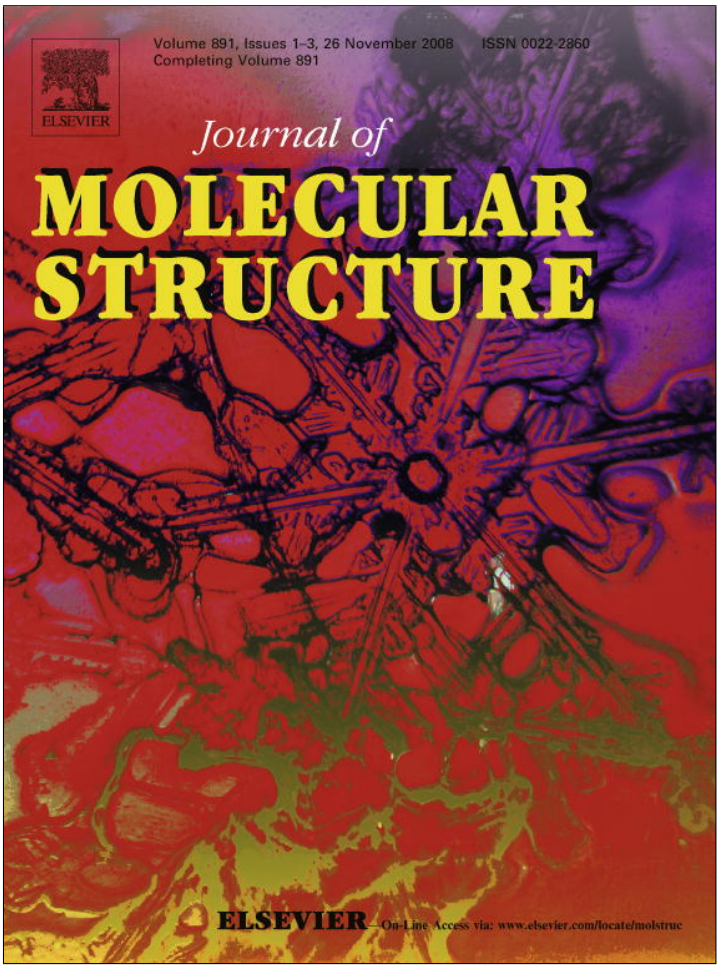

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Equilibrium and kinetic analysis on the folding of hen egg lysozyme in the aqueous-glycerol solution

Ling-zhi Wu, Bao-liang Ma, Yue-biao Sheng, Wei Wang *

National Laboratory of Solid State Microstructure and Institute of Biophysics, Nanjing University, No. 22, Hankou Road, Nanjing 210093, PR China

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ABSTRACT

In the present study, the equilibrium and kinetic analysis on the folding of hen egg lysozyme in the aqueous-glycerol solutions have been reported by means of fluorescence and circular dichroism spectra. Addition of glycerol increases the stability of lysozyme on the guanidine-induced denaturation, and the unfolding transition of lysozyme is from a two-state into a three-state mechanism. On the kinetic experiments, the folding pathway of lysozyme has been changed in the presence of glycerol. An overshot phenomenon in fluorescence spectra appears and the preformed ellipticity within the dead time is weakened. These results reveal that a new intermediate state with a non-native hydrophobic core but lack of the stable secondary structure is populated in the folding pathway of lysozyme, and the conversion of it to the native state is decelerated due to the excess of non-native hydrophobic interactions.

1. Introduction

The mechanism by which an extended and highly disordered polymer chain folds efficiently into a compact, globular protein is a center problem in molecular structural biology [1,2]. A major goal of experimental works on this mechanism is to detect and characterize partially folded states, including the transient intermediates observed in kinetic experiment, and the stable intermediates occurring under equilibrium conditions [1–4]. It is well known that the folding of some small proteins presents highly cooperative two-state behavior, while the folding of major proteins with populations of partially folded states involves multi-state process or the intermediates [4,5]. The emergence of these intermediates is not only dependent on the feature of proteins but also on the solvent environment of protein folding [6–8]. The use of cosolvents to modify the structural and functional properties of proteins is an attractive concept for the investigation of protein folding [9–11]. Organic solvents have different physicochemical properties from water molecules, e.g., dielectric constant and polarizability, which can interfere with the hydrogen bond network of solvent and the ionic group and surface hydrophobic area of proteins [10,12]. These characteristics will affect the competition between intra- and intermolecular interactions in protein folding process, even produce some stable intermediates if non-native interactions stabilize these structures [3,13]. Thus, this solvent replacement strategy will yield penetrating insights into the mechanism of protein folding, as it has into the understanding of protein–organic interactions.

