Transactions of the Materials Research Society of Japan 33[2] 479-482 (2008)

Effect of NDSB on the Protein Aggregation

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Non-detergent sulphobetains (NDSBs) are used as additives for the purification and crystallization of various proteins utilizing their stabilizing effect. However, mechanism of the action of NDSBs is still unclear. We investigated the effect of NDSB-195 on bovine serum albumin (BSA) aggregation and denaturation employing light scattering, small angle X-ray scattering, circular dichroism and fluorescence spectroscopic measurements. NDSB-195 prevented aggregate formation induced by reduction at the early stage of aggregation process. In the fluorescence measurements, novel band was observed that may originate from the local highly structured regions. Those regions might be brought by the hydrophobic interaction between β-sheet domains of adjacent BSA molecules. These facts suggest that NDSB-195 acts so as to stabilize and protect the ordered α-helical domains of BSA on the occasion of structural change.

Key words: NDSB, aggregation, secondary structure, light scattering, circular dichroism

1. INTRODUCTION

Expressions of cloned genes in bacteria have been widely used both in industry, for the production of pharmaceutical proteins, and in the structural and/or biochemical studies [1]. *Escherichia coli* is the most general host expressing various proteins due to its rapid growth and convenient handling. In the over production of proteins, however, the product of interest is frequency deposited in insoluble and inactive aggregates referred to as the inclusion bodies. Refolding of recombinant proteins expressed as insoluble inclusion bodies is not straightforward and is very troublesome. The insoluble nature of inclusion bodies may be due to their increased levels of non-native β-sheet [2]. Filamentous aggregates constituted by non-native β-sheet are known as amyloid fibrils. Formation of amyloid fibrils is associated with a large group of diseases with unrelated origins, including Alzheimer's disease, prion diseases, and type-II diabetes [3,4]. Exploitations of the methods of effective prevention of protein aggregation are therefore highly important, not only for the protein engineering but also for the clinical uses.

In order to prevent protein aggregation, many kinds of low molecular weight additives were used in the process of protein renaturation [5]. Among those additives, non-detergent sulphobetaines (NDSBs) are very useful, but are not popular yet.

NDSB is one of the zwitterionic compounds. Like zwittergents, NDSBs carry the hydrophilic head group and hydrophobic group, however, do not form micelles because of the smallness of hydrophobic group. As well as being effective in the extraction [6], stabilization, [7], and renaturation [8-10] of proteins, NDSBs have also been ascertained to enhance both the size and rate of growth of protein crystals [11]. Yet, only a limited number of proteins have been investigated, and it is probable that NDSBs may affect in different manners. The mechanism of action of NDSBs is still unclear yet.

In the present report, the additive effects of NDSB on the several protein aggregation and/or denaturation were studied. NDSB-195 [12] was used in the present study. The molecular structure is shown in Fig.1. The aggregating process of bovine serum albumin (BSA) was analyzed using light scattering, small angle X-ray scattering, circular dichroism and fluorescence spectroscopy measurements.

2. MATERIALS AND METHODS

BSA was obtained from Sigma. NDSB-195 was obtained from Merck·Calbiochem.

Lyophilized BSA was dissolved in MES buffer at pH 6.4 supplemented with or without NDSB-195. Dithiothreitol (DTT), as a reducing agent, was dissolved in milli-Q water immediately before the measurement. Measurements started at the time that DTT solutions were added to the protein solution, and carried out at 37°C. The final concentrations were as follow: 10 mg/ml BSA (light scattering) or 2 mg/ml BSA (otherwise), 20 mM MES (pH 6.4), 3 mM DTT, and with or without 0.5 M NDSB-195.

Dynamic light scattering measurements were carried out by employing ALV-5000 multiple-tau correlator to obtain the correlation function of the scattered light intensity $g^{(2)}(t)$ and the averaged scattered light intensity

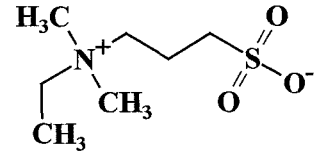

Fig. 1. Molecular structure of NDSB-195.

simultaneously. The decay time distribution function $G(\tau)$ was obtained from $g^{(2)}(t)$ by using CONTIN program. Light source was vertically polarized Ar ion laser operated at wavelength 488.0 nm and details of the apparatus were described elsewhere [13]. The measurement of $g^{(2)}(t)$ in the time course of aggregation were carried out at the scattering angle $\theta = 30^{\circ}$.

