to the $\alpha 2$ helix and the N-terminal domain of the other subunit. Thus, these data demonstrate the possibility of a multistep loss of Phb activity due to sequential disruption of the multiple contacts between the subunits in the enzyme dimer. This is in agreement with the results of the thermal inactivation studies.

Table 1 summarizes the data on thermal denaturation of oligomeric proteins where the dissociative mechanism of denaturation is fulfilled and contains information on the conformational lock number according to conclusions carried out by the authors.

Fig. 3 Contact regions in the dimeric molecule of Phb. **a** Ribbon diagram of the crystal structure of Phb (PDB 9GPB), consisting of two identical subunits, viewed perpendicular to the molecular dyad axis (depicted as a double-ended arrow). Two contact regions are encircled. The amino acid residues belonging to different subunits (subunits A and A', respectively) are shown in red and blue. **b–d** The expanded view of the contact regions I (**b**) and II (**c, d**) along the dyad axis of the dimer (**b, c**) and perpendicular to it (**d**). Residues involved in the formation of hydrogen bonds (depicted by dots) are shown by toggle wire representation and identified by the one-letter code. Residues and structural elements of the subunit A' are designated by primes. The figure is generated by using the MolBrowser 3.8-4a software (MolSoft)

Denaturation of oligomeric proteins studied by DSC

DSC is a powerful method for studying the conformational changes of the proteins induced by heating (Ibarra-Molero and Sanchez-Ruiz 2008; Johnson 2013; Lyubarev and Kurganov 2000; Privalov 2012; Sanchez-Ruiz 2010). Sanchez-Ruiz (1992) carried out the theoretical analysis of the model of thermal denaturation of an oligomeric protein including the stage of reversible dissociation of the oligomer into monomers followed by the stage of irreversible denaturation of the monomers:

where N, U and F are the native, unfolded and final (irreversibly denatured) states of the protein, respectively. Assuming

that the rate-limiting stage of the overall aggregation process is the monomer unfolding and the equilibrium $N_{\mu} \rightleftharpoons \mu U$ is initially displaced in the direction of the oligomer formation, it has been shown that the position of $T_{\max}$ ($T_{\max}$ is the temperature corresponding to the maximum point on a DSC profile) is shifted towards the higher temperatures when the protein concentrations increases.

As an example, the DSC data on denaturation of tetrameric glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit skeletal muscles (Markossian et al. 2006) are represented in Fig. 4. DSC profiles were obtained at various concentrations of the protein. Dissociative mechanism of GAPDH was supported by sedimentation velocity experiments at 45 °C (Khanova et al. 2007; Markossian et al. 2006). The analogous change in DSC profiles with varying protein concentrations were obtained for other dissociating protein

Table 1 Dissociative mechanism for irreversible denaturation of oligomeric proteins

