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Heat-induced Conversion of β_2-Microglobulin and Hen Egg-white Lysozyme into Amyloid Fibrils

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Thermodynamic parameters characterizing protein stability can be obtained for a fully reversible folding/unfolding system directly by differential scanning calorimetry (DSC). However, the reversible DSC profile can be altered by an irreversible step causing aggregation. Here, to obtain insight into amyloid fibrils, ordered and fibrillar aggregates responsible for various amyloidoses, we studied the effects on human β_2-microglobulin and hen egg-white lysozyme of a combination of agitation and heating. Aggregates formed by mildly agitating protein solutions in the native state in the presence of NaCl were heated in the cell of the DSC instrument. For β_2-microglobulin, with an increase in the concentration of NaCl at neutral pH, the thermogram began to show an exothermic transition accompanied by a large decrease in heat capacity, followed by a kinetically controlled thermal response. Similarly, the aggregated lysozyme at a high concentration of NaCl revealed a similar distinct transition in the DSC thermogram over a wide pH range. Electron microscopy demonstrated the conformational change into amyloid fibrils. Taken together, the combined use of agitation and heating is a powerful way to generate amyloid fibrils from two proteins, β_2-microglobulin and hen egg-white lysozyme, and to evaluate the effects of heat on fibrillation, in which the heat capacity is crucial to characterizing the transition.

Keywords: amyloid fibril; aggregation; dialysis-related amyloidosis; heating; heat capacity

Introduction

A differential scanning calorimeter (DSC) is a useful tool for obtaining thermodynamic parameters of the unfolding of globular proteins.[1] Beginning with the seminal calorimetric measurements reported by Privalov and colleagues,[1,2] experimental studies with DSC have focused on the reversible folding/unfolding transitions under equilibrium conditions, and the calorimetric data have been analyzed principally by assuming a two-state transition between the native (N) and unfolded (U) states (Scheme 1).[3]

$$N \Leftrightarrow U$$

Scheme 1.

On the other hand, heat-induced unfolding has been recognized to be followed occasionally by an irreversible process that induces aggregation.[4–7] Generally, the heat-induced aggregation of proteins has been modeled as shown in Scheme 2,[4–7] where A is an irreversibly unfolded protein that undergoes further reaction to form insoluble aggregates A_m composed of m monomers. The model

$$N \Leftrightarrow U \rightarrow A,$$

$$A_m + A \rightarrow A_{m+1}$$

Scheme 2.

Abbreviations used: β_2-m, β_2-microglobulin; HEWL, hen egg-white lysozyme; DSC, differential scanning calorimeter/calorimetry; $C_{p,app}$, apparent heat capacity; ThT, thioflavin T.

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involves reversible unfolding of the protein and subsequent aggregation of non-native species. In the heat-induced unfolding of globular proteins, a non-native monomer (i.e. aggregation-competent species), which is formed upon an endothermic transition, is thought to subsequently undergo exothermic inter-protein stacking, resulting in aggregation.[4–7] How the unfolding properties of a given protein influence the heat-induced aggregation, particularly the formation of amyloid fibrils, has not been fully characterized owing to the intricate kinetics during the heating process.[8,9]

Amyloid fibrils have begun to receive marked attention, because they have been recognized to be a pathological hallmark of a number of neurodegenerative (e.g. Alzheimer's, prion, Parkinson's, and Huntington's diseases) and non-neuropathic (e.g. type II diabetes, dialysis-related amyloidosis, lysozyme amyloidosis) diseases.[10,11] The main-chain backbone of amyloid fibrils is arranged in a cross-β structure oriented perpendicular to the fibril axis, which is a common characteristic of amyloid fibrils.[12] Studies *in vitro* have shown that the ability to form fibrils is a generic property of polypeptide chains, not just those involved in disease.[13] However, the mechanism by which a wide range of peptides and proteins form amyloid fibrils remains elusive.[10–13]

