Heat of supersaturation-limited amyloid burst directly monitored by isothermal titration calorimetry

Tatsuya Ikenoue a,1, Young-Ho Lee a,1, József Kardos b, Hisashi Yagi a, Takahisa Ikegami a, Hironobu Naiki c, and Yuji Goto a,2

aDivision of Protein Structural Biology, Institute for Protein Research, Osaka University, Osaka 565-0871, Japan; bDepartment of Biochemistry, Eötvös Loránd University, 1117, Budapest, Hungary; and cFaculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan

Edited* by David S. Eisenberg, University of California, Los Angeles, CA, and approved March 26, 2014 (received for review December 12, 2013)

Amyloid fibrils form in supersaturated solutions via a nucleation and growth mechanism. Although the structural features of amyloid fibrils have become increasingly clearer, knowledge on the thermodynamics of fibrillation is limited. Furthermore, protein aggregation is not a target of calorimetry, one of the most powerful approaches used to study proteins. Here, with β2-microglobulin, a protein responsible for dialysis-related amyloidosis, we show direct heat measurements of the formation of amyloid fibrils using isothermal titration calorimetry (ITC). The spontaneous fibrillation after a lag phase was accompanied by exothermic heat. The thermodynamic parameters of fibrillation obtained under various protein concentrations and temperatures were consistent with the main-chain dominated structural model of fibrils, in which overall packing was less than that of the native structures. We also characterized the thermodynamics of amorphous aggregation, enabling the comparison of protein folding, amyloid fibrillation, and amorphous aggregation. These results indicate that ITC will become a promising approach for clarifying comprehensively the thermodynamics of protein folding and misfolding.

Results

Heat for the Formation of Amyloid Fibrils Monitored by ITC. At pH 2.5, acid-denatured β2m formed amyloid fibrils in the presence of moderate concentrations of NaCl. As defined by the conformation phase diagram, fibril formation is dependent on protein and NaCl concentrations (Fig. S1) (19, 33). Spontaneous fibrillation was previously shown to be facilitated by various kinds of agitations such as stirring with a magnetic bar (34, 35) or ultrasonication (19, 36–39), leading to a burst phase of fibrillation after a lag phase. Under the conditions of persistent metastability of supersaturation, it is likely that these agitations may create seed-competent conformations. For instance, during

Significance

Although amyloid fibrils are associated with numerous pathologies, their conformational stability remains largely unknown. In particular, calorimetry, one of the most powerful methods used to study the thermodynamic properties of globular proteins, has not played a significant role in understanding protein aggregation. The aggregation of proteins following heat denaturation as monitored by differential scanning calorimetry is an infamous example demonstrating how aggregation can prevent exact analyses (25, 26). To date, few studies have investigated protein aggregation including amyloid fibrils with calorimetry (27–32). Our previous study on the exothermic heat effects accompanying fibril growth was achieved by monitoring the seed-dependent elongation of fibrils formed by β2-microglobulin (β2m), a protein responsible for dialysis-related amyloidosis, using isothermal titration calorimetry (ITC) (28).

In the present study using β2m, we succeeded in characterizing the total heat of spontaneous fibrillation and amorphous aggregation. An analysis of the heat burst associated with fibrillation or amorphous aggregation under various temperatures clarified their thermodynamic properties. The results obtained enabled the calorimetric characterization of amyloid fibrils and amorphous aggregates relative to that of the native globular structures, which opens a new field for the calorimetric study of protein aggregates.
In our studies, we used the ITC instrument to agitate the β2m solution and monitor the heat response of fibrillation. To establish supersaturation in the presence of various concentrations of β2m at pH 2.5 in the cell at 37 °C, the NaCl concentration was increased to a final value of 0.1 M by stepwise injections of a small volume of 1.0 M NaCl (Fig. 1A; Materials and Methods). After each injection, a sharp endothermic or exothermic spike, which represented the heat (q) of salt dilution, occurred and the heat flow ([=dq/dt]) returned back to the original reference power level. Notably, a marked exothermic peak with a half-width of ~2 h occurred at 0.3 mg·mL−1 of β2m at ~11 h (Fig. 1A). Similar exothermic peaks were observed at other concentrations of β2m. The lag time for the major exothermic peak (Materials and Methods) shortened (Fig. S2A) and the exothermic peak became larger with an increase in the protein concentration. When 0.5 mg·mL−1 of the β2m solution at 0.1 M NaCl was prepared in a test tube, set in the ITC cell, and followed by stirring, a similar exothermic burst with a lag time of 3.7 h was observed (Fig. S3). When we consider the time for titration of salt (3.5 h; Fig. S4), the observed lag time was independent of the methods, although the titration in the ITC cell was much simpler. The results suggested that the titration inside the ITC cell did not bring any additional effects.