Protein	Source	Initial oligomeric state, M_r	Experimental conditions	Methods used for registration of protein denaturation	Proposed kinetic mechanism	Refs
Alcohol dehydrogenase (EC 1.1.1.1)	Horse liver	Dimer, 80 kDa	50 mM pyrophosphate buffer, pH 8.8	Thermal inactivation studies at 46–55 °C, DLS, intrinsic fluorescence, ANS binding, far-UV CD	$E_2 \rightleftharpoons E_2^{(1)} \rightleftharpoons E_2^{(2)} \rightleftharpoons 2E_1 \rightarrow 2E_{den}$ Conformational lock number is equal to 2. This result was confirmed by biochemical computations.	Moosavi-Movahedi et al. (2013) [See also Poltorak et al. 1998]
Alkaline phosphatase (EC 3.1.3.1)	<i>Escherichia coli</i>	Dimer, 80 kDa	NaHCO ₃ –Na ₂ CO ₃ , pH 8.5	Thermal inactivation studies at 50–70 °C	$E_2 \rightleftharpoons E_2^{(1)} \rightleftharpoons E_2^{(2)} \rightleftharpoons E_2^{(3)} \rightleftharpoons E_2^{(4)} \rightleftharpoons 2E_1 \rightarrow 2E_{den}$ active inactive Conformational lock number is equal to 4.	Atyaksheva et al. (2009) [See also Poltorak et al. (1998)]
	Bovine and chicken intestines	Dimer, 80 kDa	NaHCO ₃ –Na ₂ CO ₃ , pH 8.5	Thermal inactivation studies at 45–55 °C	$E_2 \rightleftharpoons E_2^{(1)} \rightleftharpoons E_2^{(2)} \rightleftharpoons E_2^{(3)} \rightleftharpoons 2E_1 \rightarrow 2E_{den}$ active inactive Conformational lock number is equal to 3.	
Amine oxidase (EC 1.4.3.21)	<i>Lens esculenta</i> (lentil seeds)	Dimer, 150 kDa	100 mM potassium phosphate buffer, pH 7	Thermal inactivation studies at 52 and 57 °C	$E_2 \rightleftharpoons E_2^{(1)} \rightleftharpoons E_2^{(2)} \xrightleftharpoons{K_{dis}} 2E_1 \xrightleftharpoons{k_{den}} 2E_{den}$ K_{dis} and k_{den} were determined. Conformational lock number is equal to 2.	Moosavi-Nejad et al. (2003)
	<i>Euphorbia characias</i> latex	Dimer, 150 kDa	100 mM potassium phosphate buffer, pH 7	Thermal inactivation studies at 60 and 64 °C	$E_2 \rightleftharpoons E_2^{(1)} \rightleftharpoons E_2^{(2)} \rightleftharpoons E_2^{(3)} \rightleftharpoons 2E_1 \rightarrow 2E_{den}$ Conformational lock number is equal to 3. This result was confirmed by biochemical computations.	Amani et al. (2005)
Aminoacylase I (EC 3.5.1.14)	Porcine kidney	Dimer, 90 kDa	phosphate buffer, pH 7.2	Thermal inactivation studies at 60–65 °C	$E_2 \rightleftharpoons E_2^{(1)} \rightleftharpoons E_2^{(2)} \rightleftharpoons \dots \rightleftharpoons E_2^{(m)} \rightarrow 2E_1 \rightarrow 2E_{den}$ similar active m dimeric forms inactive	Poltorak et al. (1998)
			phosphate buffer, pH 6.5 or 7.25	Thermal inactivation studies at 55 °C	$E_2 \rightleftharpoons 2E_1 \rightarrow 2E_{den}$ active inactive	Parra and Chukhrai (2010)
