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Trifluoroethanol-induced conformational transition of hen egg-white lysozyme studied by small-angle X-ray scattering

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Abstract The trifluoroethanol (TFE)-induced conformational transition of hen lysozyme was studied with the combined use of far-UV circular dichroism (CD) and small-angle X-ray scattering. At pH 2.0 and 20°C, the addition of TFE to the native lysozyme induced a cooperative transition to an intermediate state with an increased helical content (TFE state). Small-angle X-ray scattering measurements indicated that the TFE state has a radius of gyration which is 20% larger than that of the native state and assumes a chain-like conformation with some remaining globularity. The TFE-induced transition curves obtained by CD and the small-angle X-ray scattering measurements agreed well, consistent with a two-state transition mechanism. A singular value decomposition analysis of Kratky plots of the small-angle X-ray scattering profiles indicated that two basic scattering functions reproduce the observed spectra, further confirming the validity of a two-state approximation.

Key words: α-Helix; Circular dichroism; Lysozyme; Protein folding; Solution X-ray scattering; Trifluoroethanol

1. Introduction

Understanding how proteins fold into their unique native state is one of the central problems in protein chemistry [1–4]. Partially folded equilibrium intermediate states provide a clue to understanding the mechanism of protein folding. It is possible to analyze in detail the structure and stability of these states, where this type of analysis is usually difficult with kinetic intermediates. One of the most extensively studied examples of the intermediate state is the α-helical intermediate of lysozyme, which is induced by 2,2,2-trifluoroethanol (TFE) [5–7].

Lysozyme, consisting of 129 amino acid residues and four disulfide bonds, folds into two structural domains, the α and β domains [8]. The addition of TFE to the native lysozyme at pH 2 induces a cooperative transition to an intermediate state with increased helicity (TFE state) [5–7]. In order to obtain structural information on the intermediate state, which is complementary to those obtained from the refolding kinetic measurements [6,7,9], Dobson and coworkers analyzed the TFE state using NMR techniques [5,10–12]. Buck et al. [11] have shown with ^{15}N-labeled proteins that the helical regions in the native state, as well as several neighboring residues, assume the helical structure in the TFE state. In addition, one β-sheet region and the C-terminal 3^{10} helix in the native state are converted to the helical structure in the TFE state. ^{15}N relaxation measurements have demonstrated that the α-helical preferences in the TFE state are well correlated with their restricted motion in terms of the order parameter and the effective internal correlation times [12]. The disulfide bonds have been shown to be an important factor determining the dynamics of the TFE state [12]. The general pattern of the dynamics is similar to that of the native lysozyme, suggesting the importance of local interactions in dictating the early process of lysozyme folding. Thus, the study of the TFE-induced conformational changes provides valuable insights into the mechanism of protein folding in aqueous conditions, although how TFE stabilizes the intermediate states of proteins is not clear [13,14].

Neither the dimensions nor the global topology of the TFE state have yet been established by NMR methods. Because protein folding is the process by which an extended polypeptide chain acquires maximal packing through the formation of secondary and tertiary interactions [3], such information is important to further improve our understanding of the TFE state. On the basis of the effects of TFE on various proteins measured by circular dichroism (CD), we proposed that the TFE-induced helical state assumes an open-helical structure, in which the interactions between the helical segments are weak, and many hydrophobic groups are exposed to the solvent [15] (see also [16]). Solution X-ray scattering is at present the only reliable method for addressing the size and shape of the denatured or intermediate state [17–19]. In the present study, we have characterized the TFE state of lysozyme by small-angle X-ray scattering (SAXS) and show that it does indeed have an open-helical structure, although the molecule remains relatively compact due to the presence of four disulfide bonds.

Recently, Chen et al. [20] have studied the urea-induced equilibrium unfolding transition state of the hen lysozyme by various methods including SAXS and have detected the unfolding intermediate. Other research suggests, however, that the TFE-induced conformational transition of lysozyme follows a two-state mechanism [5]. We examined the validity of the two-state approximation by comparing the transitions measured by CD and SAXS and by the singular value decomposition (SVD) analysis of the Kratky plot of scattering profiles. We detected no significant accumulation of the intermediate state, confirming a high cooperativity in the TFE-induced transition of lysozyme.

