Asian Journal of Chemistry Vol. 20, No. 2 (2008), 1503-1513

High Helical Propensity of the β-Lactoglobulin with Non-native α-Helical Intermediate: A Spectroscopic Approach

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It is generally considered that intermediates of protein folding contain partially formed native-like secondary structures. To understand the mechanism that stabilizes the non-native intermediate, it was characterized by circular dichroism the equilibrium unfolding transition of β-lactoglobulin induced by sodium dodecyl sulfate at pH 2. β-lactoglobulin is a predominantly β-sheet protein, although it has a markedly high intrinsic preference for α-helical structure. The far-UV CD spectrum of the intermediate, obtained by global fitting analysis of the CD spectra in the presence of various concentration of sodium dodecyl sulfate, was similar to the burst phase intermediate observed in the refolding thermodynamics and contained non-native α-helical structures. The effect of sodium dodecyl sulfate on the structure of native β-lactoglobulin at pH 2 was utilized to investigate the contribution of hydrophobic interactions to the stability of non-native α-helical intermediate. Addition of different concentrations of sodium dodecyl sulfate increased the helical content of the equilibrium intermediate, although the protein still assumed the native structure in the absence of sodium dodecyl sulfate. This indicate that because of the high helical preference of the amino acid sequence of β-lactoglobulin, the helical region protrudes into the boundary between the native and unfolded state, resulting in non-monomeric accumulation of the helical intermediate upon equilibrium unfolding of the native β-sheet structure. The present results suggest that a non-native α-helical intermediate accumulates during equilibrium unfolding of a predominantly β-sheet protein.

Key Words: β-Lactoglobulin, Non-native α-helical intermediate, Sodium dodecyl sulfate, Non-hierarchical mechanism, Thermodynamics, Circular dichroism.

INTRODUCTION

Although the two-state protein folding model suggested that no fragments should remain structured when isolated, several residual structures

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have now been observed in peptide fragments corresponding to regular secondary structures from native proteins[1,2]. The current consensus is that when these peptides are structured, they form secondary structures consistent with those observed in the native structure. This is known as the consistency principle and supports the framework model of protein folding[3,4].

Kinetic studies using stopped-flow circular dichroism (CD) or amide hydrogen-deuterium exchange techniques in conjunction with two-dimensional nuclear magnetic resonance (NMR) spectroscopy have indicated that intermediate structures resembling the equilibrium molten globule state accumulate before the rate-limiting step of folding[5,6]. In contrast, in a nucleation-condensation model of protein folding[7-10] the rate-limiting step is the formation of a nucleus and the folding reaction is rapid and approximated by a two-state transition. Whereas the validity of the nucleation-condensation mechanism has been argued for several small proteins, it would be difficult to expect that such a simple mechanism directly applies for a large protein consisting of multiple subdomains.

β-Lactoglobulin (β-LG) is the major whey protein found in the milk of ruminants, including cows and sheep and also monogastrics, *e.g.*, pigs, horses, dogs and cats. It is not, however, found in the milk of humans[11]. β-LG is a globular protein with a molecular weight of 18400 Dalton with each monomer of 162 amino acid residues. The secondary structure of bovine β-LG is composed of 15 % α-helix, 50 % β-sheet and 15-20 % reverse turn[12]. The tertiary structures consist of nine strands of antiparallel β-sheet, eight of which form a β-barrel that has the shape of a flattened cone or calyx[13,14]. There is a three-turn α-helix on the outer surface of the calyx. The interior of the β-barrel is hydrophobic, whereas the opening is lined with hydrophobic amino acids. The eight-stranded β-barrel is a major structural motif found in a family of proteins which have the ability to bind several amphiphilic or hydrophobic ligands[15,16] such as retinol[17], long-chain fatty[18] and sodium dodecyl sulfate[19-22].