Concanavalin A	<i>Canavalia ensiformis</i> (jack bean)	Tetramer, 125 kDa	10 mM 3,3'-dimethyl glutaric acid-NaOH buffer, 0.15 M NaCl, 0.1 M Mn ²⁺ , 0.1 M Ca ²⁺ , pH 6.9	DSC	$A_4 \rightleftharpoons 4A_{den} \rightarrow \text{aggregates}$ T_{max} value on the DSC profiles increases with increasing lectin concentration.	Schwarz et al. (1993)
Creatine kinase (EC 2.7.3.2)	Human brain	Dimer, 85 kDa	5 mM Tris–HCl buffer, pH 8.0	Thermal inactivation studies at 25–65 °C, SEC analysis	$E_2 \xrightleftharpoons{\geq 40^\circ C} 2E_1 \xrightleftharpoons{40-55^\circ C} 2E_1 \xrightarrow{\geq 55^\circ C} \text{aggregates}$	Gao et al. (2010)
Filamentous actin	Rabbit skeletal muscles	Polymer	30 mM HEPES-KOH, pH 7.3, 2 mM MgCl ₂ , 2 mM adenosine diphosphate	DSC	T_{max} on the DSC profiles is shifted to the lower temperatures as the protein concentration decreases. The model suggests that at least two reversible stages precede the irreversible thermal denaturation of F-actin. One of them is dissociation of nucleotide (ADP) from nucleotide-binding sites of actin subunits, and the other is fragmentation of actin filaments or dissociation of monomers (or short oligomers) from the filament.	Mikhailova et al. (2006)
β -Galactosidase (EC 3.2.1.23)	<i>Escherichia coli</i>	Tetramer, 465 kDa	100 mM sodium phosphate buffer, pH 7.5	Thermal inactivation studies at 45–50 °C	$E_4 \rightleftharpoons 2E_2 \rightleftharpoons 4E_1 \rightarrow 4E_{den}$ active inactive Conformational lock number is equal to 2.	Atyaksheva et al. (2008)
	<i>Kluyveromyces fragilis</i>	Tetramer, 465 kDa	100 mM sodium phosphate buffer, pH 7.5	Thermal inactivation studies at 35 °C	$E_4 \rightleftharpoons 2E_2 \rightleftharpoons 4E_1 \rightarrow 4E_{den}$ inactive active inactive Conformational lock number is equal to 2.	
Glucose oxidase (EC 1.1.3.4)	<i>Penicillium adametzii</i> , extracellular	Dimer, 160 kDa	2 mM citrate–phosphate buffer, pH 5, or distilled water	Thermal inactivation studies at 60 and 66 °C, CD	$E_2 \rightleftharpoons 2E_1 \rightarrow 2E_{den}$ The order of denaturation with respect to protein (n) is less than unity.	Eremin et al. (2001)
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)	<i>Saccharomyces cerevisiae</i>	Equilibrium mixture of dimers and tetramers with $M_r = 102$ and 204 kDa, respectively	0.2 M borate buffer, pH 8.0	Thermal inactivation studies at 40–46 °C	$E_4 \xrightleftharpoons[k_{-2}]{k_2} 2E_2 \xrightleftharpoons[k_{-1}]{k_1} 4E_1 \xrightarrow{k_{den}} 4E_{den}$ inactive active inactive The rate constants of elementary steps and dissociation constants were estimated.	Zaitzeva et al. (1996)
			0.2 M borate buffer, pH 8.0	Thermal inactivation studies at 46 °C	$E_4 \rightleftharpoons 2E_2^{(1)} \rightleftharpoons 2E_2^{(2)} \rightleftharpoons 4E_1 \xrightarrow{k_{den}} 4E_{den}$ inactive active inactive The rate constants of elementary steps and dissociation constants (K_I and K_{II}) were estimated. Conformational lock number is equal to 2.	Zaitzeva et al. (2000)

