Refolding of β-lactoglobulin studied by stopped-flow circular dichroism at subzero temperatures

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Abstract Refolding of bovine β-lactoglobulin was studied by stopped-flow circular dichroism at subzero temperatures. In ethylene glycol 45%–buffer 55% at −15°C, the isomerization rate from the kinetic intermediate rich in α-helix to the native state is approximately 300-fold slower than that at 4°C in the absence of ethylene glycol, whereas the initial folding is completed within the dead time of the stopped-flow apparatus (10 ms). At −28°C, we observed at least three phases; the fastest process, accompanied by an increase of α-helix content, is completed within the dead time of the stopped-flow apparatus (10 ms), the second phase, accompanied by an increase of α-helix content with the rate of 2 s^{-1}, and the third phase, accompanied by a decrease of α-helix content. This last phase, corresponding to the isomerization process at −15°C described above, was so slow that we could not monitor any changes within 4 h. Based on the findings above, we propose that rapid α-helix formation and their concurrent collapse are common even in proteins rich in β-structure in their native forms.

1. Introduction

During the past decade, protein folding has been extensively investigated [1,2]. Bovine β-lactoglobulin is one of the proteins for which folding processes have been extensively studied [3–8]. Kuwajima et al. [4] reported that the initial folding occurred within the dead time of their stopped-flow devices (18 ms) to form a kinetic intermediate, resembling a molten globule state, followed by formation of the native conformation within approximately 100 ms. The kinetic intermediate found is rich in α-helix, whereas the native conformation is rich in β-structure with only one α-helix. Although it is important to investigate the initial folding events, the conventional stopped-flow method could not be applied due to the time limit of the mixing.

Existence of such α-helix-rich transients is also suggested on plasminogen activator inhibitor-1 [9]. Thus, it is important to investigate initial events occurring on the folding pathway in such β-structure proteins, focused on the rapid formation of α-helix.

In the present paper, we tried to reduce the folding rate, by decreasing temperatures to subzero. The folding process was monitored by far ultraviolet circular dichroism (CD). The conversion rate of β-lactoglobulin from the molten globule state to the native state was decreased 300-fold at −15°C in the presence of 45% ethylene glycol as an anti-freeze, but the initial α-helix formation was nonetheless completed within the dead time of the stopped-flow apparatus (10 ms). At −28°C, in contrast, an observable phase of α-helical formation appeared in addition to the still existing burst phase.

2. Materials and methods

2.1. Materials

Bovine β-lactoglobulin is a major component of cow's milk. It has a predominant β-sheet, composed of two orthogonal slabs of nine antiparallel β-strands and one α-helix [10]. Bovine β-lactoglobulin A and B were separately purified from crudely purified powder purchased from Sigma (Lot 124H7045) by ion-exchange chromatography through DEAE-Sephagel (Amersham Pharmacia), as described by Cervone et al. [11]. Purification fractions of bovine β-lactoglobulin A and B were identified by native polyacrylamide gel electrophoresis, and each of them showed a single band, respectively. The purified proteins were lyophilized and stored at −20°C. Bovine β-lactoglobulin A was used in the present experiments. The concentration of bovine β-lactoglobulin A was determined spectrophotometrically using an extinction coefficient, $E^{1\%}_{1\,cm} = 9.6$, at 278 nm [12]. Guanidine hydrochloride (GuHCl) was of ultra pure reagent grade from ICN Biomedicals, Inc. (Lot 2345B). Its concentration was calibrated by refractive index measurements. All other reagents were of guaranteed reagent grade for this study.

The temperature was controlled by a controller ULT-80 by NESLAB). Viscosities were measured by a syringe viscometer and calibrated by standard solution (JS 5 and JS 20 of Nihon Grease Co.).

2.2. Stopped-flow apparatus

The stopped-flow device was constructed for special use of high viscosity and low temperature in collaboration with Unisoku Inc. The mixer is a combined one of two mixing units (4-jet and 6:1 mixer). Its dead time was estimated to be 6 ms by the test reaction of DCIP reduction by ascorbic acid (unpublished data) at 4°C. The mixing was sufficient within the limit that the viscosity is less than 200 mP and the temperature is higher than −40°C.