The α-helix to β-sheet transition of proteins is a key issue for understanding the folding and biological function of a number of proteins[23-29]. For example, the $\alpha \rightarrow \beta$ transition has been suggested to play an essential role in various conformational disease, such as prion disease or Alzheimer disease where the α-helix forms are normal and the β-sheet forms are amyloidogenic[25-27]. Bovine β-LG would be a useful model for clarifying the mechanism of the $\alpha \rightarrow \beta$ transition, since its folding process is accompanied by the $\alpha \rightarrow \beta$ transition due to the inconsistency of local and non-local interactions[30-32]. Whereas β-LG exist as a dimer at neutral pH, it dissociates into monomer below pH 3, but retains a native conformation, even in an acidic environment with a pH as low as 2[33]. During the process of β-LG refolding from a denaturant-induced unfolded state the ellipticity

at 219 nm transiently exceeds the native intensity (the overshoot phenomenon)[34]. An intuitive interpretation of this is the accumulation of a α-helical intermediate, since the circular dichroism (CD) intensity of an α-helix is greater than that of a β-sheet. Three pieces of evidence indicate that the amino acid sequence of β-LG has a markedly high preference for an α-helix conformation[35-38]. The possibility of a helical intermediate is further reinforced by the observation of a high helical preference for the β-lactoglobulin amino acid sequence, which was clarified by the addition of alcohol and also by the reduction of the disulfide bridges. A similar phenomenon was observed for cellular retinoic acid binding protein[39], but not for other β-sheet proteins.

Conformational changes and denaturation of many proteins in surfactant solutions have been studied[40-42]. Protein denaturation occurs in a numbers of ways. On the millimolar scale, sodium *n*-dodecyl sulfate (SDS) at higher concentrations is believed to be one of the most effective denaturants[43]. Moosavi-Movahedi has reviewed the folded and unfolded states of different proteins in the presence of both low and high concentrations of surfactant, respectively[44-49]. In this paper, we report the formation of a non-native α-helical intermediate of β-LG induced by *n*-alkyl sulfates as anionic surfactants at pH 2. It should be mentioned that in this paper, non-native α-helical intermediate can be induced by sodium dodecyl sulfate in β-LG at special condition and it dose not mean that intermediate state can be induced by surfactant in all proteins. The results indicate the accumulation of an intermediate as non-native α-helical state in β-LG.

EXPERIMENTAL

Bovine β-LG was purchased from Sigma. 1-Anilino-naphthalene-8-sulfonate (ANS) was obtained by Fluka. Sodium dodecyl sulfate (SDS) was purchased from Sigma. Other chemicals were of reagent grade. The concentrations of sodium dodecyl sulfates used in all experiments were under the critical micelle concentrations (CMC) and critical aggregation concentration (CAC)[50,51].

Solution preparation: The protein solution was dialyzed against buffers (20 mM HCl, pH 2). The extinction coefficients were used to calculate the concentration of the native protein at pH 2. If the initial concentration and volume of the protein solution are $[P]_0$ and V_0, respectively and the stock ligand concentration is $[L]_0$, then the total concentration of protein ($[P]_t$) and ligand ($[L]_t$) can be obtained by accounting for the total volume of the aliquot (V_c) added during the titration experiment[52]:

$$[P]_t = [P]_0V_0/(V_0 + V_c),\ [L]_t = [L]_0V_0/(V_0 + V_c) \quad (1)$$

Aliquots of *n*-alkyl sulfate were injected into the β-LG solution at 5 min intervals to allow for equilibration. Each experiment was repeated three times. The protein concentration was determined from the absorption at 278 nm using the absorption coefficient of $E_{278} = 9.6$[53].

Circular dichroism (CD) measurements: All measurements were carried out at 20 °C with thermostatically controlled cell holders. Far-UV CD spectra were measured with a Jasco spectropolarimeter (Japan), model J-720, equipped with an interface and a personal computer. The instruments were calibrated with ammonium d-10-camphorsulfonic acid[52]. The data were expressed as molar residue ellipticity [θ], which is defined as [θ] = 100 θ_{obs}/cl, where θ_{obs} is the observed ellipticity in degrees, c is the concentration is residue mol cm^{-3} and l is the length of the light path in centimetres. Typically, 50 µL of protein solution at a protein concentration of 1 mg mL^{-1}, dissolved in deionized water, was mixed with 450 µL of 20 mM HCl (pH 2) containing various concentrations of sodium dodecyl sulfates. CD spectra were recorded with a time constant of 4 s, a 2 nm band width and a scan rate of 5 nm min^{-1}, were signal-averaged over at least five scans and baseline corrected by subtracting a buffer spectrum. The CD spectra were measured with a 1 mm path length cell from 250 to 190 nm.