(Table 1 continued)

Glutathione S-transferase (EC 2.5.1.18)	<i>Schistosoma japonicum</i>	Dimer, 52 kDa	20 mM sodium phosphate buffer, pH 6.5	Thermal inactivation studies, DSC	$E_2 \rightleftharpoons 2E_{den} \rightarrow \text{aggregates}$ The DSC curves with T_{max} values dependent on protein concentration indicate that the protein unfolds in a single transition coupled with dimer dissociation.	Kaplan et al. (1997)
Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	Rabbit skeletal muscles	Tetramer, 144 kDa	10 mM potassium phosphate buffer, pH 7.5	Thermal inactivation studies at 55 °C, DSC, AUC at 45 °C	$E_4 \rightleftharpoons 2E_2 \rightleftharpoons 4E_1 \rightarrow 4E_{den}$ Inactivation kinetics is dependent on protein concentration. The order of denaturation with respect to protein (n) is less than unity. T_{max} on DSC profiles is shifted towards the lower temperatures as protein concentration decreases. AUC data evidence for the dissociation of tetramers to dimers and monomers.	Markossian et al. (2006) [See also Khanova et al. (2007) Kurganov, Agatova (1965)]
Glycogen phosphorylase <i>b</i> (EC 2.4.1.1)	Rabbit skeletal muscles	Dimer, 195 kDa	80 mM HEPES-NaOH, pH 6.8 or 30 mM HEPES-KOH, pH 7.0	Thermal inactivation studies at 44–53 °C, DSC, DLS and AUC at 46–53 °C	$E_2 \rightleftharpoons E_2^* \rightleftharpoons 2E_1 \rightarrow 2E_{den}$ active inactive Inactivation kinetics is dependent on protein concentration. Lag period appears at rather high protein concentration. T_{max} on DSC profiles is shifted towards the lower temperatures as protein concentration decreases. AUC data evidence for the dissociation of dimer to monomers.	Komilaev et al. (1997) Kurganov et al. (2000) [See also: Meremyanin et al. (2008) Eronina et al. (2009) Eronina et al. (2010)]
Hemoglobin	<i>Glossoscolex paulistus</i> , extracellular	Hetero-oligomer, 3600 kDa	20 mM phosphate–borate buffer, pH 8.0	DSC	Oligomeric dissociation of the protein during thermal denaturation manifests itself in DSC scans as a shift of T_{max} to higher temperatures as the protein concentration is increased.	Santiago et al. (2010)
Hexokinase (EC 2.7.1.1)	<i>Saccharomyces cerevisiae</i>	Dimer, 110 kDa	0.2 M borate buffer, pH 8.0	Thermal inactivation studies at 40–47 °C	$E_2 \xrightleftharpoons[k_{-1}]{k_1} 2E_1 \xrightarrow{k_{den}} 2E_{den}$ The rate constants of elementary steps and dissociation constant ($K_{dis} = k_1/k_{-1}$) were estimated.	Zaitzeva et al. (1996)
Lectin	<i>Pisum sativum</i> (pea)	Dimer, 60 kDa	20 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.4	DSC	$(AB)_2 \rightleftharpoons 2A_{den} + 2B_{den} \rightarrow \text{aggregates}$ The dissociation of pea and lentil lectins into their α and β chains is supported by the increase with lectin concentration in the T_{max} value on the DSC profiles	Schwarz et al. (1993)
	<i>Lens culinaris</i> (lentil)	Dimer, 60 kDa	10 mM 3,3'-dimethyl glutaric acid-NaOH buffer, 0.15 M NaCl, 1 mM MnCl ₂ , 1 mM CaCl ₂ , pH 6.9	DSC		
	<i>Erythrina corallodendron</i>	Dimer, 57.5 kDa	20 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.4	DSC	$A_2 \rightleftharpoons 2A_{den} \rightarrow \text{aggregates}$ T_{max} value on DSC profiles increases with increasing the protein concentration.	Surolia et al. (1996)
	<i>Psophocarpus tetragonolobus</i> (winged bean), acidic	Dimer, 54 kDa	20 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.4	DSC	$A_2 \rightleftharpoons 2A_{den} \rightarrow \text{aggregates}$ T_{max} value on DSC profiles increases with increasing the protein concentration.	Srinivas et al. (1998)
Cu,Zn-Superoxide dismutase (EC 1.15.1.1)	Bovine erythrocyte	Dimer, 32 kDa	50 mM Tris-HCl buffer, 0.1 mM EDTA, pH 7.4	Thermal inactivation studies at 75 and 77 °C, analysis of the structural data	$E_2 \rightleftharpoons E_2^{(1)} \rightleftharpoons E_2^{(2)} \rightleftharpoons 2E_1 \rightarrow 2E_{den}$ stable labile labile	Hong et al. (2005)
Triosephosphate isomerase (EC 5.3.1.1)	<i>Entamoeba histolytica</i>	Dimer, 56 kDa	20 mM potassium tetraborate buffer, 1 mM dithiothreitol, pH 8.5	ANS binding, CD, DSC, intrinsic fluorescence, proteolytic cleavage studies, SEC.	Conformational lock number is equal to 2. $E_2 \rightleftharpoons 2E_1 \rightarrow 2E_{den}$ T_{max} value on DSC profiles increases with increasing the protein concentration.	Tellez et al. (2008)

ANS 1-anilinonaphthalene-8-sulfonate; AUC analytical ultracentrifugation; CD circular dichroism; DLS dynamic light scattering; DSC differential scanning calorimetry; SEC size exclusion chromatography; UV ultraviolet

systems, namely for concanavalin A, pea and lentil lectins (Schwarz et al. 1993), *Erythrina corallodendron* lectin (Surolia et al. 1996), winged bean acidic lectin (Srinivas et al. 1998), Phb from rabbit skeletal muscles (Kurganov

et al. 2000), filamentous actin (Mikhailova et al. 2006), *Entamoeba histolytica* triosephosphate isomerase (Tellez et al. 2008) and extracellular hemoglobin of *Glossoscolex paulistus* (Santiago et al. 2010).