In a previous study,[14] we showed that, under acidic conditions, salt-induced protofibrils of β_2-m that were aggregated intentionally by agitation can be efficiently converted into well-ordered mature amyloid fibrils by heating them in a cell of the DSC instrument. DSC enabled monitoring of the heat flow accompanying the conversion. Importantly, heating itself was not sufficient to generate amyloid fibrils, but in combination with agitation it effectively triggered the formation of amyloid fibrils. The result suggested a promoting effect of moderate aggregation on the formation of amyloid templates, in which protofibrils in an aggregated state were susceptible to specific inter-molecular interactions between adjacent molecules, leading to the conformational rearrangement into a cross-β structure characteristic of mature fibrils. This finding prompted us to extend the combination of agitation and heating (i.e. agitation-heating method) to more physiological phenomena; that is, the conversion of protein molecules in the native state into amyloid fibrils at physiological pH.

We chose two globular proteins, recombinant human β_2-microglobulin (β_2-m) and wild-type hen egg-white lysozyme (HEWL), as models to study heat-induced fibrillation. *In vivo*, β_2-m consisting of 99 amino acid residues assumes a β-sandwich fold involving seven β-strands stabilized by a single disulfide bond, and associates non-covalently with the major histocompatibility complex class I heavy chain.[15] Fibrillation of β_2-m released from the major histocompatibility complex class I is a serious complication in patients receiving hemodialysis for more than ten years.[16,17] *In vitro*, β_2-m in the acid-unfolded state at pH 2.5 can be converted into amyloid fibrils seed-dependently or independently.[18–23] At neutral pH, this protein is stably folded and highly intransigent to assembly into amyloid fibrils; fibril formation has been reported to require significant destabilization of the protein, which can be achieved *via* deletion of six N-terminal residues,[24] mutation of terminal β-strands,[25] addition of copper ions,[26] and addition of fibrillation-nucleating seed fibrils,[27–29] suggesting that the species responsible for fibril formation is vary rarely populated under physiological conditions. How β_2-m forms amyloid fibrils *in vivo* is not well understood.[22,27,30]

HEWL of 129 residues is one of the most studied and best characterized proteins.[31] The native structure of this protein is composed of two domains (α and β), and is cross-linked by four disulfide bonds.[32] HEWL is homologous to human lysozyme, whose familial mutations are associated with non-neuropathic systemic amyloidosis (i.e. lysozyme amyloidosis).[33,34] Fibrillation of HEWL has been observed upon incubation at acidic pH (pH 1.6–2) and elevated temperature (57–70 °C).[35–38] Its well-documented 3D structure, thermodynamic stability, folding mechanism, and ability to form amyloid fibrils make this protein an excellent model for the study of fibrillation *in vitro*.[31,32,35–38]

With these proteins, we show that a combination of agitation and heating effectively triggers the formation of amyloid fibrils even at physiological pH, and that DSC has the advantage of being able to evaluate the effects of heat associated with the fibrillation.

Results

Heat-effects on the aggregated β_2-m

To examine the fibrillation with DSC, 30 μM β_2-m in the presence of various concentrations of NaCl was preliminarily incubated with agitation to generate aggregation in the cell of an isothermal titration calorimeter (VP-ITC) at 37 °C by stirring the solution with the attached cylinder at 310 rpm as described.[14] The pH of the protein solution in the absence of buffer was about 7.3–7.6 and not strictly controlled to eliminate the possibility that particular buffer ions could affect the aggregation process. The aggregation of proteins is usually promoted by stirring the solution.[14,39] Here, the dependence of aggregation on the concentration of NaCl and on incubation time was examined at 37 °C. As expected, the agitation facilitated the formation of larger aggregates with increases in the concentration of NaCl and the incubation time, as indicated by the increased light-scattering (see Figure 1(a) and (b)). Additionally, the increase in light-scattering intensity associated with aggregation by agitation at 25 °C was found to be about one-fourth of that obtained at 37 °C under identical conditions, suggesting that destabilization of the protein at 37 °C has a significant influence on the stirring-induced aggregation. Furthermore, as the rate of stirring decreased, the light-scattering intensity of the