Fluorescence measurements: Fluorescence measurements were performed by using a Hitachi 2500 spectrofluorimeter. The intensity at 480 nm was detected after excitation at 350 nm. Sodium dodecyl sulfate significantly affect the fluorescence of free tryptophan under the experimental conditions used. The temperature of the cell compartments was kept constant at 20 °C by water circulation.

RESULTS AND DISCUSSION

Circular dichroism (CD): The special positions of the segments with sequence corresponding to those of the synthetic peptide are shown in Table-1. The sequences of fragments 1-3 correspond to the A, D and F strands, respectively. The sequence of fragment 4 corresponds to the helix which extends from residues 130-140 and is located between strands G and I. These peptide segments were of particular interest because they were predicted to have a high helicity by secondary structure prediction methods. It determined the precise location for our fragments, we chose regions that include negatively and positively charged groups located at the N and C terminal, respectively, to counter-balance the macrodipole of the helix. The far-UV CD spectra of these fragments at pH 2 showed that the peptides retain residual helical structures even in aqueous solution (data not shown). The peptide showed similar helical preferences at pH 7. First, the helicity of fragment 4 which corresponds to the α-helix in the native β-lactoglobulin is about 17 % in water. This value is larger than those observed for the

peptides corresponding to the helices of myohemerythrin. It is, however smaller than the helicity of the 15-residue peptide corresponding to the C-terminal helix of cytochrome c or the C-peptide of RNase A, which exhibit especially high helicity even in aqueous solution. Thus, the residual helicity in fragment 4 is high but not exceptional.

TABLE 1
SEQUENCES OF BOVINE β-LACTOGLOBULIN B FRAGMENTS

Fragment	Sequence
Fragment 1 (11-28)	Ac-D I Q K V^{15} A G T W Y^{20} S L A M A^{25} A S D-NH_2
Fragment 2 (61-77)	Ac-W E N G E^{65} C A Q K K^{70} I I A E K^{75} T K-NH_2
Fragment 3 (85-101)	Ac-D^{85} A L N E N^{90} K V L V L^{95} D T D Y K^{100} K-NH_2
Fragment 4 (127-142)	Ac-Y E V V D^{130} E A L E K^{135} F D K A L^{140} K A-NH_2

The sequences of the four peptides are shown with residue numbers corresponding to those of the intact β-lactoglobulin. An additional tyrosine residue has been added to the N terminus of fragment 4 for concentration determination.

Fig. 1 shows the effects of sodium dodecyl sulfate on the far-UV CD spectra of the native state of β-LG at pH 2. The native structure of β-LG is stable as a monomer even at pH 2[33]. In the absence of sodium dodecyl sulfate in 20 mM HCl, the far-UV CD showed a spectrum with a minimum at 218 nm, consistent with the abundance of β-sheets (Fig. 1, curve 1). The addition of sodium dodecyl sulfate at different concentrations induced substantial α-helical structure as seen by the lower minima (lower $[\theta]_{208}$, $[\theta]_{222}$) at 208 and 222 nm relative to the native spectrum (Fig. 1, curve 5). A highly cooperative β-sheet to α-helix transition, with an isodichroic point at 203 nm was seen for the four panels. This suggests that β-sheet to α-helix transition is a two state process[54]. The CD spectra also show the non-native α-helical intermediate (αI) state for β-LG upon the addition of SDS (0.25 mM). When sodium dodecyl sulfate (SDS) is larger than values, the baselines increase, due to precipitation (data not shown). The shapes of the CD spectra are similar to the spectrum of β-LG in the presence of 9.8 % (v/v) TFE[55-57].

Considering these findings, the sodium dodecyl sulfate-induced conformations are not an indication of the ordinary intermediate. In fact, they are regarded as αI states of the protein with different secondary structures. The helical content in the native state of β-LG is 7 %, on the basis of the ellipticity values at 222 nm as calculated by the method of Chen *et al.*[58]. The helical content of the αI state of β-LG induced by sodium dodecyl sulfate (SDS) is 90.3 % according to this method.

