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Characterization of heat-induced aggregates of concanavalin A using fluorescent probes

Motonori Kudou, Kentaro Shiraki, Masahiro Takagi*

School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1, Asahidai, Tatsunokuchi, Ishikawa 923-1292, Japan

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Abstract

Proteins are prone to aggregate at high temperature. In order to investigate the heat-induced aggregation, Concanavalin A (Con A) was used as a model protein because disulfide formation doesn't occur throughout the aggregation process. With increasing temperature, fluorescence intensities of 8-anilino-1-naphthalenesulfonate (ANS) and thioflavin T (ThT) showed a maximum at around 45–50 °C. After the heating, the fluorescence intensities increased with decreasing temperatures. The enhancement of the fluorescence during cooling implies that the heat-induced aggregates of Con A possess porosity on this surface allowing the binding to fluorescent probes.

Keywords: Heat-induced aggregates; Concanavalin A; Thioflavin T; 8-Anilino-1-naphthalenesulfonate

1. Introduction

Proteins tend to aggregate when their exposed hydrophobic surfaces are associated with each other. In the biomedical and biotechnological fields, protein aggregation poses a serious problem during purification, refolding, and storage. Difficulty in retaining the active form, as well as controlling the formation of aggregates, is attributed to a fundamental property of proteins. In addition to the problem in large-scale production of recombinant proteins, protein aggregation is a fatal problem in living cells [1,2].

In order to understand and control the formation of aggregates, we have studied Concanavalin A (Con A) as a model protein. Below pH 6.5, Con A is a dimer with a molecular mass of 25,500 × 2 and a single carbohydrate binding site in the dimeric interface [3,4]. Con A has two metal-binding sites, S1 and S2, which bind transition metals and calcium ion. Apo-Con A is more susceptible to heat-induced aggregation than metal-binding Con A (holo-Con A). Holo-Con A is highly stable with a melting temperature at 101 °C, whereas that of apo-Con A is at 74 °C at pH 5.0 [5].

The fluorescence emission of thioflavin T (ThT) and 8-anilino-1-naphthalenesulfonate (ANS) are often used for characterizations of protein aggregates. The former and latter are specific markers for the intermolecular β-sheet conformation and hydrophobic cluster, respectively [6,7]. Kundu and Guptasarma have shown that the ANS molecules preferentially adsorb onto the hydrophobic surface with decreasing temperature, implying that aggregates of carbonic anhydrase has a porous surface [8]. In this paper, the heat-induced aggregates of Con A were characterized by ThT fluorescence, and the data was compared to ANS fluorescence.

2. Experimental procedure

2.1. Materials

Con A and ANS were purchased from Sigma Chemical Co. (St Louis, Missouri). ThT, citrate and sodium citrate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Stock solution of protein was prepared by dissolving Con A powder in deionized water at a final concentration of 3.0 mg/ml. The solution was filtrated with 0.20 μm syringe filter and stored at 4 °C.

2.2. Turbidity measurements

Sample buffer containing 100 mM Na-crirate buffer (pH 6.0) was incubated for 3 min at respective temperatures

* Corresponding author. Tel.: +81-761-51-1650; fax: +81-761-51-1655.
E-mail address: takagi@jaist.ac.jp (M. Takagi).

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before addition to Con A. The protein solution was diluted 10-fold into a final condition of 0.3 mg/ml Con A in 100 mM Na-crirate buffer (pH 6.0). Turbidities were recorded on a Jasco spectrophotometer, model V-550 (Japan Spectroscopic Company, Tokyo, Japan) using a thermostatically controlled cuvette at 35–70 °C. The turbidities were measured using an absorbance at 600 nm intensity changes.

2.3. Fluorescence measurements

The measured solutions were 0.3 mg/ml Con A, 50 μM ANS or ThT, and 100 mM Na-crirate buffer (pH 6.0). The sample was heated from 25 to 70 °C at a scan rate of 2 °C/min. The heated sample was immediately cooled from 70 to 25 °C at the same scan rate. Again, the sample was heated from 25 to 70 °C at the same scan rate. During heating and cooling, fluorescence intensity change was measured. Fluorescence emission spectra were recorded on a Jasco spectrofluorometer, model FP-6500 (Japan Spectroscopic Company) using a thermostatically controlled cuvette at 25–70 °C. ANS fluorescence was measured using an excitation wavelength at 350 nm, with the emission recorded at 480 nm. ThT fluorescence was measured using an excitation wavelength at 446 nm, with the emission recorded at 480 nm.

3. Results and discussion

3.1. Temperature-dependent aggregation

Fig. 1 shows the time course of aggregation at different temperatures. The heat-induced aggregation was investigated by turbidity at 600 nm. No aggregates were observed below 40 °C, whereas aggregates were observed above 45 °C. With increasing temperature, rate of aggregation increased. At 45 °C, the curve of aggregation had a lag phase of 100 s. The final intensity of turbidities were retained almost constant at 45–70 °C.

Appearance of lag phase and increase in turbidity are due to the following reasons. At the high temperature, native structure would be transformed into a denatured structure. The hydrophobic portions of globular proteins are exposed on protein surface under high temperature, followed by an increase in turbidity with growing aggregates. Aggregation relates to denaturation rate of protein by heating. Most likely, the increase in turbidity for the main part of the kinetic aggregation is concerned with the enlargement of aggregates depending on incubation temperature.

3.2. ANS binding

Fig. 2 shows the fluorescence intensity of ANS during heating and cooling. The fluorescence intensity showed a prominent maximum at around 40–45 °C on the heating cycle. However, this maximum disappeared on the cooling cycle. Interestingly, the fluorescence linearly increased with decreasing temperature. These data indicate that the change in ANS fluorescence is fully reversible by changing temperature.