The total heat calculated based on the peak area was normalized by the protein concentration. The normalized heat did not depend significantly on the protein concentration (Fig. 1E). Moreover, when the stirring speed was varied in the range of 200–1,000 rpm with a fixed protein concentration of 0.5 mg·mL−1, the lag time shortened with an increase in the speed (Fig. 2A and Fig. S2B). However, the total heat was independent of the stirring speed (Fig. 2B). These results suggested that the observed heat represented the enthalpy change (ΔH) of the reaction triggered by stirring. Assuming that the observed total heat was ΔH, the ΔH value at 37 °C was estimated to be −77 kJ·mol−1 from the dependence on stirring speed or −74 kJ·mol−1 from the dependence on protein concentration. The decrease in magnitude of ΔH at high protein concentrations may have been linked with the partial and transient formation of amorphous aggregates with a smaller ΔH value (see below).

After the exothermic peaks, all β2m solutions exhibited a far-UV CD spectrum with a minimum at ~218 nm, an atomic force microscopy (AFM) image of fibrils with a height of 4.5–9.0 nm and various lengths up to 1 μm, and strong thioflavin T (ThT) fluorescence (Fig. 1B–D). These results indicated that β2m solutions above 0.3 mg·mL−1 in 0.1 M NaCl at pH 2.5 were supersaturated (or metastable) and that agitation by stirring broke this supersaturation, resulting in amyloid fibrillation. We consider that the exothermic peak represents the formation of amyloid fibrils ("amyloid burst") and the observed heat gives its ΔH value. Similar effects were expected for other salts, the effectiveness of which follows the electroselectivity series (19, 33). One experiment with ammonium sulfate was shown in Fig. S4.

**Small Amyloid Burst and Excess Heat Immediately After Salt Titration.**

Careful inspection of the ITC thermograms indicated that, in all of the ITC profiles, a small exothermic peak, which we designated "small amyloid burst," appeared before the main amyloid burst (Figs. 1A, 2A, and 5A). To clarify the significance of these small peaks, we performed CD and AFM measurements and a ThT assay at several time points during the reaction at 1.0 mg·mL−1 β2m (Fig. 3B and C). Neither the CD spectrum nor the AFM image showed significant changes before and immediately after the salt injection spikes, which indicated that the dominant molecular species were still monomers. When the small exothermic peak appeared at the ~5.5-h time point, the AFM image revealed the presence of short and thin fibrils with a height of 2.6–5.3 nm. A slight change in the CD spectrum and small increase in ThT fluorescence were also observed. These results indicated that some fibrillation, possibly the formation of protofibrils, started at the point