2.3. CD measurements in equilibrium

The samples were prepared in 0.01 M phosphate-buffered saline (PBS), pH 2.0, with different concentrations of GuHCl in the absence and the presence of 45% ethylene glycol, respectively. The concentration of bovine β-lactoglobulin A was 0.44 mg/ml. CD measurements

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Abbreviations: GuHCl, guanidine hydrochloride; PBS, phosphate-buffered saline; *T*-jump, temperature jump; CD, circular dichroism

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were performed at 4°C, −15°C and −28°C, respectively, with a spectropolarimeter specially designed by Unisoku Inc. Cuvettes of 1 mm path-length were used for all measurements.

2.4. Kinetic CD measurements

The bovine β-lactoglobulin A was first unfolded in the 0.01 M PBS, pH 2.0, with 4–5 M GuHCl, and was diluted seven times with the refolding buffer (0 M GuHCl) so as to initiate the refolding. The final concentration of GuHCl is then 0.6–0.7 M. The refolding process was monitored by CD at 222 nm. Measurements were repeated and accumulated to have a good signal/noise ratio. The averaged data were normalized to give molar ellipticity. At each condition, in addition to the refolding experiments, we always performed two more experiments: mixing unfolded protein in the unfolded buffer with the same buffer. This gives us the 'initial' CD level. After the refolding experiments, we left the solution a long time at the same condition, and measured CD. This gives the 'final' level. Usually, the 'final' level gives us the level of the native conformation. However, at subzero temperatures, the 'final' level means the level stable at a few hours later.

3. Results and discussion

3.1. Stability in 45% ethylene glycol at subzero temperatures

To do experiments at subzero temperatures, we need to add anti-freeze [13]. In the present experiments, we used ethylene glycol as anti-freeze. There are several reports that alcohol solvents enhance α-helix stability [14,15]. To determine the effect of ethylene glycol on protein conformation, we first investigated the effect of ethylene glycol on the secondary structure of the protein. Fig. 1A shows the dependence of CD patterns on ethylene glycol concentration. In Fig. 1B, $[\theta]_{222}$ is plotted against the concentration of ethylene glycol. As seen in the figures, bovine β-lactoglobulin takes an α-helix-rich conformation at high concentration of ethylene glycol as in the case with other alcohols, but ethylene glycol is much milder than other alcohols [14,15]. Hereafter, we use 45% ethylene glycol as the anti-freeze, in which bovine β-lactoglobulin takes on the native conformation in the absence of GuHCl at subzero temperatures.

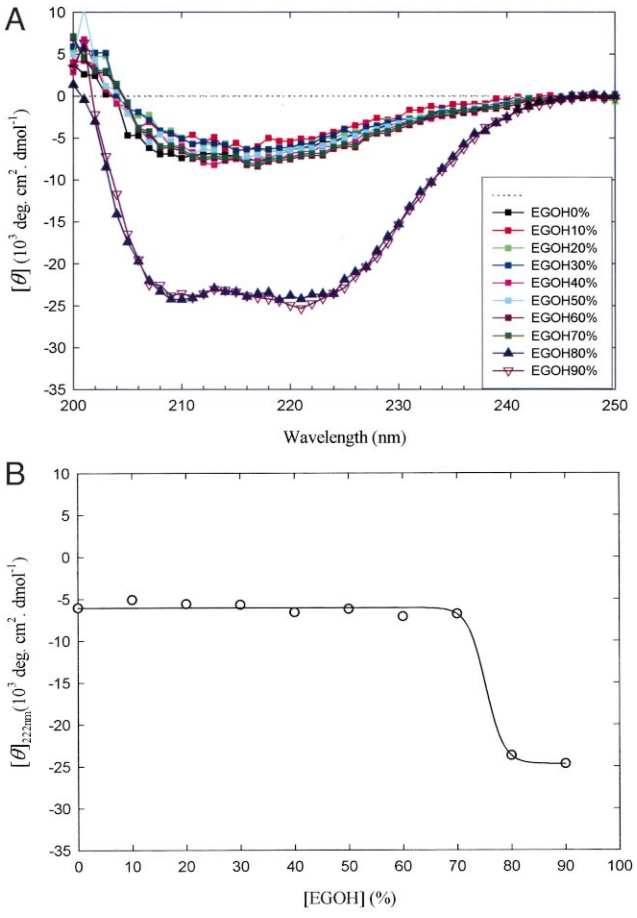

Fig. 1. A: CD spectra of β-lactoglobulin in the presence of various fractions of ethylene glycol, pH 2.0 at 4°C. Protein concentration was 0.47 mg/ml. Path-length of the cuvette was 1 mm. B: Ethylene glycol-induced equilibrium transition curve of β-lactoglobulin in 0.01 M PBS, pH 2.0 at 4°C, monitored by CD at 222 nm.