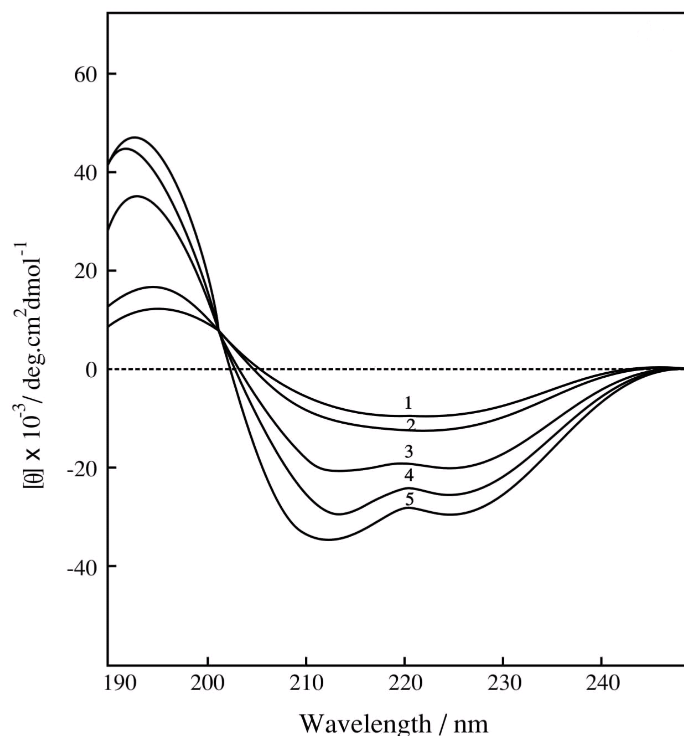

Fig. 1. Far UV CD spectra of β-LG (ellipticity, [θ]) as a function of sodium dodecyl sulfate concentration at pH 2 and and 20 °C. **1**, 0 mM SDS (native state at pH 2); **2**, 0.05 mM SDS; **3**, 0.1 mM SDS; **4**, 0.2 mM SDS; **5**, 0.25 mM SDS. Protein concentration was 27 μM

Fluorescence spectra: As in previous studies, changes in the accessibility of hydrophobic regions, changes in the accessibility of hydrophobic regions of the molecule were monitored through the binding of the fluorescence hydrophobic probe ANS. Fig. 2 shows the effect of SDS on the fluorescence spectra of the β-LG-ANS complex at pH 2. According to Fig. 2, the addition of different concentrations of SDS to the β-LG-ANS complex causes an increase in the fluorescence intensity. Here, the interaction of β-LG-ANS complex with SDS at different concentrations is consistent with the results obtain for β-LG in the presence of TFE as previously reported by Hamada *et al.*[32].

Thermodynamic analysis of non-native α-helical intermediate (αI) formation: Fig. 3 shows the sigmoidal curves (drawn by a numerical analysis method, called qubic-spline in the MATLAB program, version 6.1) for the native (N) to the non-native α-helical intermediate (αI) of β-LG upon the addition of sodium dodecyl sulfate. β-LG is a small protein with a single subunit. Therefore, a two-state analysis based on the Pace

theory was performed[59]. It is now possible to obtain equilibrium constants (K) for the N-αI states and to calculate the corresponding Gibb's free energy changes (ΔG°) as follows:

$$\Delta G^\circ = -RT \ln (A_{obs} - A_N)/(A_{\alpha I} - A_{obs}) \quad (2)$$

where R is the gas constant, T is the absolute temperature, A_N, $A\alpha I$ and A_{obs} are the physical parameters of molar ellipticity and fluorescence intensity of ANS in the presence of N, αI and any observed states, respectively.

