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Chloride-ion concentration dependence of molecular dimension in the acid-denatured state of equine β-lactoglobulin

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The chloride-ion concentration dependence of the molecular dimension in the acid-denatured state of equine β-lactoglobulin (ELG) was investigated by small-angle X-ray scattering. In the presence of chloride ion, ELG has a globular and compact conformation (the A state). The molecular dimension of ELG increases little with decreasing chloride-ion concentration. A remarkable dependence was observed for a mutant protein in which both Cys66 and Cys160 were replaced with Ala (C66A/C160A). In the presence of chloride ion, C66A/C160A has a globular and compact conformation, like the wild type. In the absence of chloride ion, however, the molecular dimension and shape was close to that in the urea-unfolded state. Previously, we have shown that the helix content in the acid-denatured state increases with decreasing chloride-ion concentration [Yamada *et al.* (2006). *Proteins Struct. Funct. Bioinf.* **63**, 595–602]. These results suggest that the secondary structure in the A state is mainly determined by non-local interactions. When they are absent in an expanded conformation, the local interactions become predominant and the amount of non-native α-helix increases.

1. Introduction

To elucidate the mechanism by which proteins fold into their unique three-dimensional structure, it is essential to examine the stabilization mechanism of the native and non-native states. Depending on the protein and/or the method of denaturation, the protein shows a variety of conformational states, *i.e.* the native state, partially folded states with several native-like features (*e.g.* the molten globule state) and a highly denatured state resembling a random coil (Ptitsyn, 1995; Kataoka & Goto, 1996; Kataoka *et al.*, 1997). In some cases, it has been shown that some residual structures exist in several proteins even when they are highly denatured (Neri *et al.*, 1992; Shortle, 1996). These remaining structures are suggested to play a key role as the potential initiation site at an early stage of protein folding kinetics (Wong *et al.*, 1996; Englander, 2000).

The molten globule state is a partially folded conformation and is characterized by the presence of substantial secondary structure, a compact shape and the absence of extensive rigid side-chain packing interactions. It is observed as an equilibrium unfolding/refolding intermediate under mildly denaturing conditions for many globular proteins such as α-lactalbumin, cytochrome *c*, apomyoglobin, ribonuclease HI and β-lactoglobulin (Kuwajima *et al.*, 1976; Griko *et al.*, 1988; Kuwajima, 1989; Ptitsyn, 1995; Dabora *et al.*, 1996). Studies on the above proteins by a stopped-flow circular dichroism (CD) technique and by a pulse-labeled hydrogen-exchange technique coupled with two-dimensional NMR spectroscopy have shown that a transient intermediate similar to the equilibrium molten globule state is formed at an early stage of protein folding. Therefore, the molten globule state at equilibrium is a good model system for the study of the stabilization mechanism of folding intermediates.

Equine β-lactoglobulin (ELG) is a major component of milk and is a member of the lipocalin family. The members of this family share an eight-stranded (labeled A-H) up-and-down β-barrel structure with an α-helix and an off-barrel strand I. ELG takes a stable molten globule state (A state) at acidic pH (Ikeguchi *et al.*, 1997). The A state has a significant amount of secondary structure including non-native α-helices. Small-angle X-ray scattering (SAXS) and analytical ultracentrifugation have shown that the protein in this state is nearly as compact as that in the native state (Ikeguchi *et al.*, 1997; Yamada *et al.*, 2005). The stopped-flow CD experiment has shown that the kinetic folding intermediate is indistinguishable from the A state (Fujiwara *et al.*, 1999). Recently, we have found that the molten globule state of ELG undergoes a conformational transition when the temperature decreases (Yamada *et al.*, 2005). The cold-denatured (C) state has been characterized by SAXS as an expanded chain-like conformational state. Nevertheless, the CD spectrum has shown that the α-helix content in the C state is much larger than that in the A state. The C state is also thermodynamically distinguishable from the A state. The temperature-dependent conformational transition of ELG at acidic pH has been interpreted as follows. In the A state, the compact conformation is mainly stabilized by the non-local hydrophobic interactions. Lowering the temperature weakens the hydrophobic interaction and results in the expansion of the polypeptide chain. Because the non-local hydrophobic interactions are absent from the expanded conformation, local interactions become predominant and the amount of non-native α-helix increases.