---

**Fig. 1.** Calorimetric observation of the amyloid burst of β2m at various protein concentrations at 37 °C. (A) Thermograms of the fibril formation of β2m at 0.3–6.7 mg·mL−1 and pH 2.5 obtained using ITC. Inset shows a close-up view of exothermic heat at 0.3 mg·mL−1 β2m. The arrowheads indicate the locations of "small burst." These also apply to the thermograms of Figs. 2, 3, and 5. (B–D) Characterization of β2m solutions after incubation in ITC cells by AFM images (B), far-UV CD spectra (C), and ThT fluorescence intensities (D). The scale bars on the AFM images indicate 1 μm, and the numbers under images are fibril height. The scale bar on the right represents the height. These also apply to AFM images in Figs. 3–5. (E) Dependences of the observed heat of the small peak (black inverted triangle), main peak (red circles), total heat including rapid heat effect (green circle), and amorphous aggregation (black circles) on the protein concentration. Inset in C shows the expansion of the heat of amorphous aggregation. The observed heats were normalized by the β2m concentration to give the ΔH values.
of the small burst, and subsequent elongation coupled with the breakage of fibrils to make new growing ends (i.e., secondary nucleation) caused the explosive amyloid burst (Fig. 3B and C). The exact position and size of the minor peaks were less dependent on the experimental conditions than those of the major peaks (Figs. 1A, 2A, and 5A, and Fig. S2). The total heat accompanying the small exothermic peak was constant (−1.5 kJ·mol⁻¹) and independent of the protein concentration (Fig. 1E). Although the observed heat contained information on the ΔH value of protofibril formation, its small fraction precludes further analysis.

We also recognized a small excess heat effect immediately after each of the stepwise addition of 1.0 M NaCl (Fig. S5). This small but notable heat effect increased with an increase in the concentration of NaCl and β2m, suggesting that it represents the formation of amorphous aggregates. However, after the completion of major amyloid burst, the formation of amorphous aggregates was evident neither from the CD spectra, ThT intensities, nor AFM images (Fig. 1B–D). Thus, it is possible that a small amount of amorphous aggregates formed after the salt injection finally transformed to the fibrils, although the exact kinetics is unknown. If this is a case, a total heat including those of rapid heat effect, small amyloid burst, and major amyloid burst should represent the ΔH value for amyloid fibrillation. Indeed, the sum of these heat effects was constant (−78 kJ·mol⁻¹) over a wide range of concentration, suggesting the validity of assumption (Fig. 1E).

**Heat of Amorphous Aggregation.** β2m formed amorphous aggregates at very high NaCl concentrations above 0.8 M at pH 2.5 (Fig. S1) (19). In analogy with the crystallization of substances, amyloid fibrils and amorphous aggregates were shown to be similar to crystals and glasses, respectively (19). In Yoshimura et al. (19), we showed that, whereas crystalline amyloid fibrils formed after a lag phase, glassy amorphous aggregates formed without a lag phase. The rapid and partial formation of amorphous aggregates after the salt titration was consistent with this view (Fig. S5).

It was difficult to increase the NaCl concentration in the cell up to ∼1.0 M by injecting the NaCl solution at a high concentration in the syringe. Thus, we performed an inverse titration: the β2m solution at a high concentration in the syringe was injected into the cell containing 1.0 M NaCl (Fig. 4). On the bases of the low CD signal, amorphous aggregates revealed by
the ITC cell with 3.6 mg of seed-dependent elongation, the current neous fibrillation obtained here were slightly smaller than those A seed-dependent elongation of amyloid fibrils of Δ obtained the Δ of fibrillation based on the relationship of ΔCp obtained the ΔCp magnitude from Δ the products observed after heat burst at all of the temperatures Δ were amyloid fibrils (Fig. 5). We also measured temperature dependence of the heat effects Δ at 37 °C and assumed to be Δ1 at 37 °C and assumed to be Δ1 (Fig. 6). Assuming that the observed heat effect represented ΔH, temperature dependence provided a heat capacity change (ΔCp) of fibrillation based on the relationship of ΔCp = ΔH/ΔT. The plot of ΔH against temperature was linear, providing a ΔCp value of −5.0 kJ·mol⁻¹·K⁻¹ (Fig. 6A and Table S1). We previously obtained the ΔH value and temperature dependence for the seed-dependent elongation of amyloid fibrils of β2m monitored by ITC (28) (Fig. 6A). Although the ΔH values for the spontaneous fibrillation obtained here were slightly smaller than those of seed-dependent elongation, the current ΔCp value was similar to that (−4.8 kJ·mol⁻¹·K⁻¹) of seed-dependent elongation (28). We also measured temperature dependence of the heat effects of amorphous aggregation (Figs. 4B and 6A, and Table S1). Although the ΔH values for amorphous aggregates at 1.0 M and various temperatures were smaller in intensity than those of mature fibrils, ΔH changed linearly against temperature, providing a ΔCp value (−3.5 kJ·mol⁻¹·K⁻¹) that was slightly smaller than that of amyloid fibrils.