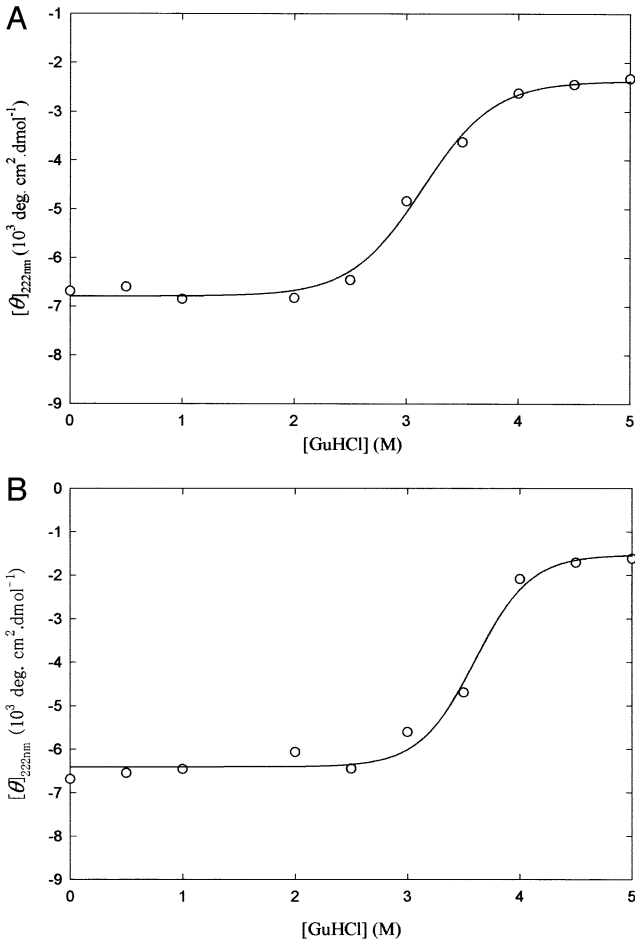

Fig. 2. GuHCl-induced equilibrium unfolding transition curves of β-lactoglobulin in 0.01 M PBS, pH 2.0, monitored by CD at 222 nm. The protein concentration was 0.44 mg/ml. A: The unfolding transition curve in the absence of ethylene glycol at 4°C at which the mid-point was at 3.2 M; B: the unfolding transition curve in the presence of 45% ethylene glycol at −15°C in which the mid-point shifted to 3.6 M.

In Fig. 2, $[\theta]_{222}$ values obtained by CD are plotted as a function of GuHCl concentration at different conditions. The protein was unfolded with the mid-point at around 3.2 M at 4°C in the absence of ethylene glycol, whereas the mid-point shifts to 3.6 M at −15°C in the presence of 45% ethylene glycol. β-Lactoglobulin was unfolded in the presence of 5 M GuHCl and 45% ethylene glycol at −28°C. Thus, we performed refolding experiments from ∼4 to 5 M to sevenfold dilution.