The expansion of the polypeptide chain may occur at a low ionic strength and at acidic pH where the electrostatic repulsion is strong. It has actually been observed for the acid-denatured cytochrome *c* and apomyoglobin (Goto *et al.*, 1990; Kataoka *et al.*, 1993; Hamada *et al.*, 1994). In the case of ELG, the chain expansion and concomitant increase of the helical content were expected at a low pH and low ionic strength. It has been found, however, that the helix formation does not occur even at a low ionic strength (Yamada *et al.*, 2006). This may be due to the presence of disulfide bonds. ELG has two disulfide bonds, Cys66–Cys160 and Cys106–Cys119. Previously, we constructed a mutant in which Cys66 and Cys160 were replaced with alanine (C66A/C160A) and the chloride-ion concentration dependence of its CD spectrum was measured at acidic pH. The CD spectrum of C66A/C160A did show the increased helix content at a low ionic strength (Yamada *et al.*, 2006). In this study, thus, we have investigated the chloride-ion concentration dependence of the molecular shape of C66A/C160A and wild-type ELG (WT) at acidic pH.

2. Materials and methods

WT was prepared from horse milk as previously described (Ikeguchi *et al.*, 1997). C66A/C160A was expressed and purified as previously described (Yamada *et al.*, 2006). Chemicals were of analytical grade from Nacalai Tesque or Wako Pure Chemical Industries (Osaka, Japan). The pH was adjusted to 1.7 with 100 m*M* phosphoric acid and the chloride-ion concentration was controlled with KCl.

2.1. Analysis of SAXS profiles

SAXS measurements were done at beamline 15A of the Photon Factory at the High Energy Accelerator Research Organization, Tsukuba, Japan. The proteins were irradiated with a monochromatic X-ray beam (1.5 Å). The intensity of the X-ray beam at the sample position was about 10^{10} photons s^{-1}. The sample cells were kept at 298 K by circulating temperature-controlled water. Data were collected with a two-dimensional charge-coupled-device-based X-ray detector. The sample-to-detector distance was about 1500 mm, which was calibrated with the meridional diffraction of dried collagen. Data were collected in the range of scattering vector Q from 0.015 to 0.2 $Å^{-1}$, where $Q = (4\pi/\lambda)\sin\theta$, λ is the wavelength and 2θ is the scattering angle. Data analysis was carried out as described previously (Arai *et al.*, 2002).

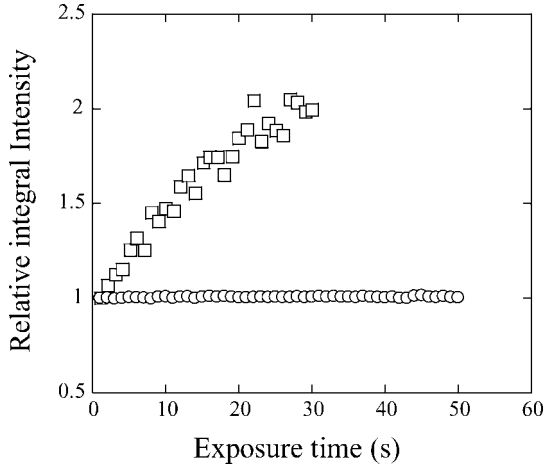

Figure 1
Change of integrated scattering intensity during the exposure of the acid-denatured ELG at pH 1.5 to X-rays. Integration was done for entire signals from the CCD detector. Using a normal static cell (squares), the integrated scattering intensity gradually increases with the exposure time. Using a static flow cell (circles), the integrated scattering intensity does not change. X-ray intensities as ion-chamber current values for the measurements using a normal static cell and a static flow cell are 69 and 87 nA, respectively.

For compact globular conformations in the native or molten globule states, a sufficiently wide Q region satisfies the Guinier approximation, $Q^2R_g^2 < 1.3$. On the other hand, the Guinier approximation is valid only in a quite narrow region for unfolded proteins, and it is difficult to obtain the radius of gyration from the slope of the Guinier plot. If the unfolded protein is regarded as a Gaussian chain, the scattering profile is better described by the Debye function in a wide Q range ($Q^2R_g^2 < 9$) (Calmettes *et al.*, 1994). In the case where the molecule is transformed from a compact globular shape to an expanded chain-like conformation, it is difficult to determine the radius of gyration during the course of such a transition. In this study, therefore, we used a third-order polynomial function to determine the apparent radius of gyration ($R_{g,app}$),

$$\ln I(Q) \simeq \ln I(0) - (R_{g,app}^2/3)x + \alpha x^2 + \beta x^3,$$

where $I(Q)$ is the scattering intensity at the scattering vector Q, and $x = Q^2$. When a Guinier plot is fitted by the third-order polynomial function, the first (zero-order) term corresponds to $\ln I(0)$, where $I(0)$ is the forward X-ray scattering intensity, and the coefficient of the second term is regarded as $-R_{g,app}^2/3$. All fits were done for data in the region $Q^2 < 0.02$.