2. Materials and methods

2.1. Materials

Hen egg-white lysozyme was purchased from Seikagaku Kogyo,

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Abbreviations: CD, circular dichroism; Rcm, disulfide bond-reduced and carboxymethylated; SAXS, small-angle X-ray scattering; SVD, singular value decomposition; TFE, 2,2,2-trifluoroethanol

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Japan, and used without further purification. The protein solution at neutral pH was prepared using 20 mM Na-phosphate buffer at pH 7.0. The solution at pH 2.0 was prepared by an appropriate concentration of HCl (usually 20 mM). Typically, stock lysozyme solutions at 60 mg ml^{-1}, which were prepared by dialysis against the respective buffers, were diluted with the same buffer and used for the measurements.

2.2. Methods

SAXS data were collected from the solution-scattering station installed at BL-10C, the Photon Factory, Tsukuba, Japan [21,22]. The sample cell was 50 μl in volume and had a 1-mm path length. The measurements were carried out at 20°C with a thermostatically controlled cell holder. The protein concentration was varied in the range of 7–30 mg ml^{-1}, and a correction was made for the concentration dependence of the scattered intensity to obtain the scattering curves at infinite dilution [22]. X-ray scattering intensities in the small-angle region are given as $I(Q)=I(0)\exp(-R_g^2Q^2/3)$, where Q and $I(0)$ are momentum transfer and intensity at 0 scattering angle, respectively [23]. Q is defined by $Q=(4\pi\sin\theta)/\lambda$, where 2θ and λ are the scattering angle and the wavelength of the X-rays, respectively. The R_g value was obtained from the slope of the Guinier plot, ln $I(Q)$ vs. Q^2. There was no aggregation problem, judging from the apparent R_g values at different protein concentrations.

CD spectra were measured with a Jasco spectropolarimeter, Model J-720 at 208C. The instrument was calibrated using ammonium *d*-10-camphorsulfonate. The results are expressed as the mean residue ellipticity, [θ], which is defined as $[\theta]=100\theta_{obs}/lc$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration of the residue in mol per liter, and l is the length of the light path in centimeters. Far-UV CD spectra were measured with a 1-mm cell at a protein concentration of 0.2 mg ml^{-1}. Near-UV CD spectra were measured with a 1-cm cell at a protein concentration of 0.5 mg ml^{-1}. The temperature was controlled at 20°C with a thermostatically controlled cell holder.

SVD analysis was done using the IGOR Pro data analysis program (WaveMetrics, Inc., USA).

3. Results and discussion

3.1. Conformational states of lysozyme measured by CD

Table 1 summarizes the CD data of the various conformational states of lysozyme. The far- and near-UV CD spectra for typical conformational states have been reported by Buck et al. [5].

According to the X-ray structure, the α-helix and β-sheet contents of lysozyme are 30.2% and 10.9%, respectively [8]. The ellipticity value at 222 nm of the native state at pH 2.0, reflecting the α-helix content, is −10 300, consistent with this helical content. Upon the addition of TFE above 15% (v/v), the negative peaks at 208 nm and 222 nm increased with a midpoint TFE concentration of 20%, representing the additional formation of helical structures (see below). The ellipticity value at 222 nm of the TFE state in the presence of 40% is −21 900, and the estimated helical content is 80% by the method of Chen et al. [24]. The near-UV CD spectrum of the native state exhibits sharp peaks (see [5]), indicating that the side chains are fixed in a unique tertiary structure. On the other hand, the near-UV CD intensities of the TFE state were weak and monotonous, showing that the side chains are flexible. These CD data as well as the recent NMR results with ^{15}N-labeled lysozyme [11,12] provide the following picture of the TFE state. In the presence of TFE, the compactly packed native structure is disordered. However, the native-like helices as well as some additional helices exist, but the regions connecting the helices are disordered and flexible. Consequently, the global topologies of the helices are not fixed.