**Temperature Dependency of Aggregation Heat.** ITC measurements of the amyloid burst at 1.0 mg·ml⁻¹ were performed at various temperatures between 31 and 43 °C (Fig. 5A). The lag time shortened (Fig. 2C) and the exothermic peak became larger with an increase in temperature. The ΔH value increased in magnitude from −41.3 to −101.1 kJ·mol⁻¹ (Fig. 6A and Table S1). We confirmed using far-UV CD, AFM, and ThT assays that the products observed after heat burst at all of the temperatures were amyloid fibrils (Fig. 5B–D).

**Evaluation of Thermodynamic Parameters.** To comprehensively understand the thermodynamics of aggregation, we have to know the changes in free energy (ΔG) and entropy (ΔS) in addition to the ΔH and ΔCp terms, which are directly determined by calorimetry (24). Although the detailed mechanical models of fibril formation remain elusive (16), a simplified model will still be valid for describing the equilibrium between monomers (M) and fibrils (P) (13–15, 28):

\[
(P) + (M) = \frac{k_1}{k_{-1}} (P), \tag{1}
\]

where \(k_1\) and \(k_{-1}\) are the apparent rate constants for polymerization and depolymerization, respectively. The elongation of fibrils is defined by the equilibrium association constant (K) as follows:

\[
K = \frac{[P][M]}{[P][M]} = k_1/k_{-1}, \tag{2}
\]

where [P] is the concentration of fibrils and [M] is the concentration of monomers. The equilibrium is clearly independent of [P]. Hence, we obtain the equilibrium monomer concentration \([M]_e\) as follows:

\[
[M]_e = k_{-1}/k_1 = 1/K. \tag{3}
\]

([M]e is referred to as the “critical concentration” (13, 15) because fibrils form when the concentration of monomers exceeds \([M]_e\). By determining \([M]_e\), we can calculate the apparent free energy change of fibrillation (ΔGapp) by the following: ΔGapp = −RTlnK = RTln[M]e, where \(R\) and \(T\) are the gas constant and temperature, respectively. Combined with the ΔH value directly obtained from the ITC measurements, we can obtain the ΔS by ΔGapp = ΔH − TΔS. Although mechanism 1 might not be exactly true for amorphous aggregation, we assumed that it is also a reversible process determined by solubility and thus is approximated by mechanism 1.

We used an ELISA (SI Text) to determine the \([M]_e\) value under various conditions (Table S2). We then estimated the...
ΔG_{app} and ΤΔS for fibrillation and amorphous aggregation. We also estimated the temperature dependencies of these parameters as well as those of ΔH, in which we used ΔG_{app} values at 37 °C to link the ΔH and ΤΔS functions. These functions were compared with those for folding to the native state (Fig. 6).

The ΔG_{app} value for fibrillation (−45.0 kJ·mol⁻¹) at 37 °C and pH 2.5 was the same as that of amorphous aggregation (−45.4 kJ·mol⁻¹) under the same conditions (Fig. 6C). These values were significantly larger in intensity than that (−21.0 kJ·mol⁻¹) of the native state at pH 7.0 (28), although distinct pH values preclude a direct comparison. Although a small range of temperatures used for the experiments makes the extrapolation less accurate at this stage, separation of ΔG_{app} into the enthalpy and entropy terms indicated that both amyloid fibrils and amorphous aggregates are stabilized enthalpically above 40 °C, whereas they are stabilized entropically below 20 °C.