Small angle X-ray scattering measurements were performed (wavelength = 0.149 nm) at the photon factory of High Energy Accelerator Research Organization (BL-10C, KEK-PF). Radii of gyration were determined by the Guinier-plot analyses of the scattering functions at low scattering angles. Distance distribution function was calculated from the experimental scattering functions.

Circular dichroism measurements were carried out using Jasco J-720 spectropolarimeter. The UV CD data were acquired at 20°C in 0.1 mm path-length cell. CD data were expressed as the mean residue ellipticity [θ] in deglcm^2/dmol. The spectra were analyzed for the content of secondary structures by using protein CD analysis program, CONTINLL [14,15].

Fluorescence spectra were obtained by using Hitachi F-4010 fluorescence spectrophotometer at the Vv geometry. Excitation wavelength was 330 nm.

3. RESULTS AND DISCUSSION

BSA is a protein that has a lot of disulfide bond in native state. Aggregation takes place due to the destabilization induced by reduction of disulfide bond. Figure 2 shows the temporal evolution of the hydrodynamic diameter of BSA at 10 mg/ml with or without 0.5 M NDSB-195. Increase of diameter corresponds to the growth of BSA aggregation. Beginning of the increase at the time of 30 min in the absence of NDSB and 50 min in the presence of NDSB was observed. Fairly less and delayed increase was clearly observed in the presence of NDSB than that in the absence of NDSB. Radii of gyration, Rg, were evaluated by the Guinier-plot of the scattering functions of the SAXS measurements. The ratios of Rg to Stokes radii calculated from the decay time distribution were less than unity suggesting the compact aggregate formation. It was ascertained, too, by the fact that Kratky-plot showed a marked appearance of maximum against the scattering wave vector. That is, the temporal evolution after the addition of DTT proceeds as the aggregate growth of BSA molecules. Figure 3 depicts the distance distribution function P(r) of BSA without and with NDSB-195 at 25°C. Only a slight sharpening of P(r) is observed by the addition of NDSB-195. This fact means that NDSB-195 does not modify the intact conformation of BSA, but affects to maintain the ordered state.

Figure 4 illustrates far-UV CD spectra after the addition of DTT. The analyses of secondary structure employing CONTINLL program showed that α-helical content decreases (simultaneously, increase of β-sheet content) notably just after the addition of DTT for the case without NDSB-195, but such a decrease was inhibited substantially by NDSB-195. These results suggest that NDSB-195 prevented aggregate formation of BSA molecules at the early stage of aggregating process, and slowed down its progress by affecting it so as to maintain the ordered α-helical state of native BSA.

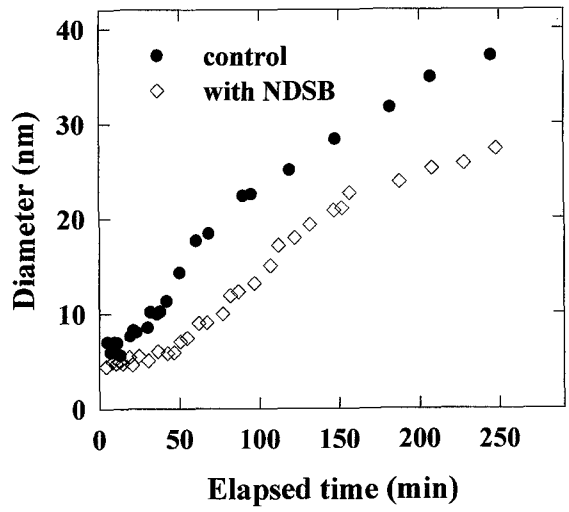

Fig. 2. Temporal growth of the hydrodynamic diameter of BSA aggregates after addition of DTT. Control means the absence of NDSB.