Fig. 4 Temperature dependences of excess heat capacity (C_p^{ex}) of GAPDH (10 mM sodium phosphate buffer, pH 7.5) at various concentrations of the protein: 1 0.5, 2 1.5 and 3 3.0 mg/mL. C_p^{ex} was calculated per GAPDH tetramer. The heating rate was 1 °C/min. Adapted from Markossian et al. (2006)

Thermal aggregation of oligomeric proteins and testing anti-aggregation activity

Irreversible denaturation of oligomeric proteins is accompanied by the formation of aggregates (Fink 1998; Markossian and Kurganov 2004). The suppression of aggregation of non-native proteins in the cell is provided by the protective systems functioning with the participation of molecular chaperones and osmolytes (Fonin et al. 2016; Papp and Csermely 2006; Tanguay and Hightower 2015). To elucidate the mechanism of the anti-aggregation activity of chaperones and to select the agents possessing the high protective ability, in vitro test systems of different classes are used (Kurganov 2015). Among these test systems, those based on thermal aggregation of client proteins are of special interest. The distinctive feature of the test systems of such a class is that the aggregation stage is preceded by the stage of unfolding of the protein molecule. To substantiate the using of a test system for these purposes, the kinetic mechanism of denaturation and aggregation processes should be known.

The order of aggregation with respect to the protein (n^{agg}) is determined by the kinetic regime of protein aggregation. If the rate-limiting stage of the overall aggregation process is the unfolding of the protein molecule, the n^{agg} value coincides with the order of denaturation with respect to the protein. This reasoning allows us to understand the fact that the order of aggregation with respect to the protein for thermal aggregation of Phb at

48 °C was found to be 0.5 (Eronina et al. 2016). According to Eq. (5), the order of denaturation with respect to the protein becomes equal to 0.5 for the dissociating system $D \rightleftharpoons 2 M$ at rather high protein concentrations.

If the rate-limiting stage of the overall aggregation process is the aggregation of the unfolded protein molecules, the n^{agg} value is equal to 2 (Curto et al. 2012; Tarus et al. 2005). When studying the kinetics of protein aggregation at various temperatures, it was demonstrated that, at relatively low temperatures, the kinetic regime where the rate-limiting stage is unfolding of the protein molecule is realized ($n^{\text{agg}} = 1$) and elevation of the temperature results in switching to the kinetic regime where the rate-limiting stage is the aggregation of the unfolded protein molecules ($n^{\text{agg}} = 2$) (Borzova et al. 2016; Fedurkina et al. 2006; Nikolaidis and Moschakis 2017).

Denaturation and aggregation of oligomeric proteins under crowded conditions

Biochemical processes are highly sensitive to intracellular crowded conditions, arising from the presence of high concentrations of macromolecules. The latter can occupy up to 40 % of the total cellular volume, resulting in the decrease in the available volume. Such media are referred to as “crowded” (Ellis 2001; Ellis and Minton 2003; Minton 2001). Macromolecular crowding exerts influence on the equilibrium and kinetics of macromolecular reactions in which there exists a significant difference between the volume excluded to reactants and the volume excluded to products (Minton 2001). Such reactions include protein isomerization, folding/unfolding and protein–protein interactions (Chebotareva 2007; Chebotareva et al. 2001, 2004, 2010, 2013, 2015a, b; Ellis 2001; Ellis and Minton 2003; Gruebele et al. 2016; Hall and Minton 2003; Kurganov and Chebotareva 2013; Kuznetsova et al. 2014; Minton 2001; Roman et al. 2011, 2016; Sluchanko et al. 2015; Wills and Winzor 2011; Zhou et al. 2008). Macromolecules (proteins, polysaccharides, neutral polymers) as well as low-molecular-weight compounds (for example, osmolytes) can be used in model experiments as crowding agents (Chebotareva et al. 2001, 2004, 2010; Davis-Searles et al. 2001; Ellis 2001; Jiao et al. 2010; Roman et al. 2011; Sluchanko et al. 2015).

When interpreting the effect of crowding on self-association of proteins, we should take into account the existence of isomerization stages for individual oligomeric forms of the protein (Chebotareva et al. 2001; Harris and Winzor 1988). Consider, for example, self-association of Phb induced by allosteric activator AMP. In the

Scheme 1 The general scheme of thermal aggregation of oligomeric proteins proceeding under crowded conditions. Oligomer* is an oligomer with weak conformational lock [intermediate $E_2^{(m)}$ in Eq. (6)]

presence of AMP, the relatively compact T-state is transformed to the relaxed R-state which reveals an ability to associate to the tetrameric form R_4 :

Chebotaeva et al. (2001) showed that the experimentally observed effect of the crowding agent, trimethylamine N-oxide, was significantly less than the theoretically predicted effect. This result was explained by the displacement of the isomerization state $T_2 \leftrightarrow R_2$ towards the formation of the compact T_2 -state.