The increase in the fluorescence during cooling is not related to an increase in hydrophobic surfaces for ANS binding [8]. Moreover, the hydrophobic clefts located on the surface of aggregate are quite sensitive to temperature; i.e. the decreased temperature results in an increased propensity to bind hydrophobic dye [9]. These results suggest that heat-induced aggregate of Con A could be porous structure allowing the binding to ANS.

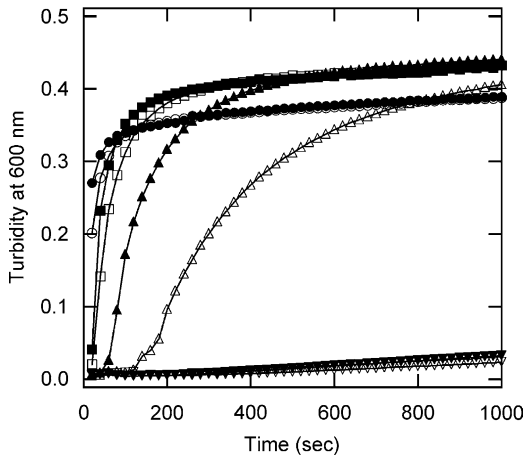

Fig. 1. Timecourse for aggregation of Con A at 70 °C (closed circles), 65 °C (open circles), 60 °C (closed squares), 55 °C (open squares), 50 °C (closed triangles), 45 °C (open triangles), 40 °C (closed inverted triangles) and 35 °C (open inverted triangles). The signal were monitored by absorbance at 600 nm intensity change.

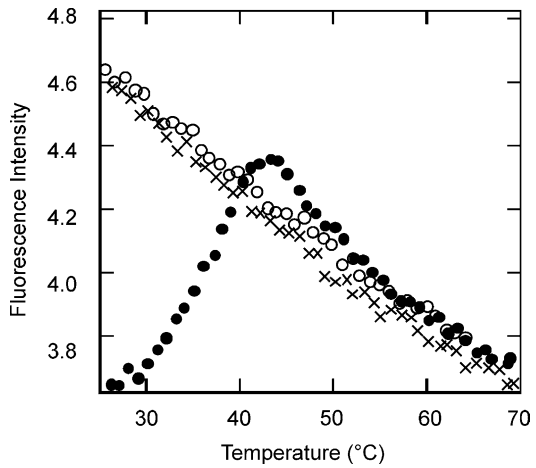

Fig. 2. Fluorescence intensity of ANS as a function of temperature. Heating (closed circles), cooling (open circles) and reheating (crosses). The fluorescence was monitored by 480 nm excited at 350 nm.

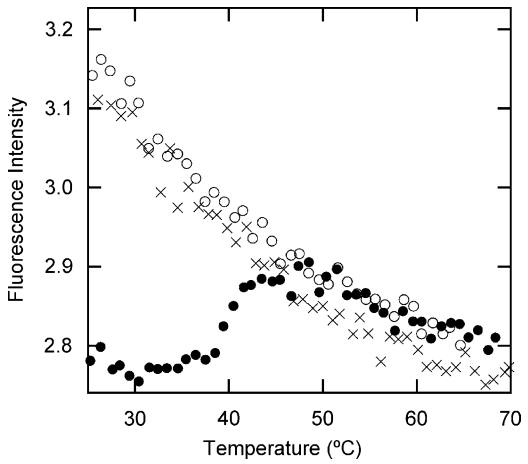

Fig. 3. Fluorescence intensity of ThT as a function of temperature. Heating (closed circles), cooling (open circles) and reheating (crosses). The fluorescence was monitored by 480 nm excited at 446 nm.

3.3. ThT binding

Fig. 3 shows the fluorescence intensity of ThT during heating and cooling. The temperature-dependence of fluorescence intensity of ThT intensity showed the similar profile to that of ANS. This maximum disappeared on the cooling cycle. These data indicate that the fluorescence change of ThT was fully reversible by changing temperature.

It should be noted that ANS and ThT bind to the different regions of proteins, hydrophobic cluster and intermolecular β-sheet, respectively. However, the similar profiles were obtained as shown in Figs. 2 and 3. Maeda et al., have shown that the heat-induced aggregates of Con A contains large amount of β-sheet [10], implying that the aggregates of Con A may be stabilized by β-sheet interactions as well as hydrophobic interactions. Most likely, the increase in fluorescence of ThT during cooling is attributed to the enlargement of β-sheet content on the surface of aggregates. This is highly related to the increase in size of pore on this surface (Fig. 2).

3.4. Property of heat-induced aggregates

The present paper revealed that the heat-induced aggregates of Con A contains large amount of pore on the surface. Theoretically, overall porosity of aggregates decreases with increasing hydrophobicity of proteins [11]. This indicates that the porosity of aggregates may depend on pH and ionic strength. From an experimental data, there are no ANS-accessible pores measured at the isoelectric pH [9]. The pH- and temperature-dependent porosity implies that aggregates contain large amount of refolded molecules. As proteins refold, the pores were formed on surface of aggregates.

4. Conclusion

In this paper we studied the heat-induced aggregates of Con A using fluorescence probes ANS and ThT. The following conclusions were proposed. (1) Aggregates of Con A were observed by the heat treatment above 45 °C at pH 6.0. (2) Fluorescence intensity of ANS increased with decreasing temperature. The intensity change was fully reversible. (3) Temperature-dependence of fluorescence intensity change of ThT was similar to that of ANS. These fluorescence data are related to an aggregate surface of Con A.

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