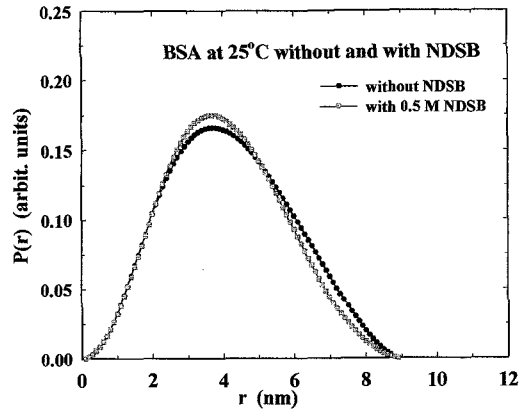

Fig. 3. Distance distribution function of BSA without and with NDSB obtained by the Fourier transform of scattering functions of SAXS measurements.

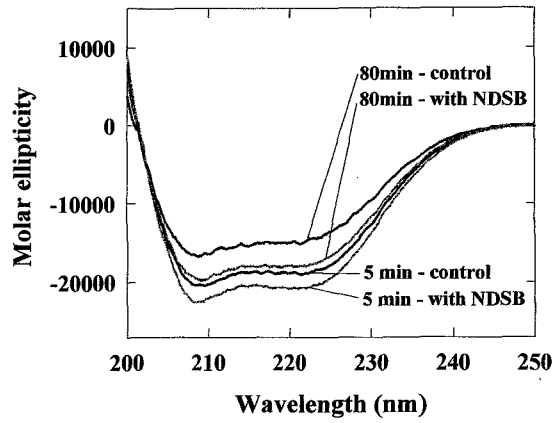

Fig. 4. Far-UV CD spectra of BSA after addition of DTT. The overall profile corresponds to α-helical structure even at 80 min, where the aggregate growth proceeded significantly.

On the other hand, fluorescence spectra exhibit very unique behavior. In accordance with the marked increase of hydrodynamic diameter (formation of aggregates), bell shaped band (band-X) appeared accompanying very broad blind fluorescence modes. This band shows a systematic wavelength shift with varying the excitation wavelength, and this fact suggests that band-X does not originate from the fluorescence mode. The spectra represented as a function of frequency shift from the excitation light is shown in Fig. 5. A sharp band near 3500 cm^{-1} corresponds to the Raman mode of water. No band was observed near 6000 cm^{-1} at 0 min. At 60 min, band-X appeared slightly. Band-X is clearly observed around 6000 cm^{-1} at 300 min after the addition of DTT for both samples of the control (without NDSB) and that of with NDSB, where aggregation of BSA proceeds very much. The spectrum at 60 min for the sample with NDSB is almost indistinguishable form that at 0 min of control sample. as for the origin of band-X, the magnitude of frequency shift of band-X varies with the variation of excitation wavelength and the origin is not clearly defined yet at the present stage.

The spectra were decomposed to the contributions of Rayleigh component, Raman mode of water and blind fluorescence mode, and the band-X was extracted. Peak frequency shift of the extracted band was about 6500 cm^{-1}. Temporal growth of its peak intensity is shown in Fig. 6. The curve profiles are quite similar to those of Fig. 2, and it is suggested that band-X is related to the aggregate formation.

In order to clarify this hypothesis, similar fluorescence spectroscopic measurements were carried out for NiPAM gel beads. NiPAM gel beads were synthesized by emulsion polymerization with [NiPAM] / [BIS] = 140, where NiPAM and BIS are N-isopropylacrylamide and N,N'-methylene-bis-acrylamide as a cross-linking agent, respectively. Distinct volume phase transition took place around 32°C. The spectra represented as a function of frequency shift is shown in Fig. 7. Similarly to Fig. 5, bell shaped band near 6500cm^{-1} was observed, and it grew with the decrease of temperature. At low temperature, such a band is very weak. With the shrinking of gel beads, peak intensity grows markedly in a very narrow temperature range, and it shows plateau behavior at further higher temperature. Such a behavior agreed well with the density variation of gel beads. The similar results were obtained for the gelling process of polysaccharide (data not shown). For NiPAM gel beads, volume phase transition occurs due to hydrophobic interaction and the formation of local densely packed structure. The appearance of distinct volume phase transition (not the precipitation) in poly(NiPAM) chains originates from the globule-coil transition in a single chain and the aggregation of micro-clusters (crumpled globules) due to hydrophobic interaction [16,17]. Those crumpled globules result in a large size change of the chain. Amide groups adjacent to isopropyl group form tight hydrogen bonds extensively in that process. Those hydrogen bonded region could form a local regular structure, and such a structure grows up with the increase in temperature. Band-X could be brought about by the cooperative vibration in that structure.