The far- and near-UV CD intensities of the unfolded state in the presence of 4 M Gdn-HCl were weak, showing that the protein is substantially unfolded. The reduction of the disulfide bonds in 4 M Gdn-HCl did not notably affect the CD spectra.

3.2. Size and shape of the TFE state

Fig. 1a shows the Guinier plots of the scattering curves for the various conformational states of lysozyme. The slope of the Guinier plot corresponds to the square of the radius of gyration (R_g). The R_g values obtained from the scattering curves are 18 Å for the native state, 38 Å for the disulfide-reduced and carboxymethylated (Rcm) states, 21 Å in the presence of 40% TFE, and 23 Å in the presence of 4.0 Gdn-HCl (Table 1). Interestingly, the TFE state is as much expanded as the Gdn-HCl-induced denatured state, although the secondary structural contents in these states do differ from one another. The R_g value in the presence of 4 M Gdn-HCl was smaller than that expected for a random coil conformation of 129 amino acid residues (44 Å [25]). This discrepancy may be attributed to the presence of four disulfide bonds, which restrict the free motion of the chain. Consistent with this idea, the reduction of the disulfide bonds in 4 M Gdn-HCl resulted in the large expansion in the R_g, as observed for the Rcm lysozyme (Table 1).

The Kratky plot, $I(Q)\times Q^2$ versus Q plot, is a useful expression of the scattering profile to describe the structural characteristics of a chain molecule [17–19]. The scattering profile from a typical chain molecule has a region at moderate angles where the scattering intensity is proportional to Q^{-2}, and a region at higher angles where the scattering intensity tends to be proportional to Q^{-1}. The Kratky plot of such a profile shows a monotonously increasing curve with a plateau. On the other hand, the scattering profile from a globular protein has a region where the scattering intensity obeys Porod's law, that is, $I(Q)\propto Q^{-4}$ [23]. Therefore, the Kratky plot should have a peak, and the peak position should depend on the R_g. For protein folding, a peak in a Kratky plot indicates a compact globular structure, and the absence of a peak is an indication of a loss of compactness or globularity.

Fig. 1b shows Kratky plots of lysozyme in various dena-

Table 1
Structural properties of the various conformational states of lysozyme at pH 2.0 measured by far-UV CD and SAXS

	[θ] at 222 nm	% of helix	R_g (Å)
Intact lysozyme			
Native state	−10 300	26.3	17.6 ± 0.5
TFE state in 40% TFE	−21 900	64.6	21.0 ± 0.6
Unfolded state in 4.0 Gdn-HCl	−2 700	1.2	22.9 ± 1.0
Rcm lysozyme			
Unfolded state	−5 700	11.1	37.9 ± 1.0
Unfolded state in 4 M Gdn-HCl	−2 500	0.5	35.8 ± 0.5