Discussion

Amyloid formation occurs in supersaturated solutions via a nucleation-dependent manner (18, 40, 41), analogous to crystallization of substances (17, 42). Under the conditions of persistent metastability, nucleation does not occur in practice (14, 18). However, various kinds of agitations can break supersaturation, leading to the formation of fibrils. We used ITC for stirring the solution and for monitoring the accompanying heat effects. The results showed that we can perform calorimetric measurements of amorphous fibrillation of β2m as well as amorphous aggregation revealing the ΔH and ΔC_p values. By combining these values with ΔG obtained from the solubility of β2m monomers, we can address the thermodynamics of protein aggregation (Fig. 6 and Tables S1 and S2). The methodology is straightforward and can be applied to study various amyloid fibrils as well as amorphous aggregates.

The heat capacity change upon protein unfolding has been primarily determined by the hydration of polar and apolar groups and to a much lesser extent by the disruption of internal noncovalent interactions such as van der Waals interactions, H bonds, and ionic interactions (21, 24). The magnitude of the magnitude of the hydration of the buried hydrophilic and polar groups and to a much lesser extent by the disruption of internal interactions such as van der Waals interactions, H bonds (21, 24). The magnitude of the hydration of the buried hydrophilic and polar groups and to a much lesser extent by the disruption of internal interactions such as van der Waals interactions, H bonds (21, 24).

Two main effects have been shown to be responsible for the ΔH of protein unfolding: the hydration of the buried hydrophobic and polar groups that become exposed in the unfolded state, and the disruption of internal interactions such as van der Waals interactions, and H bonds (21, 24). The magnitude of the ΔH of amyloid fibrils (normalized by protein concentration) was significantly less than that of the folding of native β2m (Fig. 6 and Table S1). The ΔH values for amorphous aggregation were even smaller in intensity. From the observed similarity of the ΔC_p values, we assumed a similar contribution of the hydration of the buried groups between native and fibril conformations. Therefore, the observed decrease in ΔH appeared to be the result of different internal interactions (28). It is generally accepted that there is a stronger and more persistent backbone H-bond network in the amyloid structure than there is in the globular fold of proteins, leading to an increase in the β-sheet content (3, 12, 43). However, H bonds should increase the magnitude of the ΔH value, which is inconsistent with the results.

Thus, a reasonable explanation for the ΔH order in magnitude of “native structure > amyloid fibril > amorphous aggregate” is that it dominantly represents side-chain packing in folded or misfolded structures (Fig. 6B). The overall side-chain packing in the amyloid form cannot be as optimal as that in the native state because the structure is determined by extensively H-bonded β-structured backbones (11, 12). The loss of tight packing may be more serious for amorphous aggregation.

The separation of overall stability of amyloid fibrils (ΔG) into the ΔH and ΤΔS terms illustrates that the contributions of the two terms vary depending on temperature. Fibrillation is determined by the favorable entropic term at −20 °C at which ΔH is close to zero. ΔG is minimal at −35 °C, at which the fibrils exhibit maximal stability and, thus, ΤΔS is zero because ΤΔS = dΔG/dΤ. Fibrillation is then determined by the favorable enthalpy term at 35 °C. Thus, the temperature-dependent enthalpy–entropy interplay determines the stability of amyloid fibrils. To understand this interplay, we have to estimate amyloid-specific factors such as the entropy loss resulting from a rigid H bonding of backbones and a reduction in the number of monomers as well as the enthalpy gain obtained from numerous molecular contacts.

In conclusion, we showed that quantitative calorimetric analysis with ITC was indeed possible for the supersaturation-limited amyloid fibrillations. Stirring inside the ITC cell can break persistent supersaturation, which triggers fibrillation. Compared with the single crystals of substances, amyloid fibrils retain a thin and linear morphology. Moreover, the shear forces of stirring...
keep fibrils dispersed in solution and fragment fibrils, which accelerate seed-dependent propagation. These enabled accurate calorimetric measurements of the amyloid burst, making the thermodynamic characterization of fibrillation possible. By carefully adjusting these conditions, we can also monitor the heat of amorphous aggregation. Accordingly, ITC will become a promising approach for clarifying the thermodynamic properties of protein aggregates.

