Thermal Denaturation and Aggregation Assays in Analytical Biochemistry

Boris I. Kurganov*

Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow, Russia

In recent years, to screen the compounds revealing affinity to the proteins, the methods based on the registration of the change in thermal stability of proteins in the presence of the compounds being tested are widely used. In such experiments thermal denaturation is studied in the regime of heating of the protein solution at a constant rate. To control unfolding of the protein molecules, different physical methods are used, such as Differential Scanning Calorimetry (DSC) [1-9], intrinsic fluorescence [7,9,10-13], extrinsic fluorescence based on the measurements of the emission from extrinsic fluorescent dyes (8-anilino-1-naphthalenesulfonic acid, 4,4′-bis(1-anilino-8-naphthalenesulfonic acid), SYPRO Orange, Nile red) [14-27] and circular dichroism [10,28-30]. When denaturation of the protein is accompanied by irreversible aggregation of denatured protein molecules, the denaturation process can be followed by monitoring the increase in the light scattering of the protein solution [10,20,31-36] or the increase in apparent absorbance in the visible region [7,11,30]. The screening procedures using these physical methods can be automatized [3,10,14,18,20,21,28,29,32,37-39] resulting in acceleration of searching the compounds which are of practical importance, for example, compounds that reveal affinity to protein target and act as potential pharmaceutical products.

The screening systems based on the registration of the increment of the light scattering intensity on heating of the protein solution at a constant rate are of special interest. It is evident that such test systems are applicable for searching the compounds specifically interacting with the proteins and for the rough estimate of the stability of the complex protein–agent under test. Many investigators have restricted themselves to such an aspect of using of the screening systems. However one should take into account that the agents under study may affect not only the stage of protein denaturation but also the stage of aggregation of denatured molecules. In such cases the interpretation of the obtained results becomes complicated and the additional experiments should be performed to elucidate what stage of the general process of aggregation (stage denaturation or stage of aggregation) is affected by the agent under test. It is significant that, if for the screening systems, which are based on registration of excess heat capacity, fluorescence or ellipticity of the protein, aggregation is a factor complicating the interpretation of the results, for the screening systems based on registration of the light scattering intensity the investigator will have a chance of testing the compounds which exert their action exclusively on the stage of protein aggregation. The case in point is an array of protein chaperones and low-molecular-weight chemical chaperones.

First of all, consider the principles of analysis of the dependences of the light scattering intensity on temperature for the screening systems in which the protein undergoes denaturation on heating at a constant rate. When studying aggregation of interleukin-1β and its mutant forms, Wetzel with coworkers [40,41] used aggregation temperature ($T_{\text{agg}}$) to characterize the dependences of the light scattering intensity ($I$) on temperature ($T$). Parameter $T_{\text{agg}}$ was defined as a length cut off on the abscissa axis by continuation of the linear part of the dependence of $I$ on $T$ (Figure 1A). The advantage of this approach is that only one parameter is used for characterization of the propensity of the protein to aggregation, namely parameter $T_{\text{agg}}$. There is a correlation between parameter $T_{\text{agg}}$ characterizing thermal stability of sequence variants of interleukin-1β and the $\Delta\Delta G^\circ_{\text{ref}}$ value characterizing thermodynamic stability (the difference between the free energies of unfolding for mutant and for the wild type) for each sequence variant, as determined in reversible unfolding experiments in guanidine hydrochloride monitoring the fluorescence of the single tryptophan at position 120.

The dependence of the light scattering intensity ($I$) on temperature ($T$) has a sigmoid shape. At rather high temperatures the light scattering intensity reaches a limiting value ($I_{\text{lim}}$). To characterize thermostability of a protein, Senisterra and coworkers [31,33,35] used the temperature ($T_{\text{agg}}$) corresponding to the middle point of the transition, i.e., a temperature at which $I = I_{\text{lim}}/2$ (Figure 1B). Parameters $I_{\text{lim}}$ and $T_{\text{agg}}$ are determined by fitting of the experimental dependence of $I$ on temperature with the following empiric equation, analogous to the Boltzmann equation:

$$I = \frac{I_{\text{lim}}}{1 + \exp[(T_{\text{agg}} - T)/B]},$$

where $B$ is a constant. According to Senisterra and coworkers parameter $T_{\text{agg}}$ may be considered as a measure of protein thermostability and the change in the $T_{\text{agg}}$ value in the presence of a ligand characterizes the effect of the latter on protein thermostability. To substantiate this conclusion, the authors used DSC, which is a source of direct information on the protein resistance to high-temperature exposure. The correlation between the $T_{\text{agg}}$ value and the position of the maximum on the DSC profiles ($T_{\text{max}}$) was demonstrated [31,33].