As an important model system for understanding protein folding, hen egg white lysozyme has been studied in great detail by a combination of different techniques [1,4,14,15]. It is a two-domain globular protein with 129 amino acids. The α-domain is composed of three α-helices and one C-terminal 3_{10} helix, and the β-domain is composed of anti-parallel three-stranded β-sheet and one loop. Besides, there are also six tryptophane (Trp) residues within the globular protein, which makes lysozyme easy for fluorescence studies. Under equilibrium condition, hen egg white lysozyme is often considered as a typical example of two-state unfolding mechanism. But at extreme pH conditions, a populated equilibrium intermediate appears in the denaturation of lysozyme [1]. A significant feature of lysozyme folding pathway is the existence of kinetic heterogeneity in the folding process, which has been attributed to the presence of kinetic traps generated in the initial stages of folding [4,16,17]. One of the interesting issues in the folding of lysozyme is that fully reduced and unfolded lysozyme can correctly be reoxidized and refolded in glycerol containing varying amounts of water with an efficiency comparable with that in aqueous solution [8,18]. Glycerol is known to enhance the activity of enzymes and the structural stability of proteins [12,19–21]. Despite lysozyme can refold correctly in glycerol, the effects of glycerol on the folding pathway of lysozyme is few reported, especially in the earlier stage of protein folding. In our work, equilibrium and kinetic analysis on the folding of hen egg lysozyme in the aqueous-glycerol solution have been systematically studied by means of fluorescence and circular dichroism spectra. Addition of glycerol increases the stability of lysozyme on the GdnCl-induced denaturation, and the unfolding transition is changed into a three-state mechanism with the accumulation of the partially unfolded

* Corresponding author. Tel.: +86 25 83686031; fax: +86 25 83595535.
E-mail address: wangwei@nju.edu.cn (W. Wang).

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equilibrium state. The refolding kinetics of lysozyme has been investigated by stop-flow measurements. It is found that the glycerol decelerates the refolding of lysozyme as a result of the excessive hydrophobic interactions.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme was purchased from Sigma Chemical Co. (St. Louis, USA). High pure glycerol (>99%) and ultra pure guanidine hydrochloride (GdnCl) were purchased from AMRESCO Cop (USA). All other chemical reagents used in our experiment were of analytical grade without further purified. All buffer solutions were prepared with Milli-Q super purified water. Sodium acetate was added to these buffers at the concentration of 0.05 M/L. In the case of the glycerol buffer solutions, the distilled water solution was replaced by a glycerol/water mixture with an appropriate volume fraction. The pH of the solvent buffers was adjusted with acetate acid and sodium hydroxide to be within ±0.05 pH units of the desired value of 4.0 at 25 °C in all cases. Protein samples were prepared in 0.05 M/L sodium acetate buffers (pH 4.0). The concentration of proteins was determined spectrophotometrically, and the concentration of GdnCl was determined from refraction index measurements [2].

2.2. Equilibrium experiments

The two stock solutions of native protein (2 mg/ml) and GdnCl (8 M/L) were prepared in sodium acetate buffer containing different proportions of glycerol (0%, 10%, 20%, 30%, 40% and 50% (v/v)), respectively. In the GdnCl-induced denaturation experiment, different amounts of GdnCl samples were added to protein solutions for getting the desired concentrations of denaturants. The final concentration of proteins was 0.2 mg/ml in the buffer with different GdnCl concentration. These solutions were incubated for enough time (>5 h) at 25 °C and used for the following spectral measurements.

The intrinsic fluorescence measurements were performed on fluorescence spectrophotometer (Jasco FP-6500, Tokyo, Japan) with a cuvette of 1 cm light path. The excitation wavelength of fluorescence spectrum was set at 280 nm, and the emission spectra were recorded in the wavelength range of 295–400 nm. The bandwidths of excitation and emission slits were set as 3 nm and 5 nm, respectively. The circular dichroism (CD) spectra were recorded on a Jasco-810 (Tokyo, Japan) spectrophotometer under constant nitrogen flush at 25 °C. The steady-state far-UV CD spectra of proteins were recorded from 190 to 250 nm using a 1 cm path-length cuvette under constant nitrogen flush. Spectra were collected with a scan speed of 200 nm/min and a response time of 1 s. Each spectrum was the average of 5 scans. The analytical method on these data of the fluorescence and CD spectra were described in the previous publication in detail [22].