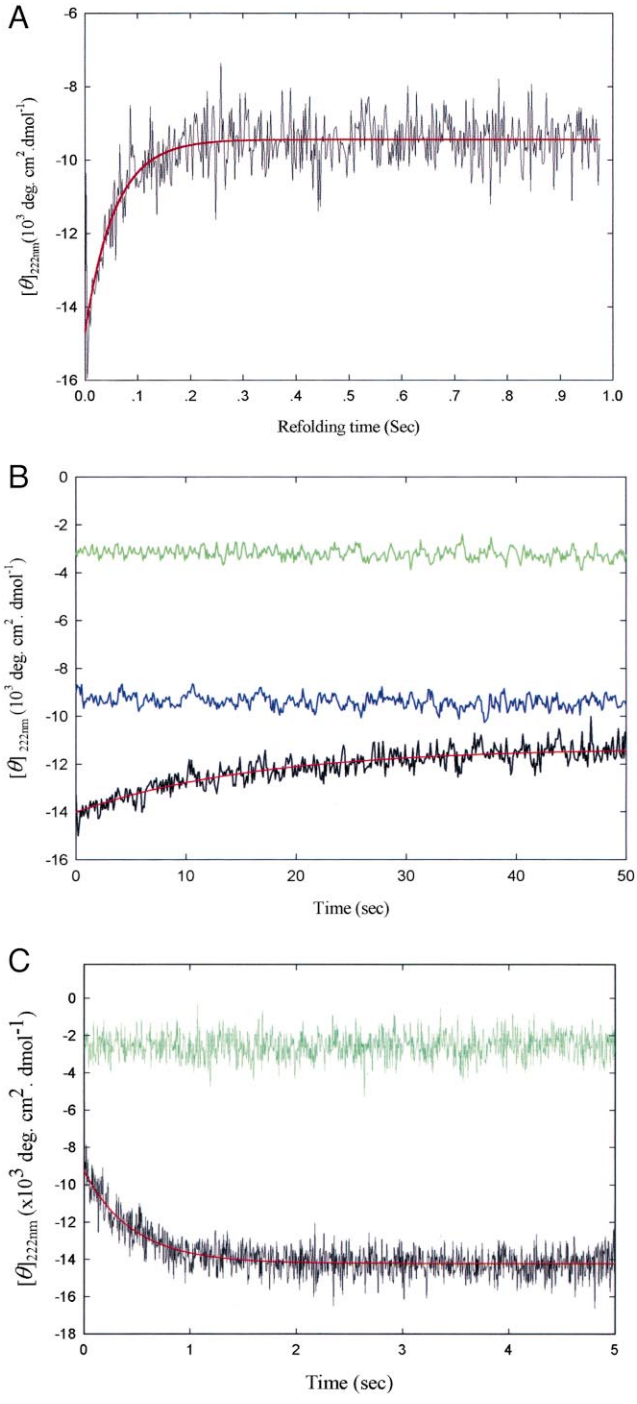

Fig. 3. Kinetic refolding curves of β-lactoglobulin in PBS 0.01 M, pH 2.0, monitored by CD at 222 nm. The refolding reaction was initiated by a concentration jump of GuHCl from 4∼5 M to 0.6∼0.7 M. A: The kinetic refolding curve of β-lactoglobulin in the absence of ethylene glycol at 4°C. The red smooth line represents a simulated kinetic curve as a single exponential, which gives the rate constant of 17.6 s^{-1}. B: The kinetic refolding curve (black) of β-lactoglobulin in the presence of ethylene glycol at −15°C. Red smooth curve is a simulated one to a single exponential, which gives the rate constant of 0.061 s^{-1}. The green line represents the initial level of the refolding processes, and the blue line shows the final level which was measured 10 min after the initiation of the refolding. C: The kinetic refolding curve (black) of β-lactoglobulin in the presence of ethylene glycol at −28°C. Concentration of GuHCl was jumped from 5.0 M to 0.7 M. The green curve represents the initial level. The red smooth curve shows the simulated one to a single exponential, which gives the rate constant of 2.175 s^{-1}.

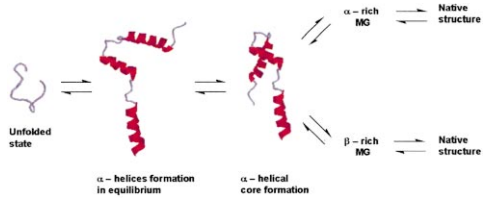

Fig. 4. A proposed scheme of the initial events of protein folding, suggesting the importance of α-helical core formation in case of β-rich proteins as well as α-helix-rich proteins.

3.2. Refolding of β-lactoglobulin at subzero temperatures

First, we performed refolding experiments at 4°C in the absence of anti-freeze. In Fig. 3A, a concentration jump from 4.0 M to 0.56 M is shown at 4°C in the absence of ethylene glycol. As seen in the figure, an isomerization process was observed with a decay time of 56.9 ms at 4°C in the absence of ethylene glycol. This process accompanies the decrease in α-helix content, in agreement with previous reports [3–5]. The process was slowed down to 153.4 ms at 4°C in the presence of 45% ethylene glycol, and was slowed down more drastically at −15°C in the presence of 45% ethylene glycol, as shown in Fig. 3B. At this temperature, the process is 300-fold slower than that at 4°C in the absence of ethylene glycol. In Fig. 3B, the initial and the final levels of CD are also shown. The CD value after 100 s does not fit to the final level, indicating there is another even slower phase after the isomerization process observed. The CD value did not recover to the final value even 2 h after the concentration jump (data not shown). It is also clear from Fig. 3B that there is a burst phase finished within the dead time of the stopped-flow apparatus (10 ms). We have also performed refolding experiments by decreasing temperature. However, we could only see the initial α-helix formation within the dead time of the stopped-flow apparatus at −20°C and −25°C.