Vedadi and coauthors [20] applied both fluorescence- and light-scattering-based approaches to measure the thermal stability of 221 recombinant proteins from humans and human parasites in the presence and absence of a range of chemicals. Purified proteins were subjected to gradually increasing temperature in both methods, and the temperature shift between the melting temperature ($T_{\text{m}}$ for fluorescence measurements or $T_{\text{agg}}$ for light-scattering measurements) in the presence and absence of a bound ligand was measured. The extent of the temperature shift is believed to be proportional to the affinity of the ligand for a given protein. The fluorescence and light-scattering approaches were applied to recombinant proteins in two experimental formats. In the first, the proteins were screened against a set of "generic" solution conditions designed to identify stabilizing conditions comprising salts, pH, and simple additives, such as

*Corresponding author: Boris I. Kurganov, Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow, Russia, Tel: +7(495)952-5641; Fax: +7(495)954-2732; E-mail: kurganov@inbi.ras.ru

Received November 06, 2013; Accepted November 07, 2013; Published November 11, 2013

Citation: Kurganov BI (2013) Thermal Denaturation and Aggregation Assays in Analytical Biochemistry. Biochem Anal Biochem 2: e143. doi: 10.4172/2161-1009.1000e143

Copyright: © 2013 Kurganov BI. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
When trying to estimate parameter $I_{\text{lim}}$, we should take into account that the "true" limiting level of the light scattering intensity may not be reached because of precipitation of the large-sized aggregates formed at high temperatures. Such a precipitation results in the decrease in the light scattering intensity, and the real experimental dependence of $I$ on temperature looks like a curve passing through a maximum. The maximum value of the light scattering intensity may be lower than the $I_{\text{lim}}$ value calculated from Eq. (1). Besides, the correlation between the increment of the light scattering intensity and the degree of protein denaturation should be controlled not only by checking the correlation between parameters $T_{\text{agg}}$ and $T_{\text{m}}$, but by strict analysis of the relationship between turbidimetric and calorimetric data, the latter supplying direct information on the degree of protein denaturation.

To avoid the uncertainty in the estimation of parameter $I_{\text{lim}}$, Eronina et al. [36] proposed to analyze the initial parts of the dependences of $I$ on temperature using the following empiric equation:

$$I = K_{\text{agg}} (T - T_0)^2, \quad (T > T_0)$$

In this equation $T_0$ is the initial temperature of aggregation, i.e., the temperature at which the light scattering intensity begins to increase (Figure 1C), and $K_{\text{agg}}$ is a constant with the dimension of $\text{(counts/s)}^{1/2} \text{°C}^{-2}$. To demonstrate the applicability of this equation, the data on thermal aggregation of glycogen phosphorylase b (EC 2.4.1.1), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and creatine kinase (EC 2.7.3.2) from rabbit skeletal muscles and bovine liver glutamate dehydrogenase (EC 1.4.1.3) were used [36]. The measurements of the hydrodynamic radius of the protein aggregates showed that the initial temperature of aggregation ($T_0$) indicates the moment of origination of the start aggregates. The hydrodynamic radius of the start aggregates ($R_{\text{agg}}$) is several tens of nanometers. The analysis of the data obtained by Golub et al. [42] shows that analogous situation takes place for thermal aggregation of aminotransferase (EC 2.6.1.1) from pig heart mitochondria ($T_0 = 54.6 \pm 0.5$ °C and $R_{\text{agg}} = 60 \pm 1$ nm). As demonstrated in [36], the $T_0$ value decreases with increasing the protein concentration whereas parameter $K_{\text{agg}}$, which can be considered as a measure of the aggregation rate, increases as the protein concentration increases.

Aggregation systems under discussion may be used for testing the compounds affecting protein stability as a result of direct binding to the native protein molecule (for example, substrates and modifiers of the enzymes). Besides, test systems based on thermal aggregation of model proteins in the regime of heating at a constant rate allow the anti-aggregation properties of the compounds possessing chaperone-like activity (for example, small heat shock proteins) to be characterized [43-49]. Strictly speaking, if we want to select agents affecting exclusively the stage of denaturation, we should demonstrate that these agents have no effect on the stage of aggregation. To solve this problem, the investigator should have at his disposal the screening systems which allow the effect of the agents on the stage of protein aggregation to be tested. In test systems of such a type the preliminary denatured protein should be used. For example, test systems based on aggregation curve-associated temperature ($T_{\text{agg}}$) to characterize the propensity of the protein to aggregation. The lower the $T_{\text{agg}}$ value, the higher is the propensity of IL-1ra to aggregation. Using this approach, the acceleration of aggregation of IL-1ra with increasing protein concentration was demonstrated.

When studying thermal aggregation of human interleukin-1 receptor antagonist (IL-1ra), Raibekas [32] also used the midpoint aggregation curve-associated temperature ($T_{\text{agg}}$) to characterize the propensity of the protein to aggregation. The lower the $T_{\text{agg}}$ value, the higher is the propensity of IL-1ra to aggregation. Using this approach, the acceleration of aggregation of IL-1ra with increasing protein concentration was demonstrated.

When studying thermal aggregation of human interleukin-1 receptor antagonist (IL-1ra), Raibekas [32] also used the midpoint aggregation curve-associated temperature ($T_{\text{agg}}$) to characterize the propensity of the protein to aggregation. The lower the $T_{\text{agg}}$ value, the higher is the propensity of IL-1ra to aggregation. Using this approach, the acceleration of aggregation of IL-1ra with increasing protein concentration was demonstrated. It is evident that the accuracy of determining parameter $T_{\text{agg}}$ is connected with the reliability of the estimation of parameter $I_{\text{lim}}$.
and aggregation processes. This study was funded by the Russian Foundation for Basic Research (grant 11-04-00932-a) and the Program “Molecular and Cell Biology” of the Presidium of the Russian Academy of Sciences.

References