2.3. Kinetics experiments

Refolding reactions of lysozyme induced by GdnCl were performed by a Biologic SFM-3 stop-flow apparatus. The unfolded protein in 6 M/L GdnCl was diluted by buffer solutions (0.05 M sodium acetate, pH 4.0) containing appropriate amounts of GdnCl with a mixing ratio of 1:10 to achieve final concentration of GdnCl in the range of 0.8–2.5 M. The final protein concentration is 0.2 mg/ml. The dead times of the refolding measurements were <5 ms. The temperature was controlled at 25 °C by circulating water. The intrinsic fluorescence spectrum was monitored by 320 nm cutoff filter at an excitation wavelength of 280 nm. The far-UV Circular dichroism spectrum was recorded on 222 nm under constant nitrogen flush. The path lengths of the optical cell were 1.5 mm (FC-15) and 1.0 mm (TC-100/10) for fluorescence and CD measurements, respectively. The time courses of unfolding and refolding curves were fitted by Biokine software to obtain the unfolding and refolding rate constants as a function of GdnCl as follows:

$$A(t) = A(\infty) + \sum_{i=1}^{n} A_i \times \exp(-k_i t) \quad (1)$$

where t is the time, $A(t)$ and $A(\infty)$ were the observed values of the fluorescence and ellipticity spectrum at time t and infinite time, k_i and A_i were the apparent rate constant and the amplitude of the ith kinetic phase, respectively. Whatever the kinetic model used for the determination of kinetic constants, the determination of the starting point of the reaction and the baseline parameters in the region of the graph where the rate constant evaluation is made must be performed ahead. Each kinetic experiment was repeated for 30 times and the accumulated kinetic data were averaged.

3. Results and discussion

3.1. Equilibrium unfolding of lysozyme in the presence of glycerol

The addition of cosolvents to aqueous of proteins is known to modify the protein structure in variety of ways. In order to make sure the conformational changes of lysozyme in the presence of glycerol, the intrinsic fluorescence and far-UV CD spectra of lysozyme in aqueous-glycerol solutions are measured as shown in Fig. 1A and B, respectively. For fluorescence spectra in Fig. 1A, the fluorescence intensity is decreased largely in the presence of glycerol, and the emission peak gradually shifts to the shorter wavelength with the increment of glycerol from 0% to 50% as seen in the insert of Fig. 1A. The fluorescence spectrum is sensitive to the solvent accessible surface area and the surrounding polarity of the fluorophores in solutions [2]. The intensity decrease and blue shift of the emission peaks indicate that lysozyme in the aqueous-glycerol solution presents a more fixed tertiary structure with the burial of Trp residues. In contrast, for CD spectra in Fig. 1B, the ellipticity spectra of lysozyme in the presence of glycerol are mostly unchanged compared to that in pure aqueous solutions. Considering the far-UV CD spectra as a probe of secondary structure of proteins, little change of the ellipticity signals suggests the secondary structure in the mixture is similar to that of lysozyme in water.

Glycerol stabilizes proteins on the thermal denaturation [20]. To characterize the stabilization of glycerol in chemical denaturation, the GdnCl-induced unfolding of lysozyme in the aqueous-glycerol solution (from 0% to 50%) is measured by fluorescence and far-UV CD spectra. The fractions of unfolded molecules, f_u, obtained from fluorescence and CD spectra of lysozyme with varying volumes of glycerol are shown as a function of GdnCl concentration in Fig. 2A and B. These unfolding transition curves of lysozyme in water and glycerol solutions all follow sigmoidal transitions. At low concentration of GdnCl, f_u is zero and a majority of proteins are at the native states. Once the concentration of GdnCl is over 3 M/L, the native structure of proteins start to be destroyed and the unfolded fraction of proteins becomes more and more. Till the higher concentration of GdnCl (6 M/L), f_u is to 1 and all proteins are unfolded. In the presence of glycerol, the unfolding transition curves of fluorescence and ellipticity spectra all shift to the higher concentration of GdnCl, and the protein is more stable against GdnCl with the increments of glycerol. Even at high concentration of GdnCl, the helix structure of lysozyme in ellipticity spectra retains some residual structure at 50% glycerol, although the tertiary structure of lysozyme monitored by fluorescence spectra is totally destroyed in that case. These measurements suggest that glycerol