Materials and Methods

Assays of Amyloid Fibrils. Expression and purification of human β2m are described in SI Text. The formation of fibrils and amorphous aggregates was characterized by various methods including ThT fluorescence, AFM, CD, and ELISAs. The details are described in SI Text.

ITC Measurements. ITC measurements for the spontaneous fibrillization of β2m at 0.3–6.7 mg·mL⁻¹ dissolved in 10 mM HCl solution (pH 2.5) were performed with a VP-ITC instrument (GE Healthcare) at the desired temperatures (31–43 °C). The consecutive injections of 20 μL of the 10 mM HCl solution containing 1 M NaCl in the syringe into the β2m solution in the cell were conducted following a 60-min initial delay for complete equilibration. To minimize the heat effects caused by the difference in temperature, the consecutive injections were required because the temperature of the solution inside injection syringe was not controlled except 20 μL in the needle. The first titration of 2 μL was adopted to minimize the influence of residual bubbles and imperfect solution filling the syringe. Nine salt titrations in real time with 10 μcL⁻¹ of reference power. The reaction cell was continuously stirred at 600 rpm. Lag time was defined by a period between the time starting the measurement under stirring and the time of major heat effect occurred as shown in Fig. 3A. To examine the effects of the stirring speed on fibrillation, the stirring speed was changed from 200 to 1,000 rpm. To monitor amorphous aggregation, 3.5 mg·mL⁻¹ β2m in 10 mM HCl solution without salt was inversely titrated into 10 mM HCl solution containing 1 M NaCl at the desired temperatures (31–43 °C). The parameters for ITC measurements except for shortening the initial delay to 30 min were identical to those used for fibril formation. The total heat effects, which were shown to be equal to the ΔH values, were calculated using peak areas after subtracting the heat of dilution and baseline corrections.

ACKNOWLEDGMENTS. We thank Ms. Kyoko Kigawa for the expression and purification of β2m. This work was supported by the Japanese Ministry of Education, Culture, Sports, Science, and Technology. J.K. is supported by Bolyai János fellowship of the Hungarian Academy of Sciences and Hungarian Scientific Research Fund (OTKA; Grant 18950).

Supporting Information

Ikenoue et al. 10.1073/pnas.1322602111

SI Text

Proteins and Reagents. Recombinant human β2-microglobulin (β2m) protein with an additional methionine residue at the N terminus was expressed in Escherichia coli and purified as previously reported (1). The concentration of β2m was determined by measuring absorbance using a molar extinction coefficient of 19,300 M⁻¹·cm⁻¹ at 280 nm (1). Thioflavin T (ThT) was purchased from Wako Pure Chemical Industries. All other reagents were obtained from Nacalai Tesque.

Fluorescence Assay. The formation of β2m fibrils was observed by a fluorometric assay with ThT at 37 °C. Excitation and emission wavelengths were 445 and 485 nm, respectively. Five-microliter aliquots were taken from the isothermal titration calorimetry (ITC) cell after incubation and mixed with 1.0 mL of 5 μM ThT in 50 mM glycine-NaOH buffer (pH 8.5). The individual intensities of ThT fluorescence were normalized using the intensities of 6.7 mg·mL⁻¹ β2m (Fig. 1A), at ∼12 h (Fig. 3A) or 34 °C (Fig. 4C). ThT fluorescence spectra were measured using a F4500 fluorescence spectrophotometer (Hitachi).

CD Spectroscopy. Far-UV CD spectra of β2m before and after incubation in the ITC instrument were measured with a J-820 spectropolarimeter (Jasco) using a cell with a light path of 1 mm. Sample solutions contained 0.1 mg·mL⁻¹ β2m in 10 mM HCl (pH 2.5) and 100 mM NaCl. CD signals between 195 and 250 nm were expressed as the mean residue ellipticity [θ] (degrees-square centimeters per decimole). Temperature regulation was performed using a PTC-423L Peltier unit (Jasco).