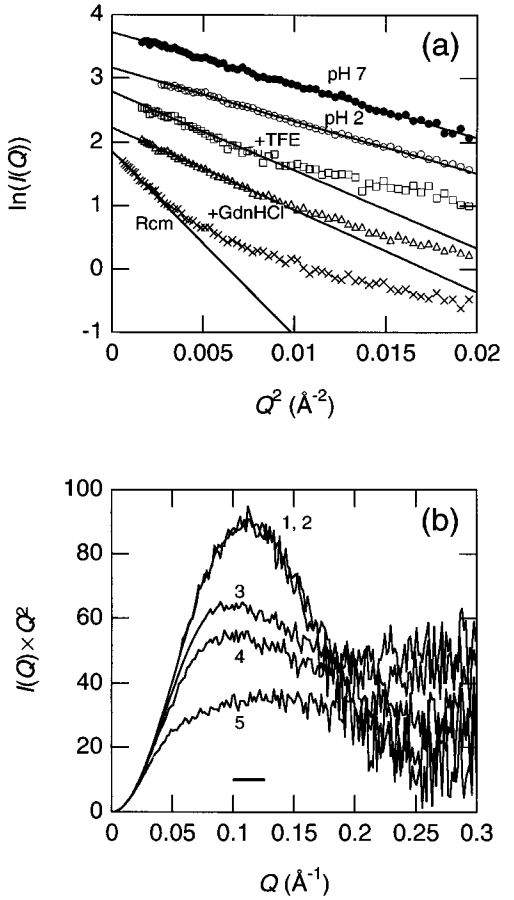

Fig. 1. Guinier (a) and Kratky (b) expression of scattering profile of various conformational states of lysozyme at 20°C. a: Symbols indicate raw data and lines are the result of curve fitting analysis in the region of $R_gQ < 1.3$. ●, intact lysozyme at pH 7.0; ○, intact lysozyme at pH 2.0; □, intact lysozyme in 35% (v/v) TFE at pH 2.0; △, intact lysozyme in 4.0 M Gdn-HCl at pH 2.0; ×, Rcm lysozyme at pH 2.0. b: 1, intact lysozyme at pH 7.0; 2, intact lysozyme at pH 2.0; 3, intact lysozyme in 35% (v/v) TFE at pH 2.0; 4, intact lysozyme in 4.0 M Gdn-HCl at pH 2.0; 5, Rcm lysozyme at pH 2.0.

tured states. In the absence of a denaturant, the spectrum has a clear peak at the region of $Q = 0.11$ Å, indicating that the protein molecule is globular. By contrast, the spectrum of the disulfide-reduced form (Rcm) has a plateau instead of a peak, suggesting that the molecule is chain-like rather than globular. The TFE-induced denatured state showed a spectrum similar to that of the Gdn-HCl-induced denatured state. These spectra of the disulfide-intact denatured states were intermediate between those of the native state and the disulfide-reduced state, indicating that the molecules are less globular than the native state but more globular than the disulfide-reduced state. These results again suggests that the TFE- and Gdn-HCl-induced denatured states would be intrinsically as disordered as the random coil-like state, but the connection by four disulfide bonds prevents the molecule from expanding freely (Table 1).

3.3. Cooperativity of the TFE-induced transition

The TFE-induced conformational change of lysozyme was followed by SAXS. Fig. 2a,b shows the Guinier and Kratky plots of the scattering curves of lysozyme in the presence of various concentrations of TFE at pH 2.0 and 20°C. With an increase in the concentration of TFE, the slope in the Guinier plot became steeper, showing that the R_g value increased and the molecule expanded. The increase in TFE concentration also changed the spectra in the Kratky plot. The height of the peak became lower and the peak position shifted to a lower Q value. The shift of the peak position indicates again that the R_g value became larger with an increase in the TFE concentration.

We compared the transition curves on the basis of SAXS measurements with that of the CD. As a transition measured by CD, the ellipticity at 222 nm was plotted against the TFE concentration (Fig. 3a). The transition was highly cooperative and the midpoint TFE concentration was 20%. The transition curve was simulated by assuming a two-state transition between the native and TFE states, and a linear dependence of the free energy change of the conformational transition, ΔG, upon molar TFE concentration, [TFE]:

$$\Delta G = \Delta G_0 + m[\mathrm{TFE}] \quad (1)$$

where ΔG_0 is ΔG in the absence of alcohol and m is a measure of cooperativity. The observed ellipticity values were converted to ΔG assuming the native and TFE state baselines. Although the validity of the linear dependence on TFE con-