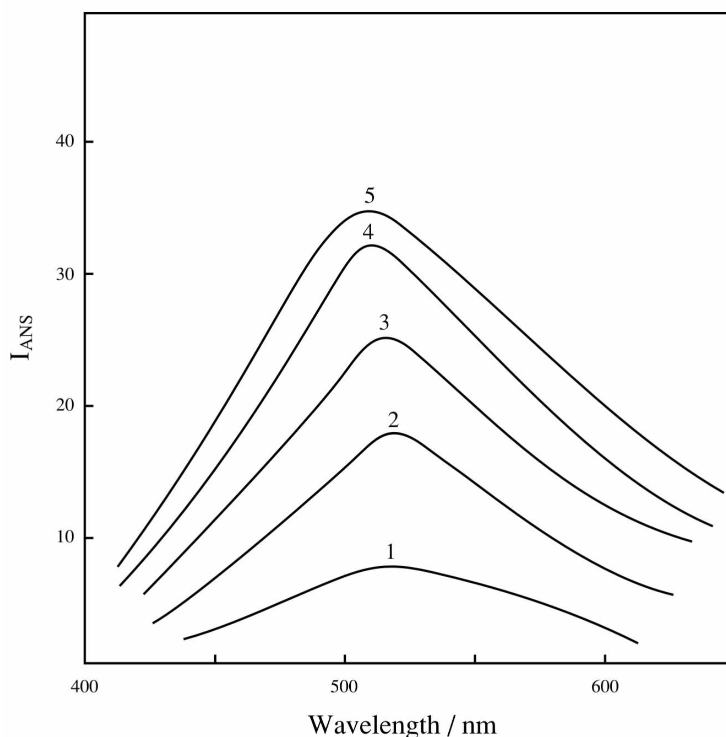

Fig. 2. Fluorescence spectra of 250 μM ANS in the present of following: **1**, free ANS at pH 2; **2**, native state of β-LG at pH 2; **3**, β-LG upon the addition of 0.05 mM SDS; **4**, β-LG upon the addition of 0.1 mM SDS; **5**, β-LG upon the addition of 0.25 mM SDS. The ratio of molar concentrations [ANS]/[protein] = 250/1

Fig. 3 shows the plot of ΔG° against total sodium dodecyl sulfate concentration ([sodium dodecyl sulfate]$_{total}$ = [sodium dodecyl sulfate]$_{free}$ + [sodium dodecyl sulfate]$_{bound}$). The free energie of αI formation in the absence of sodium dodecyl sulfate, ΔG° (H_2O), was calculated by the least-squares method from the following equation[59]:

$$\Delta G^\circ = \Delta G^\circ (H_2O) - m \text{ [sodium dodecyl sulfate]} \quad (3)$$

where m is the slope of linear curve reflecting the cooperativity and also hydrophobicity of the transition state. The m-value correlates strongly with the amount of protein surface exposed to the solvent upon unfolding[59-62]. A similar dependence on accessible surface area has been found for the heat capacity change (ΔC_p), which was confirmed for a set of proteins[62,63]. The m-values and heat capacity changes correlate well with each other and also ΔC_p of the protein is linearly related to the fraction of hydrophobic residues[64]. Thus, for proteins that undergo a simple two-state unfolding mechanism, the amount of surface exposed to solvent upon unfolding is a main structure determinant for m-values, ΔC_p and hydrophobicity. The free energy value shown as the dotted linear line in Fig. 3.

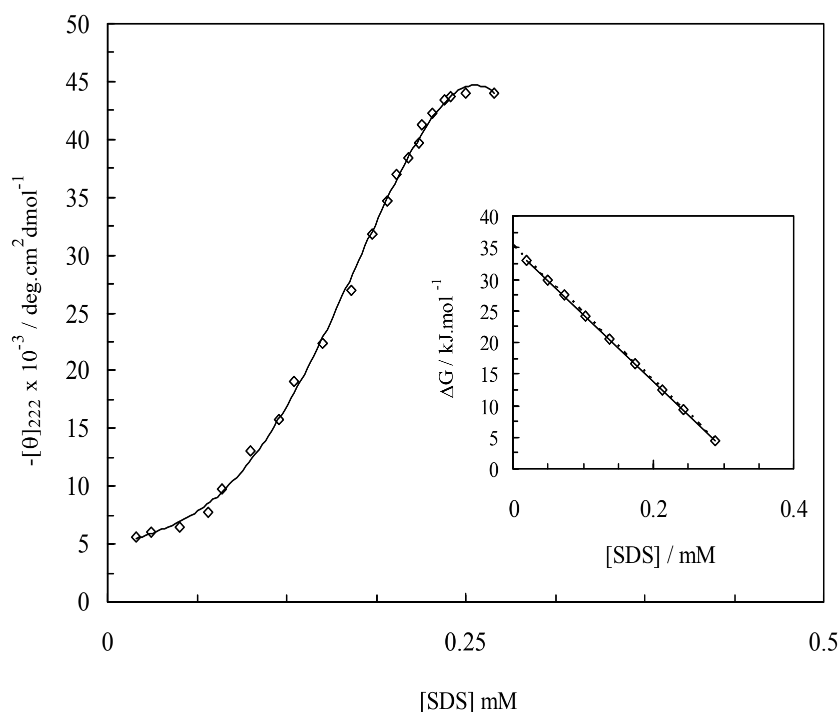

Fig. 3. Plots of molar ellipticity ($[\theta]_{222}$) *vs.* concentrations of SDS at pH 2 and 20 °C. Inset: Free energy values (ΔG°) *vs.* concentrations of SDS