ELISA to Determine the Remaining Monomer Concentrations. According to a model of the supersaturation-dependent formation of fibrils, the remaining monomer concentration after the formation of fibrils is equal to the equilibrium monomer concentration (i.e., critical concentration). This is also true for the formation of amorphous aggregates. To quantify the amount of residual β2m monomers after the formation of amyloid fibrils or amorphous aggregates, immunosorbent analyses using a commercial immunoassay kit (Human β2m ELISA test; MD Bioproducts) were conducted using aggregates produced in ITC as described above. Supernatants of the sample solutions after centrifugation at 72,000 × g with a CS 120GX ultracentrifuge (Hitachi) for 30 min at the same temperature as the formation of aggregates were recovered and used for the ELISA. A series of diluted samples of the supernatants were assayed using standard β2m solutions as references. The standard solutions confirmed a sensitive and quasilinear concentration dependence in the 0–200 ng·mL⁻¹ range.


Fig. S1. Conformation phase diagram of β2m at 37 °C and pH 2.5. The regions of unfolded monomers (blue), amyloid fibrils (red), and amorphous aggregates (magenta) are shown. Conformational states were determined in this study (▲, ●, X) and also in our previous studies (▲, △, ○, *) (1, 2). Lines are boundaries between the phases. The boundary between the unfolded soluble states and amyloid fibrils defines the critical concentration, which is equal to the equilibrium solubility of unfolded monomers. The critical concentrations from this study (X) and our previous study (*) (1).

Fig. S2. Dependencies of the observed lag time of heat peaks on the protein concentration or stirring speed at pH 2.5 and 37 °C. (A) The lag times of the main and small heat peaks shown in Fig. 1 were plotted against the protein concentration. (B) The lag time for the major heat peak observed at 0.5 mg·mL⁻¹ as shown in Fig. 2 was plotted against the stirring speed. (C) The lag times of the main and small heat peaks at 1.1 mg·mL⁻¹ β2m and 600 rpm at various temperatures shown in Fig. S4 were plotted against the temperature. The solid lines were drawn to guide the eye. It is noted that the lag times indicate the period from the start of heat monitoring (i.e., time 0 in the ITC thermogram) and the time of the amyloid burst.

Fig. S3. Amyloid burst of supersaturated β2m prepared outside the ITC cell and then monitored by ITC. (A) Thermogram of fibril formation at 0.5 mg·mL⁻¹, 0.1 M NaCl, and 37 °C. The stirring speed was 600 rpm. The lag time was 3.7 h with a dead time of 55 min, where the dead time refers to a time between the sample preparation outside the ITC cell and the setup inside the cell with stabilization of the heat capacity signal. The ΔH value of the major peak was 79.8 kJ·mol⁻¹. (B and C) Characterization of the β2m solution after the heat burst by the far-UV CD (B), and ThT fluorescence and AFM (C). The normalized ThT intensity after incubation in the ITC cell with (+) and without (−) salt injections is shown. The scale bar on the AFM image indicates 1 μm, and the numbers under images are fibril height.
Fig. S4. Thermogram for the formation of amyloid fibrils in the presence of 5 mM sodium sulfate at 37 °C. A volume of 1.4 mL of β2m at 1.0 mg mL⁻¹ was titrated with 300 mM (NH₄)₂SO₄ by nine titrations in total with the final concentration of 3 mM. Total heat effects including the peaks at 12 and 17 h were 38.5 kJ mol⁻¹. The value was smaller than the value obtained for the titration with NaCl.