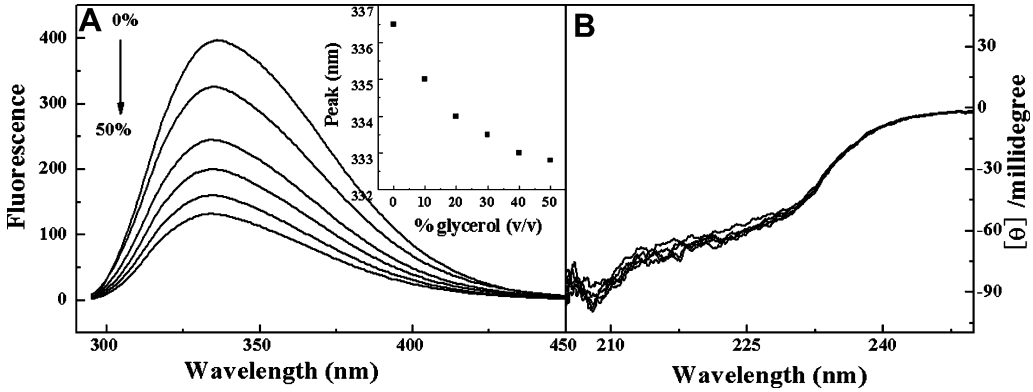

Fig. 1. The effect of glycerol on the conformational changes of lysozyme at pH 4.0 and 25 °C, monitored by the intrinsic fluorescence (A) and the far-UV CD spectra (B) in aqueous-glycerol solutions from 0% to 50% (v/v). The peak shift of emission spectra as a function of the glycerol concentration is shown as an insert of (A).

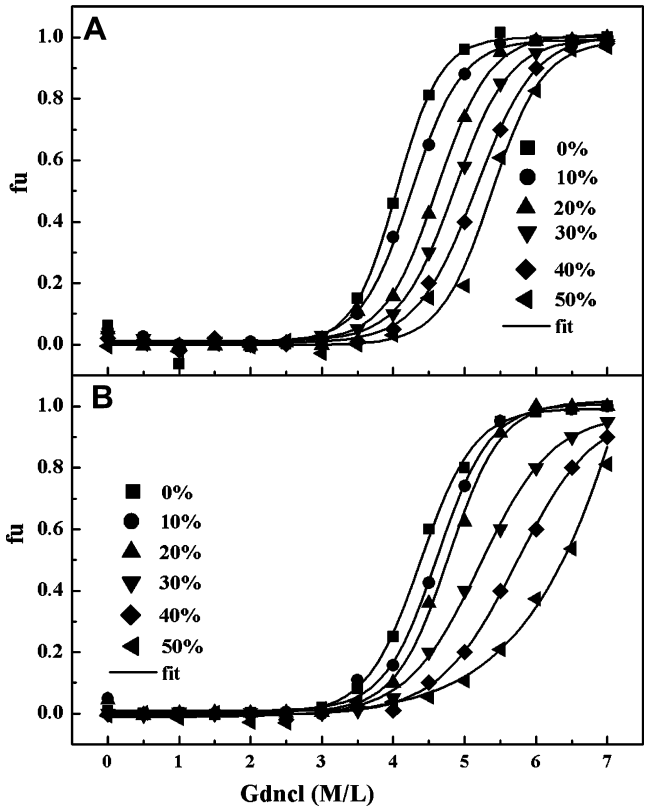

Fig. 2. The GdnCl-induced equilibrium unfolding transition curves of lysozyme monitored by fluorescence spectra (A) and CD spectra (B) in the presence of glycerol at pH 4. The concentration of glycerol is from 0% to 50%. Here f_u is the fraction of unfolded lysozyme molecules in GdnCl-induced denaturation, obtained using the equation, $f_u = (y_n - y)/(y_n - y_u)$, where y is the observed fluorescence intensity or ellipticity at a given GdnCl concentration, and y_n and y_u are the values of fluorescence intensity (or ellipticity) at the folded and unfolded states extrapolated from the pre- and post-transition baselines to the GdnCl concentration under consideration, inferred from Ref. [22].

stabilizes lysozyme on the chemical denaturation, but the extent of stabilization by glycerol on the tertiary and secondary structure is different, which are also inferred from the thermal dynamic parameters obtained from the unfolding process of lysozyme. For quantitative analysis, the change of free energy (ΔG), the midpoint of chemical denaturation (C_m) and the solvent-exposed surface (m) based on the GdnCl-induced unfolding process are obtained as listed in Table 1 [1,2,22]. The values of ΔG_{H_2O} and C_m are increased compared to that of lysozyme in aqueous solution, while that of *m* is decreased due to the loss of the solvent-exposed surface in the presence of glycerol. Moreover, the increase of C_m in fluorescence spectra is about 1.3 M/L from 0% to 50% of glycerol, while it is 2.0 M/L for that of CD spectra.