The similar order was reported for the alcohol-induced α-helix formation of β-LG[56]. Alcohol-induced denaturation of proteins has been considered to arise from the low polarity of the solvent which decreases the hydrophobic interactions stabilizing the compact native structure of proteins. Therefore it is plausible to expect that variation of the effectiveness of alcohol may be related to the polarity of the solvent. In solvents of low

polarity, hydrophobic interactions stabilizing the native structure are weaken and simultaneously the local hydrogen bonds are strengthened, resulting in denaturation and stabilization of the extended α-helical structures. The far-UV CD spectra in the presence of various concentrations of *n*-alkyl sulfates show that, whereas β-LG is unfolded at pH 2, the addition of sodium dodecyl sulfate stabilizes the helical conformation. This indicates that the denaturant of a sodium dodecyl sulfate is closely correlated with its potential for stabilizing the helical conformation in the β-LG. Although the anionic head of sodium dodecyl sulfate is an important factor determining the SDS effects, we consider that the direct interaction between hydrophobic tails of SDS and hydrophobic groups of proteins is responsible for the SDS effects. When SDS at high concentrations as denaturant ligands interact with native state of β-LG, the interior hydrophobic groups of protein expose to solvent, then the polarity around the β-LG will decrease. This leads to stabilization of the interamolecular hydrogen binds and consequently the formation of a helical conformation. Strengthening the hydrogen bonds is responsible for the *n*-alkyl sulfate effects on the helix formation of proteins, suggesting that the mechanism of the *n*-alkyl sulfates-induced helix formation of proteins are distinct from that of the *n*-alkyl sulfates-induced denaturation of proteins in which hydrophobic effects arising from *n*-alkyl sulfates molecules are important.

It is known that globular protein polypeptide chains include both hydrophilic and hydrophobic side chains. Moreover, the secondary structure of proteins forms cluster from hydrophobic side chains that, in turn, from the hydrophobic core of globular proteins. Some of the native proteins have a stronger affinity to ANS because they have hydrophobic sites exposed to the solvent (for example, active centres and sites for heme or substrate binding)[64-67]. Fig. 2 represents the fluorescence spectrum of ANS in the presence of β-LG at different concentrations of SDS. The affinity of ANS to the β-LG increases significantly when the rigidity of protein tertiary structure is disrupted, therefore the addition of SDS at different concentrations to the native state of β-LG at pH 2 cause an increase in the fluorescence intensity.

The spectroscopic properties of the αI state strongly support the view that SDS at different concentrations stabilize the αI state of β-LG. The αI state in contrary to native state of β-LG shows highly amounts of α-helix. The spectroscopic properties of the αI state closely resemble the ones, as reported by Hirota *et al.*[57], thus implying a close structural similarity. In conclusion, we show that the helical propensities of β-LG in the presence of sodium dodecyl sulfate is not related to its native structure. Furthermore, the helical propensity of the β-LG in the presence of sodium dodecyl sulfate must be related to the intrinsic helical propensity as was observed

for proteins[30]. The results suggest a case of non-hierarchical protein folding, in which the α-helical intermediate accumulates during the formation of the native β-sheet structure.

ACKNOWLEDGEMENTS

The financial support of the Research Council of the Islamic Azad University, Mashhad Branch, Mashhad, Iran and Glasgow University, U.K. are gratefully acknowledged.