Fig. S5. Amorphous aggregation immediately after the salt titration. (A) Expanded thermograms for injection of NaCl at various β2m concentrations. The data are the same as shown in Fig. 1A. (B) Dependences of the observed heat on the NaCl concentration at various β2m concentrations. Crosses are the observed heat for a reference titration in the absence of protein, representing the heat effects of buffer dilution. (C) Dependence on the β2m concentration of the total excess heat where the difference between the peak areas in the presence and absence of β2m were summed up to 0.1 M NaCl. It is noted that the excess heats were not normalized by the β2m concentration. We consider that the excess heats arise from the transient formation of amorphous aggregates (see the text).
Table S1. Enthalpy and heat capacity changes with the folding and misfolding of β2m at various temperatures

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>$\Delta H_{\text{folding}}^*$, kJ mol$^{-1}$</th>
<th>Seeded fibrillation*</th>
<th>Spontaneous fibrillation</th>
<th>$\Delta H_{\text{amyloid}}$, kJ mol$^{-1}$</th>
<th>$\Delta H_{\text{amorphous}}$, kJ mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>-142.6</td>
<td>-85.1</td>
<td>-41.3 ± 3.3</td>
<td>-19.8</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>-159.4</td>
<td>-96.7</td>
<td>-56.9 ± 2.2</td>
<td>-27.5</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>-176.3</td>
<td>-119.2</td>
<td>-73.6 ± 5.8</td>
<td>-42.8</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>-193.1</td>
<td>-123.2</td>
<td>-86.0 ± 6.0</td>
<td>-49.6</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>-210.0</td>
<td>-145.2</td>
<td>-101.1 ± 3.9</td>
<td>-68.8</td>
<td></td>
</tr>
<tr>
<td>Δ$C_p$</td>
<td>-5.6 ± 0.4</td>
<td>-4.8 ± 0.2</td>
<td>-5.0 ± 0.2</td>
<td>-3.5 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

*Data taken from our previous study (1).


Table S2. Thermodynamic parameters of the folding and misfolding of β2m obtained by the ELISA

<table>
<thead>
<tr>
<th>Type of aggregates</th>
<th>Temperature, °C</th>
<th>$[M]_e^*$, nM</th>
<th>$K^*$, μM$^{-1}$</th>
<th>$\Delta G_{\text{app}}^*$, kJ mol$^{-1}$</th>
<th>$-T\Delta S^*$, kJ mol$^{-1}$</th>
<th>$\Delta H^*$, kJ mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amyloid fibrils</td>
<td>31</td>
<td>37.7</td>
<td>26.5</td>
<td>-43.2</td>
<td>-1.9</td>
<td>-41.3 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>25.8</td>
<td>38.8</td>
<td>-45.0</td>
<td>28.6</td>
<td>-73.6 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>2.4</td>
<td>421.4</td>
<td>-51.7</td>
<td>34.3</td>
<td>-86.0 ± 6.0</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>15.5 (42.4 μM)</td>
<td>64.5</td>
<td>-46.3</td>
<td>27.7</td>
<td>-74</td>
</tr>
<tr>
<td>Amorphous aggregates</td>
<td>31</td>
<td>19.3</td>
<td>51.8</td>
<td>-44.9</td>
<td>-25.1</td>
<td>-19.8</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>21.4</td>
<td>46.8</td>
<td>-45.5</td>
<td>-2.7</td>
<td>-42.8</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>17.3</td>
<td>57.8</td>
<td>-46.5</td>
<td>3.1</td>
<td>-49.6</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>22.7 (42.4 μM)</td>
<td>44.0</td>
<td>-45.4</td>
<td>-2.6</td>
<td>-42.8</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>29.3 (169.5 μM)</td>
<td>34.1</td>
<td>-44.7</td>
<td>-1.9</td>
<td>-42.8</td>
</tr>
</tbody>
</table>

*The concentrations of the residual monomers determined by the ELISA. The initial concentration was 84.7 μM (1.0 mg mL$^{-1}$), except for those indicated in parentheses.

The values of $K$ and $\Delta G_{\text{app}}$ were determined from experimentally determined $[M]_e$ using the relationship: $K = 1/[M]_e$ and $\Delta G_{\text{app}} = -RT\ln K$ (see text). The values of $-T\Delta S$ were determined by $\Delta G_{\text{app}} = \Delta H - T\Delta S$. $\Delta H$ values were obtained directly by ITC (Table S1).