A combination of CD and fluorescence spectroscopy is often used to demonstrate whether denaturant-induced equilibrium unfolding of proteins is a two-state or three-state behavior with the stable intermediate states [13]. The unfolding transition curves of the fluorescence and CD spectra of lysozyme at 0%, 20% and 50% of glycerol are compared in Fig. 3A–C, respectively. In aqueous solution, the unfolding transition curves of fluorescence and CD spectra coincide within the range of experimental uncertainty, indicating that the tertiary and secondary structure of lysozyme unfold simultaneously in a cooperative process. Thus the GdnCl-induced unfolding of lysozyme at pH 4 follows a two-state transition model. However, in the presence of 20% glycerol, the two transition curves no longer coincide, indicating the presence of partially unfolded equilibrium states as an uncooperative behavior. When the concentration of glycerol is to 50%, a more separation from the unfolding transition curves of the tertiary and second structure occurs, in which a relatively stable intermediate is obviously populated in the unfolding process. Therefore, the unfolding transition of lysozyme in the presence of glycerol is a three-state mechanism (i.e., native, intermediate and unfolded state). The equilibrium intermediate state is characterized by the loss of native tertiary structure, but with some remaining of secondary structure as observed from the fluorescence and CD spectra in Fig. 3.

3.2. Folding kinetics of lysozyme in the presence of glycerol

Equilibrium studies permit identification of the protein conformations that have the lowest energy levels, but more information on the level of the transition states and the corresponding rate-limiting steps in protein folding are provided in the kinetic folding process [14,15]. The folding kinetics of lysozyme in the presence of glycerol has been studied by stop-flow fluorescence and CD spectroscopy. The emission spectra are monitored above the wavelength of 320 nm, while the CD spectra are monitored at 222 nm. The representative time course of the fluorescence spectra of lysozyme refolding in aqueous and glycerol solution containing 1.2 M GdnCl is shown in Fig. 4. After dilution the samples from 6 to 1.2 M GdnCl, the refolding trace in aqueous solutions displays a substantial fluorescence quenching within the dead time of the apparatus and a slow reaction with the decreasing of the fluorescence intensity into the native state. The refolding process could be fitted by two exponential functions, suggesting that lysozyme refolding consists of two kinetic phases. By contrast, the profile

Table 1
The GdnCl-induced unfolding transition thermodynamic parameters of lysozyme denaturation monitored by fluorescence spectra

% Glycerol (v/v)	0	10	20	30	40	50
ΔG_{H_2O} (kJ mol^{-1})	25.83 ± 0.95	29.45 ± 1.02	30.52 ± 1.35	31.17 ± 1.39	31.98 ± 1.57	32.31 ± 1.66
C_m (M/L)	4.05 ± 0.11	4.26 ± 0.09	4.60 ± 0.13	4.86 ± 0.12	5.14 ± 0.15	5.35 ± 0.17
m (kJ mol^{-1} M^{-1})	6.58 ± 0.18	6.60 ± 0.21	6.45 ± 0.19	6.33 ± 0.23	6.21 ± 0.27	6.06 ± 0.33

These data are obtained from the unfolding transition curves of fluorescence spectra, ΔG_{H_2O} is the free energy change in the absence of denaturant, C_m is the midpoint of chemical denaturation, m is the solvent-exposed surface of proteins on the GdnCl-induced unfolding process.