REFERENCES

1. C.B. Anfinsen, *Science*, **181**, 223 (1973).
2. K. Kuwajima, *Proteins: Struct. Funct. Genet.*, **6**, 87 (1989).
3. O.B. Ptitsyn, in ed.: T.E. Creighton, The Molten Globule State, In Protein Folding, W.H. Freeman and Company, New York, p. 243 (1992).
4. P.A. Jennings and P.E. Wright, *Science*, **262**, 892 (1993).
5. A.R. Fersht, *Proc. Natl. Acad. Sci. USA*, **92**, 10869 (1995).
6. R.L. Baldwin, *J. Biochem. NMR*, **5**, 103 (1995).
7. L.S. Itzhaki, D.E. Otzen and A.R. Fersht, *J. Mol. Biol.*, **254**, 260 (1995).
8. S. Pervaiz and K. Brew, *Science*, **228**, 335 (1985).
9. A.M. Gutin, V.I. Abkevich and E.I. Shakhnovich, *Biochemistry*, **34**, 3066 (1995).
10. D.V. Waterhouse and W.C. Johnson, *Biochemistry*, **33**, 2121(1994).
11. S.G. Hambling, A.S. McAlpine and L. Sawyer, *Adv. Dairy Chem.*, **1**, 141 (1992).
12. L.K. Creamer, D.A.D. Parry and G.N. Malcolm, *Arch. Biochem. Biophys.*, **227**, 98 (1983).
13. M. Papiz, L. Sawyer, E. Eliopoulos, A. North, J. Findlay, R. Sivaprasadarao, T. Jones, M. Newcomer and P. Kraulis, *Nature*, **324**, 383 (1986).
14. S. Brownlow, J.H.M. Cabral, R. Cooper, D.R. Flower, S.J. Yewdall, I. Polikarpov, A.C.T. North and L. Sawyer, *Structure*, **5**, 481 (1997).
15. D.R. Flower, A.C.T. North and T.K. Attwood, *Protein Sci.*, **2**, 753 (1993).
16. L. Banaszak, N. Winter, Z. Xu, D.A. Bernlohr, S. Cowan and T.A. Jones, *Adv. Protein Chem.*, **45**, 89 (1994).
17. S. Futterman and J. Heller, *J. Biol. Chem.*, **247**, 5168 (1972).
18. A.E. Spector and J.E. Fletcher, *Lipids*, **5**, 403 (1970).
19. T.L. McMeekin, B.D. Polis, E.S. Dellamonica and J.H. Custer, *J. Am. Chem. Soc.*, **71**, 3606 (1949).
20. T.S. Seibles, *Biochemistry*, **8**, 2949 (1969).
21. M.N. Jones and A. Wilkinson, *Biochem. J.*, **153**, 713 (1976).
22. E. Lamiot, E. Dufour and T. Haertle, *J. Agric. Food Chem.*, **42**, 695 (1994).
23. J. Mottonen, A. Strand, J. Symersky, R.M. Sweet, D. Danley, K.F. Geoghegan, R.D. Gerard and E.J. Goldsmith, *Nature*, **355**, 270 (1992).
24. D.L. Minor and P.S. Kim, *Nature*, **380**, 730 (1996).
25. S.B. Prusiner, *Science*, **278**, 245 (1997).
26. H. Mihara and Y. Takahashi, *Curr. Opin. Struct. Biol.*, **7**, 501 (1997).
27. R.W. Carrel and D.A. Lowas, *Lancet*, **350**, 134 (1997).
28. D.R. Booth, M. Sunde, V. Bellotti, C.V. Robinson, W.L. Hutchinson, P.E. Fraser, P.N. Hawkins, C.M. Dobson, S.E. Radford, C.C.F. Blake and M.B. Pepys, *Nature*, **385**, 787 (1997).
29. D. Eliezer, J. Yao, H.J. Dyson and P.E. Wright, *Nature Struct. Biol.*, **5**, 148 (1998).
30. K. Shiraki, K. Nishikawa and Y. Goto, *J. Mol. Biol.*, **245**, 180 (1995).