At −28°C, in contrast, we observed another phase accompanying the increase of α-helices with the relaxation time of 459.8 ms, as shown in Fig. 3C. An initial burst, showing that the increase of α-helices still exists as in the case at higher temperatures, which was followed by the process accompanying the increase of α-helices. After these two processes, the $[\theta]_{222}$ did not change as long as 4 h, which indicates that the isomerization process, corresponding to the process observed at higher temperatures, did not occur as long as 4 h. The observed phase accompanying the increase of α-helices is very sharp dependent on the measured temperature. At −25°C, no corresponding phases were observed as described above, while at −27.3°C, the relaxation time of α-helical increase is 71.1 ms.

From the findings above, we can conclude the following: in case of bovine β-lactoglobulin, the initial formation of α-helices is very rapid, comparing the isomerization process of α-helix-rich conformation to β-rich conformation. If both processes have the same temperature dependence, the initial α-helical formation should be faster than 2 μs at 4°C in the absence of ethylene glycol.

3.3. Rate of α-helix formation

Ballew et al. reported that apomyoglobin shows at least three phases (the fastest one is shorter than 1 μs, the second one is several μs and the third one is on the order of subseconds) by means of temperature jump (*T*-jump) study [16].

They ascribed the first one to α-helix formation, the second one to A-G-H helix core formation, and the last one to the conversion of the molten globule state to the native state as previously reported by Jennings and Wright [17]. For the first and the second steps, our results are in very good coincidence with them. It is then probable to assign the initial burst to the instantaneous α-helical formation, and the second phase to core formation. However, Clarke et al. reported, from the stopped-flow CD study, that α-helical formation rate of peptide AK16 (alanine-rich peptide) is as slow as 67 ms at 0°C, and those of poly-glutamic acid and poly-L-lysine are also detectable in this region by a stopped-flow device [18]. They ascribed the apparent differences to: *T*-jump starts from the incomplete unfolded state. Therefore the rate which Ballew et al. observed is the propagation rate of α-helices and not the initiation of α-helices, and they concluded the initial folding step should be as slow as several tens of ms [18]. We performed the same type of GuHCl concentration jump experiments for α-helix-forming C17 fragment of α-lactalbumin and poly-glutamic acid (data not shown). In both cases, we could observe only burst phases at 4°C. The slowness of α-helix formation in case of poly-alanine might be due to its lower stability compared to C17 fragment. However, we could not understand the discrepancy in case of poly-glutamic acid. It might be due to experimental conditions, or otherwise due to incomplete mixing efficiency of their stopped-flow equipment.

3.4. Folding core formation

It is most interesting that we could observe a phase loading to increased α-helix content at −27.3°C and −28°C. As the solvent (ethylene glycol 45%–buffer 55%) is so viscous, we could not decrease the temperature lower than this. Instead, we observed the same phase in a ternary solvent system (ethylene glycol 30%–methanol 20%–buffer 50%) at even lower temperatures (data not shown). This phase has a sharp dependence on the measured temperatures. Although we need more detailed studies, it is probable that this indicates folding core formation even in β-rich proteins, as illustrated in Fig. 4. This model is very consistent with the folding scheme of α-helix-rich protein, apomyoglobin [10]. Recently, we observed the folding of a β-rich protein, ubiquitin, which also showed the initial burst having high α-helical conformations (unpublished data). The scheme shown in Fig. 4 would be general in many proteins including β-rich proteins as well as α-helix-rich proteins. In the scheme, the most important step in protein folding is the initial core formation, initiated by the collapse of α-helices.

Recently, Chikenji and Kikuchi reported UDβ (up-and-down β-barrel) proteins, including β-lactoglobulin, have a strong probability of forming α-helix-rich intermediates in their refolding process [19]. It is very plausible that the scheme in Fig. 4 is widely applicable to such UDβ proteins.

3.5. Comparison with continuous flow experiments

Recently, Kuwata et al. reported the initial step of folding of bovine β-lactoglobulin is ca. 50 μs by means of the continuous flow technique combined with fluorescence [6]. We have also performed concentration jump experiments with the present stopped-flow equipments with a fluorescent probe. At 4°C, we could see some phases occurring continuously. At lower temperature in the presence of anti-freeze (ethylene glycol 45%), we also observed some phases. However, at −28°C, we could not see change in fluorescence within 10 s. This might indicate that the fluorescence change which Kuwata et al. observed is not the initial step, but some continuous change, which does not accompany with the change in CD.

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