The general process of thermal aggregation of oligomeric proteins is represented in Scheme 1. This process includes destruction of the conformational lock, dissociation of oligomers into monomers, denaturation of monomers and aggregation of unfolded monomers. All the stages of thermal aggregation are greatly affected by the crowded medium. According to present views, crowding stabilizes compact conformations of native proteins and therefore interferes with the dissociation of the protein oligomer and protein denaturation. In contrast, crowded conditions stimulate the aggregation of unfolded proteins. These effects of crowding are demonstrated in Scheme 1.

When studying the aggregation of Phb induced by guanidine hydrochloride, it was shown that proline acts as a crowding agent enhancing protein aggregation (Eronina et al. 2005). The suppression of the dissociation of protein oligomers and protein denaturation under the action of 1 M proline was demonstrated for thermal denaturation of Phb at 48 °C (Eronina et al. 2009). Under given conditions, the rate-limiting stage for Phb aggregation was the unfolding of the protein molecule. One can expect that 1 M proline would enhance Phb aggregation. However, owing to the high stabilizing effect of 1 M proline, the overall action of this osmolyte is the suppression of the aggregation process. These data demonstrate that, when interpreting the effect of aggregation suppressors or crowding agents on the aggregation of target oligomeric proteins, consideration must be given to the nature of the rate-limiting stage of the aggregation process.

Conclusions

In the present review, attention has been focused on the mechanisms of the thermal denaturation of oligomeric proteins involving the stages of the reversible dissociation of oligomers into monomers. A convenient method for detecting these mechanisms is DSC. The change in the shape of the DSC profile for oligomeric proteins by varying the protein concentration (shift of $T_{\max}$ position) is indicative of the fulfillment of the dissociative mechanism of thermal denaturation.

When analyzing the kinetics of the thermal denaturation of oligomeric proteins or thermal inactivation of oligomeric enzymes, particular attention should be given to the analysis of the kinetic curves obtained at various concentrations of the protein. It has been just such an approach which can be used to substantiate the denaturation mechanism involving the stages of the reversible dissociation of the protein oligomer. However, nowadays, the theoretical basis applicable for the analysis of the series of the kinetic curves obtained at various protein concentrations for the denaturation of dissociating protein systems is practically lacking. The elaboration of appropriate approaches to analyze thermal denaturation kinetics for oligomeric proteins is one of the important problems of modern theoretical biophysics.

It is significant that the possible effects of crowding on the thermal denaturation of dissociable oligomeric proteins should be taken into account. Crowding can affect the stages of oligomer dissociation, denaturation and aggregation of unfolded protein molecules. It must be emphasized that the opposite effects may be observed for the action of crowding on the dissociation and denaturation stages on the one hand, and on the aggregation stages, on the other hand.

Acknowledgments We are very glad to congratulate Prof. Donald Winzor warmly on his 80th birthday. We wish him good health, many happy years of scientific activity and friendship of his many friends the world over. We appreciate greatly all that he has done to advance our understanding on the way macromolecules behave in the non-ideal environment. Since our review is devoted to the behavior of dissociating

enzymes, it is relevant to remember that Prof. Donald Winzor and his colleagues were among those who started to elaborate the theory of allosteric regulation based on the displacement of the equilibrium between oligomeric enzyme forms under the influence of allosteric effectors (Frieden 1967; Kurganov 1967; Nichol et al. 1967). Further investigations of Prof. Donald Winzor concerning the role of the protein isomerization induced by the binding of specific ligands or the presence of small inert cosolutes in fulfillment of the biological functions of proteins were of great interest to us, and we were glad to carry out the joint study of the effects of crowding on the allosteric properties of glycogen phosphorylase *b* (Chebotareva et al. 2001, 2005). It was a very exciting experience for me (N.A.C.) to talk to Prof. Donald Winzor and to discuss out experimental results.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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