31. D. Hamada and Y. Goto, *J. Mol. Biol.*, **269**, 479 (1997).
32. D. Hamada, Y. Kuroda, T. Tanaka and Y. Goto, *J. Mol. Biol.*, **254**, 737 (1995).
33. R.G. Futage and P.S. Song, *Biochim. Biophys. Acta*, **625**, 28 (1980).
34. K. Kuwajima, H. Yamaya, S. Miwa, S. Sugai and T. Nagamura, *FEBS Lett.*, **221**, 115 (1987).
35. K. Nishikawa and T. Noguchi, *Methods Enzymol.*, **202**, 21 (1991).
36 Y. Kuroda, D. Hamada, T. Tanaka and Y. Goto, *Folding & Design*, **1**, 243 (1996).
37. E. Dufour, H.C. Bertrand and T. Haertle, *Biopolymer*, **33**, 589 (1993).
38. K. Kuwata, M. Hoshino, V. Forge, S. Era, C.A. Batt and Y. Goto, *Protein Sci.*, **8**, 2541 (1999).
39. Z.P. Liu, J. Rizo and L.M. Gierasch, *Biochemistry*, **33**, 134 (1994).
40. A.A. Moosavi-Movahedi, G.A. Naderi and B. Farzami, *Thermochim. Acta*, **239**, 61 (1994).
41. M.R. Housaindokht and A.A. Moosavi-Movahedi, *Int. J. Biol. Macromol.*, **16**, 77 (1994).
42. A.A. Moosavi-Movahedi, *IUPAC*, **66**, 71 (1994).
43. A.A. Moosavi-Movahedi and S. Ghobadi, *Thermochim. Acta*, **189**, 201 (1991).
44. A.A. Moosavi-Movahedi and M.R. Razeghifard, *Thermochim. Acta*, **188**, 183 (1991).
45. D. Ajloo, A.A. Moosavi-Movahedi, G.H. Hakimelahi, A.A. Saboury and H. Gharibi, *Colloid and Surfaces B: Biointerfaces*, **26**, 185 (2002).
46. M.R. Dayer, A.A. Moosavi-Movahedi, P. Norouzi, H. Ghourchian and S. Safarian, *J. Biochem. Mol. Biol.*, **35**, 364 (2002).
47. A.A. Moosavi-Movahedi, Thermodynamics and Binding Properties of Surfactant-Protein Interactions in Encyclopedia of Surface and Colloid Science, Marcel Dekker, New York, p. 5344 (2002).
48. A.A. Moosavi-Movahedi, J. Chamani, Y. Goto and G.H. Hakimelahi, *J. Biochem.*, **133**, 93 (2003).
49. A.A. Moosavi-Movahedi, J. Chamani, M. Gharanfoli and G.H. Hakimelahi, *Thermochim. Acta*, **409**, 137 (2004).
50. A. Dominguez, A. Fernandez, N. Gonzalez, E. Iglasias and L. Montenegrol, *J. Chem. Educ.*, **74**, 1227 (1997).
51. E. Iglasias, *J. Phys. Chem.*, **100**, 12592 (1996).
52. T. Takakuwa, T. Konno and H. Meguro, *Anal. Sci.*, **1**, 215 (1985).
53. D.M. Byler, H. Susi and M.M. Farrel, *Biopolymer*, **22**, 2507 (1983).
54. T.Y. Tsong, *Biochemistry*, **14**, 1542 (1975).
55. D. Hamada, S. Segawa and Y. Goto, *Nature Struc. Biol.*, **3**, 868 (1996).
56. N.H. Nakaoka and Y. Goto, *Bioorg. Med. Chem.*, **7**, 67 (1999).
57. N. Hirota, K. Mizuno and Y. Goto, *Protein Sci.*, **416**, 6 (1997).
58. Y.H. Chen, J.T. Yang and H. Martinez, *Biochemistry*, **11**, 4120 (1972).
59. K.M.G. Oliveira, V.L. Valente-Mesquita, M.M. Botelho, L. Sawyer, S.T. Ferreira and I. Polikarpov, *Eur. J. Biochem.*, **268**, 477 (2001).
60. C.N. Pace, *Methods Enzymol.*, **131**, 266 (1986).
61. A.A. Moosavi-Movahedi, A.E. Wilkinson and M.N. Jones, *Int. J. Biol. Macromol.*, **9**, 327 (1987).
62. J.K. Myers, C.N. Pace and J.M. Scholtz, *Protein Sci.*, **4**, 2138 (1995).
63. D. Shorrtle, *Adv. Protein Chem.*, **46**, 217 (1995).
64. D. Laurent, K. Marly, M. Donald, Jr. Kurtz, A. Robert and T.S. Eugene, *Curr. Separat.*, **79**, 17 (1998).
65. M.K. Gilson and B.H. Honig, *Biochemistry*, **25**, 2097 (1986).
66. J.W. Pitra, M. Falta and F. Gunsteren, *Biophys. J.*, **80**, 2546 (2001).
67. D.C. Turner and L. Brand, *Biochemistry*, **7**, 3381 (1968).

(*Received*: 3 April 2007; *Accepted*: 10 October 2007) AJC-5999