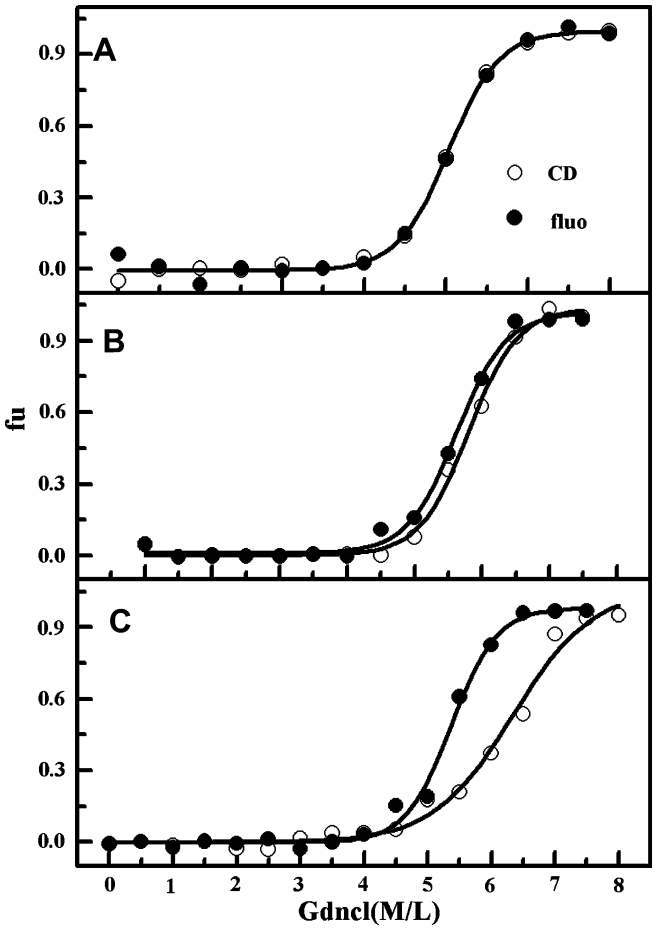

Fig. 3. The comparison of the unfolding fraction of GdnCl-induced unfolding of lysozyme from fluorescence and CD spectra in the presence of glycerol (v/v): (A) 0%, (B) 20% and (C) 50%. Here f_u is the fraction of unfolded lysozyme molecules in GdnCl-induced denaturation.

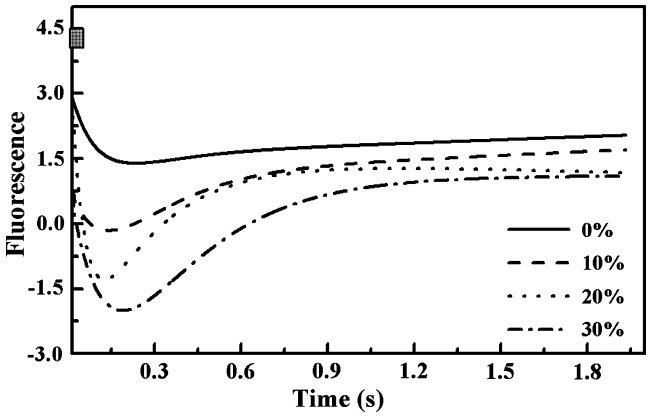

Fig. 4. The time course refolding of lysozyme is monitored by fluorescence spectra at pH 4 in the presence of glycerol. The concentration of glycerol (v/v) is 0%, 10%, 20% and 30%. The reaction is initiated by GdnCl concentration jumping from 6 to 1.2 M/L. The fluorescence spectra are detected by 320 nm cutoff filter at an excitation wavelength of 280 nm.

of lysozyme in presence of glycerol is complex and characterized by at least three phases. Firstly, a substantial decrease of fluorescence intensity occurs in the dead time of the experiments. Secondly, the fluorescence intensity is further decreased in a fast phase, and reaches a minimum of the reactions. Lastly, the fluorescence intensity is gradually restored to the native state in a slow step. With the increments of glycerol concentration, a deeper overshot appears and the fluorescence intensity at the last phase is decreased, which suggests that the refolding behavior of lysozyme is changed in addition of glycerol.

As a represent of the hydrophobic residues in proteins, the change of tryptophan fluorescence is sensitivity to the development of the hydrophobic cores [16]. After dilution of the initial denatured state, the hydrophobic collapse drives compaction of the protein and embeds tryptophan into the hydrophobic interior of proteins, so fluorescence intensity decreases rapidly. It seems that the collapse is already substantial within the first few milliseconds of the refolding. Next, the fluorescence quenching is developed into an overshot phenomenon in the fast phase, which can be a rate-limiting step of kinetic intermediates in the subsequent refolding events [7,19]. To further explore the effect of glycerol on the lysozyme folding, the observed rate constants of fast and slow phases depending on the concentration of glycerol are obtained as shown in Fig. 5. Compared to that in the pure aqueous solution, the folding of fast phase is accelerated by glycerol initially. However, the refolding of lysozyme is not increased all the time with the increments of glycerol. The rate constant reaches a maximum at about 20% of glycerol, and turns to decrease in higher concentration of glycerol. It is coincident with the change of the fluorescence spectra in Fig. 4. With the increment of glycerol, the appearance of the overshot phenomenon of fluorescence spectra becomes earlier from 0% to 20% of glycerol, but this trend is changed at 30% glycerol. It is obvious that a deeper overshot phenome-