The exact assignment of band-X is of course needed. However, it can be considered analogously that unstruc-

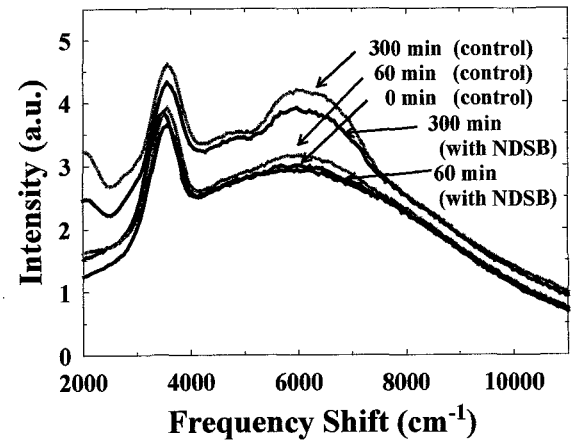

Fig. 5. Spectra of BSA represented as a function of frequency shift.

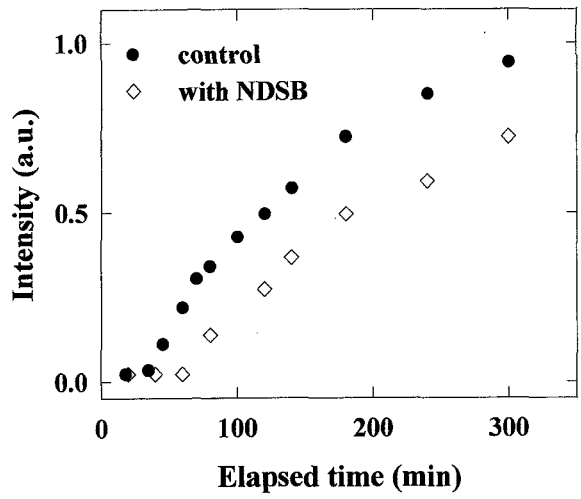

Fig. 6. Temporal growth of band-X. Intensity denotes the peak intensity at 6500cm^{-1}.

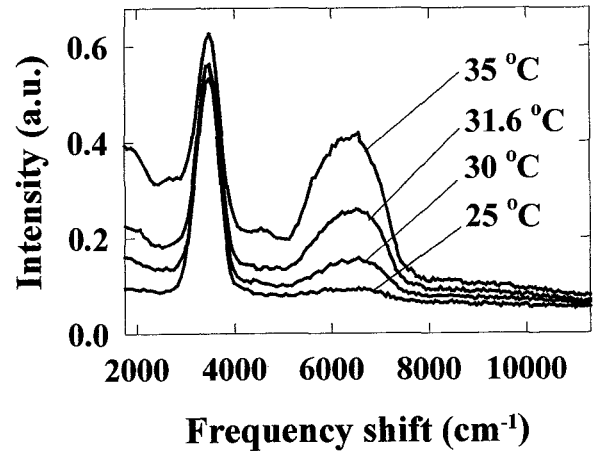

Fig. 7. Spectra of NiPAM gel beads at various temperatures represented as a function of frequency shift.

tured regions (and the neighboring turn or loop regions) and random coil regions resulted from the reduction of S-S bonds by DTT may interact with each other in case of BSA. In fact, it is shown by the secondary structure analyses that β-sheet content increases simultaneously with the decrease of α-helical content. Those β-sheet domains, newly formed in this manner, become to interact hydrophobically and result in tightly packed and local structured regions. Therefore, band-X should be regarded as the result of building up of such a region. NDSB-195 acts to inhibit the destruction of α-helical structure and the hydrophobic interaction between β-sheet domains. Therefore, the retardation of aggregation could occur in the presence of NDSB-195.

If it was taken into account that NDSB can be easily removed by dialysis and do not inhibit protein activity [18], NDSB prevents protein aggregation not by interacting with (binding to) BSA molecules directly, but by affecting the solvent-atmosphere (state of solvation) surrounding the surface of BSA molecules. In fact, the additive effect of NDSB appears at the NDSB concentration much larger than the protein concentration. Therefore, it is suggested that NDSB may prevent protein aggregation by the screening effect of hydrophobic (and electrostatic) interaction accompanied by the conformational change of protein.