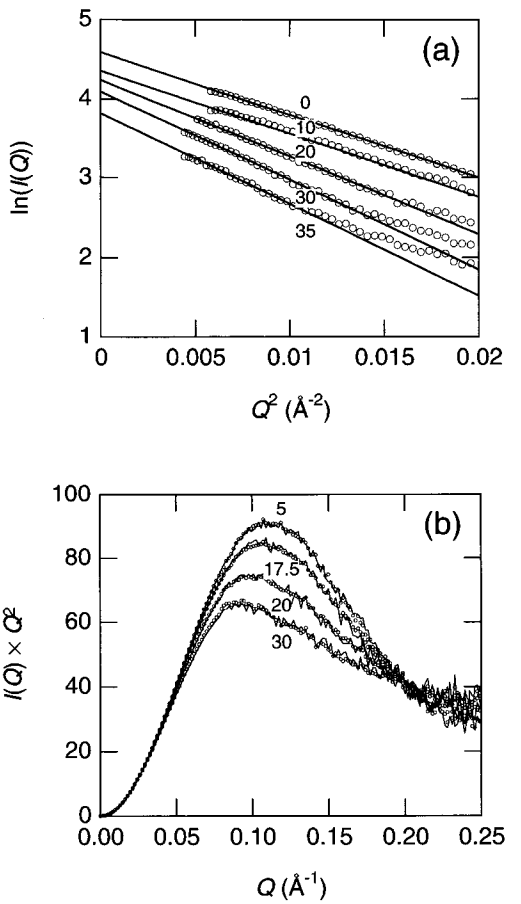

Fig. 2. Dependence on TFE concentration of Guinier plots (a) and Kratky plots (b) of lysozyme at pH 2.0 and 20°C. Solid lines in b indicate the raw data and small circles indicate the SVD reconstructed curves using the first two components. The numbers refer to the TFE concentration in percentage by volume.

centration is unknown [13], it corresponds well with the transition curve.

We used two different methods to obtain the transition curve measured by SAXS. First, R_g^2, the slope of the Guinier plot, was plotted against TFE concentration. However, there is some ambiguity in this approach because, in cases where the molecules are heterogeneous in size as expected for the two-state mechanism, R_g^2 does not strictly reflect the fractions of the molecules in these two states [17]. Therefore, the transition curve was also obtained from the Kratky plot. We selected the integrated intensity around the peak of the native state ($0.1 < Q < 0.125$), as indicated by a bar in Fig. 2b, to follow the transition.

From these spectral sets, we obtained three different transition curves, which were compared by superimposing the values for the native and TFE states (Fig. 3a). As can be seen, these transition curves agree well with each other, suggesting that the two-state approximation is valid and that the increase in helix content occurs simultaneously with the loss of compactness and globularity.

3.4. SVD analysis

To examine the validity of a two-state transition from a different viewpoint, we analyzed the Kratky plots by SVD.

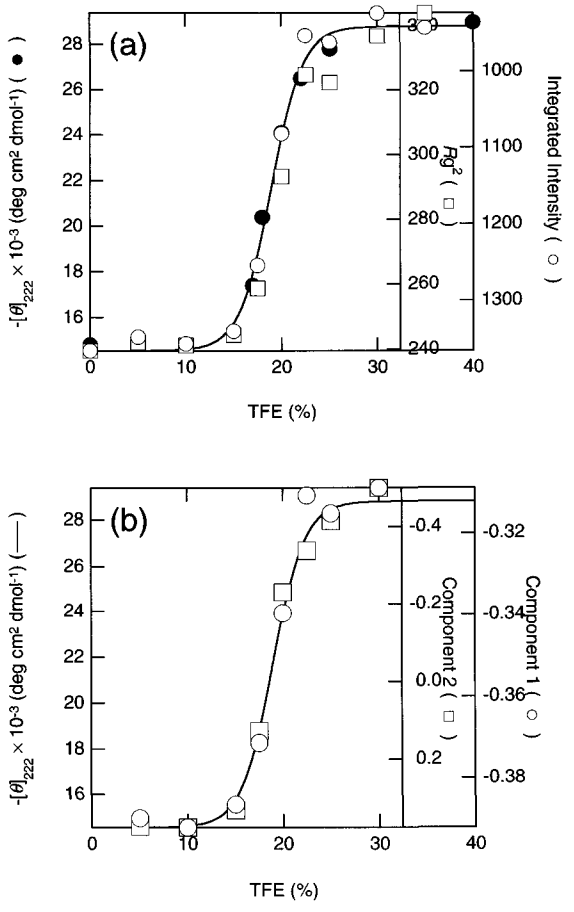

Fig. 3. Transition curves between the native and TFE states of lysozyme at pH 2.0 and 20°C. a: ●, ellipticity at 222 nm; ○, integrated intensity of Kratky plot as indicated by bars in Fig. 2b; □, R_g^2. b: Dependence of the intensity of the first two components of SVD analysis on the TFE concentration. The lines are a theoretical curve for the CD transition with Eq. 1 and following parameters: $\Delta G_0 = 27.8$ kJ/M, $m = -1.47$ kJ/M/[% of TFE].