3. Results

It is well known that long exposure probably causes some chemical damage in the protein so that the damaged protein becomes liable to aggregate. At acidic pH, ELG is also susceptible to radiation damage. This is apparent from Fig. 1, which shows the change in the integration of scattering intensity during the exposure to X-rays. Using a normal static cell (a mica-windowed cell with a 1 mm path length), the integral intensity increases with the exposure time. In this study, therefore, we used a static flow cell (Kihara & Nagamura, 1999). The flow rate was adjusted in such a way that protein molecules would be exposed to the X-ray beam for less than 1.5 s. As a result, using a static flow cell, the integral scattering intensity does not change during the long exposure (Fig. 1). Therefore, it is possible to average the scattering data over a long time.

Fig. 2 shows the Guinier plot of WT and C66A/C160A at pH 1.7. The protein concentration is as low as 0.3–0.5 mg ml^{-1}, which is comparable to the protein concentration used for the previous CD study (Yamada *et al.*, 2006). The Guinier plot of WT is nearly linear both in the absence and presence of chloride ion, although the deviation from linearity is remarkable in the absence of chloride ion. The scattering curves of C66A/C160A are different in the presence and absence of chloride ion. In the presence of 80 m*M* chloride ion, C66A/C160A gives a nearly linear Guinier plot. In the absence of chloride ion, however, the Guinier plot shows a curvature like that observed for unfolded proteins.

Fig. 3 shows the chloride-ion concentration dependence of $R_{g,app}$. The $R_{g,app}$ of C66A/C160A increases with decreasing chloride-ion concentration. Although a similar tendency was observed for WT, its change is much smaller than that observed for C66A/C160A. These results indicate that C66A/C160A undergoes a conformational transition from a compact form to an expanded form with decreasing chloride-ion concentration.

Fig. 4 shows Kratky plots of WT and C66A/C160A in the presence and absence of chloride ion. In the presence of 80 m*M* chloride ion,

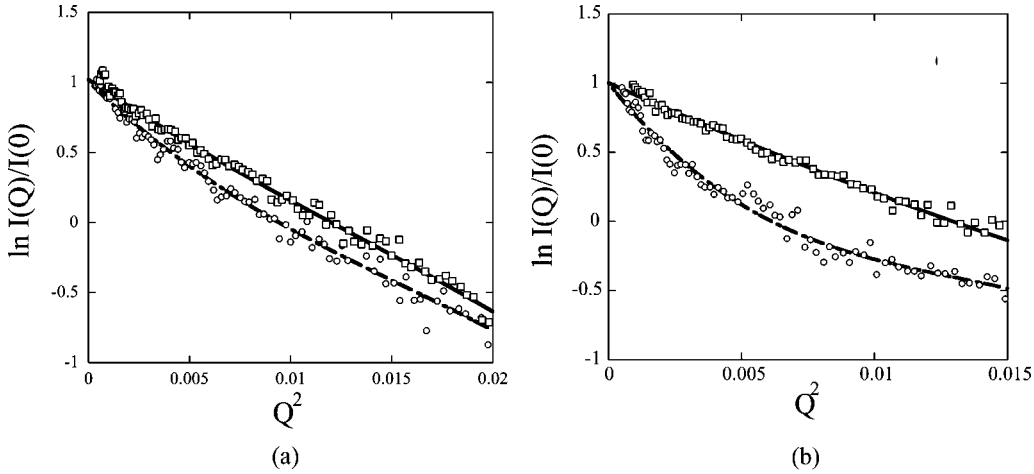

Figure 2
The normalized Guinier plots of WT (*a*) and C66A/C160A (*b*) in the acid-denatured state. The open circles show data in the absence of chloride ion. The squares show data in the presence of 80 m*M* chloride ion. The solid and broken lines show best-fit curves of the polynomial fitting function.

Kratky plots of both C66A/C160A and WT have a clear peak around Q = 0.09 Å^{-1}, indicating the globular shape. In the absence of chloride ion, the Kratky plot of WT still has a peak around Q = 0.09 Å^{-1}, although the peak height is lower than that in the presence of chloride ion. On the other hand, the Kratky plot of C66A/C160A has no peaks in the absence of chloride ion, suggesting that the molecule has lost its globularity. This pattern of Kratky plot is similar to that in the cold-denatured or urea-unfolded states of ELG.