Stabilizing and protecting effects similar to those of NDSBs are known in several small stress-responsive molecules, e.g. ectoine [19]. In a recent computational study, it has been reported that those effects are due to keeping the water structure near the protein surface unchanged against external change of circumstances [20]. The protective effect of ectoine appears at rather high concentration as 50~100 mM similar to the case of NDSB [21]. That is, NDSB distributes around protein molecule together with water molecules and condensates where the hydration state varies accompanied by the disordering and unstructuring of amino acid sequences. Those actions should proceed to the α-helical structure preferably to β-sheet state. Of course, this hypothesis is not proven enough, and energetic evaluation is desirable. However, weak hydrophobic property and tendency to form weak cluster, which is observed actually in aqueous NDSB solutions, should be essential properties for those stabilizing agents.

4. CONCLUSION

Present study demonstrated that NDSB-195 prevents aggregate formation of BSA at the early stage of aggregation process. NDSB-195 retained α-helical structure in the aggregating process and delayed the aggregation.

ACKNOWLEDGMENTS

A part of this work has been performed under the approval of the Photon Factory Program Advisory Committee (Proposal No. 06G394). One of the authors (K.K.) is grateful for the support in the SAXS measurements.

REFERENCES

[1] E. D. B. Clark, *Curr. Opin. Biotechnol.* **9**, 157–163 (1998).
[2] E. D. B. Clark, *Curr. Opin. Biotechnol.* **12**, 202–207 (2001).
[3] C. M. Dobson, *Nature* **426**, 884–890 (2003).
[4] Y. Porat, Y. Mazor, S. Efrat and E. Gazit, *Biochemistry* **43**, 14454-14462 (2004).
[5] T. Arakawa and K. Tsumoto, *Biochem. Biophys. Res. Commun.* **304**, 148–152 (2003).
[6] L. Vuillard, C. Braun-Breton and T. Rabilloud, *Biochem. J.* **305**, 337–343 (1995).
[7] T. Collins, S. D. Amico, D. Georlette, J. C. Marx, A. L. Huston and G. Feller, *Anal.Biochm.* **352**, 299-301 (2006).
[8] M. E. Goldberg, N. Expert-Bezançon, L. Vuillard and T. Rabilloud, *Folding Des.* **15**, 21–27 (1995).
[9] L. Vuillard, T. Rabilloud and M. E. Goldberg, *Eur. J. Biochem.* **256**,128–135 (1998).
[10] N. Expert-Bezançon, T. Rabilloud, L Vuillard and M. E. Goldberg, *Biophys. Chem.* **100**, 469-479 (2003).
[11] L. Vuillard, R. Leberman, C. B. Colominas, S. Cusack and T. Rabilloud *FEBS Lett.* **353**, 294-296 (1994).
[12] K. D. Koclega, M. Chruszcz and W. Minor, *Acta Cryst.* **E62**, o5757-o5759 (2006).
[13] R.Takahashi, M.Akutu, K.Kubota and K.Nakamura, *Progr. Colloid. Sci.* **114**, 1-7 (1999).
[14] N. Sreerama and R. W. Woody, *Anal. Biochem.* **287**, 252-260 (2000).
[15] W. C. Johnson, *Proteins: Structure, Function, and Genetics* **35**, 307-312 (1999).
[16] T. M. Birshtein and V. A. Pryamitsyn, *Macromolecules*, **24**, 1554-1560 (1991).
[17] X. Wang, X. Qiu and C. Wu, *Macromolecules*, **31**, 2972-2976 (1998).
[18] H. Kogure, Y. Masuda, T. Ishii, K. Wakamatsu, K. Kubota and M. Ochiai, *Trans. MRS-J.* **31**, 787-790 (2006).
[19] R. Talibart, M. Jebbar, G. Gouestbet, S. H-Kabbab, H. Wroblecki, C. Blanco and T. Berbard, *J. Bacteriol.* **176**, 5210-5217 (1994).
[20] I. Yu and M. Nagaoka,*Chemical Physics Letters* **388**, 316-321 (2004).
[21] M. Kanapathipillai, G. Lentzen, M. Sierks and C. B. Park, *FEBS Lett.* **579**, 4775-4780 (2005).

(Recieved March 6, 2008 ; Accepted March 12, 2008)