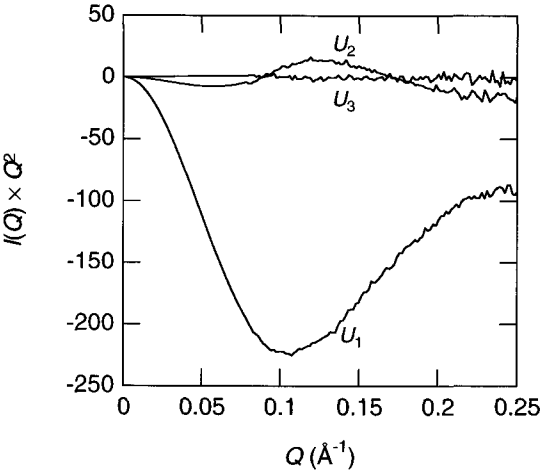

Fig. 4. The basis scattering functions, U_1, U_2, and U_3, in Kratky form from SVD analysis. The curves are weighted by their singular values, 1874.3 for U_1, 127.1 for U_2 and 28.1 for U_3.

SVD analysis is a useful approach for determining the minimum number of structural states in the transition by using a single set of spectra [20,26]. The advantage of this method is that it is not necessary to know the conformational properties of the native or unfolded state. Analysis of a set of Kratky plots at various concentrations of TFE resulted in two large singular values, with the others being negligible (Fig. 4). Reconstructed Kratky plots using only the first two components are very similar to the original Kratky plots (Fig. 2b). The global χ^2 values from original spectra do not differ between the two- and three-component reconstructions. Fig. 3b shows the dependence of the intensity of these two components on the TFE concentration, which corresponds with the transition curve measured by the ellipticity at 222 nm. Because the intensities of these components represent the fraction of the first and second components in each Kratky plot, this agreement further supports a two-state mechanism.

3.5. Conclusion

Chen et al. [20] have studied the equilibrium unfolding transition of hen egg-white lysozyme induced by urea at pH 2.9 by various methods, including near- and far-UV CDs and solution X-ray scattering. The transition curves measured by these methods were not consistent, suggesting the accumulation of an intermediate state. SVD analysis of solution X-ray scattering data provided convincing evidence of the accumulation of the partially unfolded intermediate. These results [20] contrasted with those observed here, where similar methodologies indicated an absence of the intermediate for the TFE-induced transition. This distinction suggests that the TFE-induced conformational transition is more cooperative than the urea-induced denaturation, resulting in the negligible accumulation of an intermediate. Indeed, whereas the TFE-induced transition occurs in a narrow range of TFE concentration, the urea-induced transition is apparently less cooperative [20]. Accumulation of the intermediate states was detected for horse cytochrome *c* during the methanol-induced denaturation [16]. This suggests that the intermediate could be detected during the transition induced by alcohols with less denaturing potential than TFE. Although such possibilities should be investigated, the presents results confirm that a two-state ap-

proximation is valid at least for the TFE-induced transition of lysozyme at pH 2 and 20°C.

Examination of the effects of TFE on various proteins including β-lactoglobulin and lysozyme indicate that the helical propensities in TFE are determined predominantly by local interactions, suggesting that, in TFE, proteins assume an open-helical structure, in which the interactions between the helical segments are weak and many hydrophobic groups are exposed to the solvent [14,27]. The open-helical structure is distinct from the compact molten globule structure under aqueous conditions, which is stabilized by weak but significant interhelical hydrophobic interactions [28]. The present results demonstrate that the TFE state of lysozyme is indeed an open helical structure with exposed hydrophobic surfaces, but it is restricted to a relatively compact conformation due to the presence of four disulfide bonds.