4. Discussion

The secondary structures in the molten globule state are stabilized not only by short-range interactions but also by non-local interactions (Arai & Kuwajima, 2000). It has been shown for apomyoglobin, cytochrome *c* and α-lactalbumin that the helices in the molten globule state are destabilized when the molecule is expanded at low temperatures, where the hydrophobic contraction is weak, or at low ionic strength, where the electrostatic repulsion is strong. We have shown that the cold-denatured ELG takes an expanded chain-like conformation at lower temperatures (Yamada *et al.*, 2005). In this study, we have also confirmed that C66A/C160A is expanded at low anion concentration. As expected, the molecular dimension is well correlated with the helix content. Previously, we have shown that the cold-denatured ELG shows a CD spectrum indicating a significant amount of α-helix (Yamada *et al.*, 2005) and that C66A/C160A shows a helical CD spectrum at low ionic strength (Yamada *et al.*, 2006). Thus, the helix content in the acid-denatured state increases when the molecule is expanded. In contrast to helices in the molten globule states of other proteins, the helix content in the A state increases when the molecule is expanded. This is explained as follows. In the A state, the secondary structure is mainly determined by non-local interactions. When they are absent in the expanded conformation, local interactions becomes predominant and the amount of non-native α-helix increases.

It is interesting that C66A/C160A is significantly expanded in the absence of chloride ion while WT is not. The Cys66–Cys160 disulfide

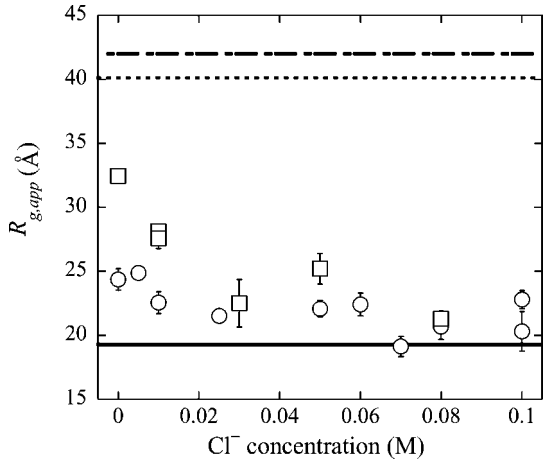

Figure 3
The chloride-ion concentration dependence of $R_{g,app}$ in the acid-denatured states of C66A/C160A (squares) and of WT (circles). The R_g of the native ELG (solid line), of the unfolded ELG in 8 *M* urea (pH 8.7) (dotted line) and of the unfolded C66A/C160A in 8 *M* urea (pH 8.7) (broken line) are shown.

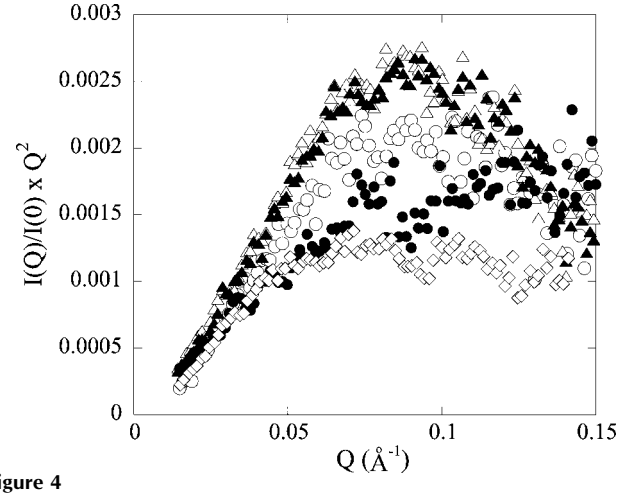

Figure 4
The normalized Kratky plots in the acid-denatured states of WT (open symbols) and C66A/C160A (filled symbols). The triangles and circles show a Kratky plot in the presence and absence of 80 m*M* chloride ion, respectively. The open diamonds shows a Kratky plot of WT in the unfolded state at pH 4.0 in the presence of 8 *M* urea.

bond would suppress the expansion of the polypeptide chain so that WT is not expanded even in the absence of chloride ion. At low temperatures, however, WT is expanded although it has intact disulfide bonds. This result indicates that the electrostatic repulsion is not sufficient for expanding the disulfide-containing molecule and the low temperature is effective for expanding it. In fact, C66A/C160A is not expanded to the same degree as the cold-denatured state. Even in the absence of chloride ion, $R_{g,app}$ of C66A/C160A (32 Å) is smaller than that in the cold-denatured ELG (37 Å). Unfortunately, the poor signal-to-noise ratio (Fig. 4) does not allow detailed comparison between the scattering curves of C66A/C160A in the absence of chloride ion and of the cold-denatured ELG.

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