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References

[1] Dobson, C.M. (1995) Nature Struct. Biol. 2, 513–517.
[2] Baldwin, R.L. (1995) J. Biomol. NMR 5, 103–109.
[3] Dill, K., Bromberg, S., Yue, K., Fiebig, K.M., Yee, D.P., Thomas, P.D. and Chan, H.S. (1995) Protein Sci. 4, 561–602.
[4] Dill, K.A. and Chan, H.S. (1997) Nature Struct. Biol. 4, 10–19.
[5] Buck, M., Radford, S.E. and Dobson, C.M. (1993) Biochemistry 32, 669–678.
[6] Radford, S.E. and Dobson, C.M. (1995) Phil. Trans. R. Soc. Lond. Biol. 348, 17–25.
[7] Radford, S.E., Dobson, C.M. and Evans, P.A. (1992) Nature 335, 700–704.
[8] Artymiuk, P.J. and Blake, C.C.F. (1981) J. Mol. Biol. 152, 737–762.
[9] Dobson, C.M., Evans, P.A. and Radford, S.E. (1994) Trends Biochem. Sci. 19, 31–37.
[10] Yang, J.J., Buck, M., Pitkeathly, M., Kotik, M., Haynie, D.T., Dobson, C.M. and Radford, S.E. (1995) J. Mol. Biol. 252, 483–491.
[11] Buck, M., Schwalbe, H. and Dobson, C.M. (1995) Biochemistry 34, 13219–13232.
[12] Buck, M., Schwalbe, H. and Dobson, C.M. (1996) J. Mol. Biol. 257, 669–683.
[13] Hirota, N., Mizuno, K. and Goto, Y. (1997) Protein Sci. 6, 416–421.
[14] Hirota, N., Mizuno, K. and Goto, Y. (1997) J. Mol. Biol. (submitted).
[15] Shiraki, K., Nishikawa, K. and Goto, Y. (1995) J. Mol. Biol. 245, 180–184.
[16] Kamatari, Y.O., Konno, T., Kataoka, M. and Akasaka, K. (1996) J. Mol. Biol. 259, 512–523.
[17] Kataoka, M., Hagihara, Y., Mihara, K. and Goto, Y. (1993) J. Mol. Biol. 229, 591–596.
[18] Kataoka, M., Nishii, I., Fujisawa, T., Ueki, T., Tokunaga, F. and Goto, Y. (1995) J. Mol. Biol. 249, 215–228.
[19] Kataoka, M. and Goto, Y. (1996) Folding Design 1, R107–R114.
[20] Chen, L., Hodgson, K.O. and Doniach, S. (1996) J. Mol. Biol. 261, 658–671.
[21] Ueki, T., Hiragi, Y., Kataoka, M., Inoko, Y., Amemiya, Y., Izumi, Y., Tagawa, H. and Muroga, Y. (1985) Biophys. Chem. 23, 115–124.
[22] Kataoka, M., Head, J.F., Vorherr, T., Krebs, J. and Carafoli, E. (1991) Biochemistry 30, 6247–6251.
[23] Glatter, O. and Kratky, O. (1982) Small Angle X-ray Scattering, Academic Press, New York.
[24] Chen, Y.H., Yang, J.T. and Martinez, H.M. (1972) Biochemistry 11, 4120–4131.
[25] Rowe, G. and Pineiro, A.L. (1990) Biophys. Chem. 36, 57–64.
[26] Shrager, R.I. and Hendler, R.W. (1982) Anal. Chem. 54, 1147–1152.
[27] Hamada, D., Kuroda, Y., Tanaka, T. and Goto, Y. (1995) J. Mol. Biol. 254, 737–740.
[28] Nishii, I., Kataoka, M. and Goto, Y. (1995) J. Mol. Biol. 250, 223–238.