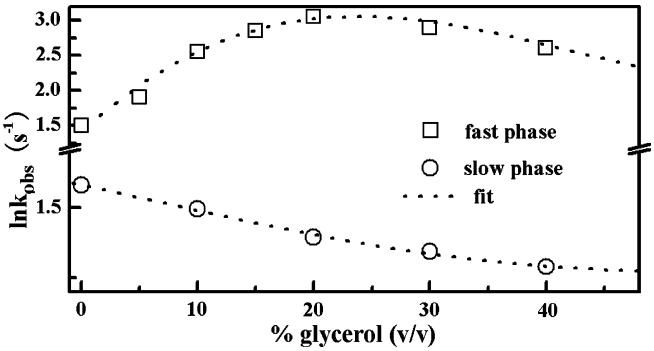

Fig. 5. Glycerol concentration dependence of the natural logarithm of the observed folding rate constants of lysozyme monitored by fluorescence spectra in the fast and slow phase. The folding rate constants per second are obtained from the time course of the folding curves by Eq. (1), labeled as k_{obs} (s^{-1}).

non is delayed because a large amount of non-native hydrophobic interactions occur. Meanwhile, the rate constant in slow phase is decreasing at all times, indicating that the folding of the slow phase is retarded due to the reorganization of hydrophobic interactions in the folding process [5,16].

To explore more information about the folding of helix structure in addition of glycerol, the folding reactions are monitored by far-UV CD at 222 nm as shown in Fig. 6. The denatured protein is induced for the refolding by diluting the GdnCl concentration from 6 to 1.2 M/L in 0.05 M/L sodium acetate buffer solutions containing various concentrations of glycerol using the stop-flow mixing apparatus. In aqueous, a more negative ellipticity at 222 nm than that of native state is preformed in the first phase, and is reduced to native state with a relatively slow phase. At 10% glycerol, the excess ellipticity of lysozyme preceded by a burst phase is reduced, and then restored to the native state slowly. When the concentration of glycerol is to 20% and 30%, the helix structure developed in the dead time is less than that of the native state, and the signal is gradually increasing to the value of the native state.

The preformed helix structure for lysozyme in water is agreed with the previous observations under corresponding conditions [13,16,23]. The acquisition of the secondary structure is associated with the rapid formation of local hydrogen bonding in the refolding process. However, the excess ellipticity is reduced in the presence of glycerol. It seems that the addition of glycerol destabilizes the helix structure in the first phase. To make sure whether the disappearance of excess helix structure will accelerate or decelerate the sequential formation of secondary structure as the protein folds, the folding rate constants for the ellipticity recovery in the slow phase are obtained by fitting the observed reaction curves of ellipticity spectra with Eq. (1). The plots of the natural logarithm of the folding rate constants versus GdnCl concentration in varying proportion of glycerol are shown in Fig. 7. Clearly, compared to that in aqueous solution, the folding of lysozyme is decelerated in addition of glycerol. Thus, there must be some rate-limiting steps in the folding of lysozyme with glycerol even if the non-native helix structure is absence.

4. Conclusions

The emergence of intermediate states in the folding of hen egg white lysozyme has been focused on the studies of protein folding [1,4,5]. As a global protein with two distinct domains (α and β), the folding of lysozyme involves a number of well-defined steps and the population of specific intermediates. Specially, in majority of lysozyme molecules, the helical part of the α-domain folds more rapidly than the β-domain under certain conditions [4,16]. Hence, a transiently accumulating partially folded state with non-native helical structure formed in α-domain is located on a slow pathway, as described in our studies. Interestingly, the folding pathway of lysozyme has been changed in addition of glycerol. On the kinetic experiments, an overshot phenomenon in fluorescence spectra appears as a consequence of the hydrophobic collapse interactions, and the excess helix structure has been reduced from CD spectra. These results reveal that a new intermediate state with a non-native hydrophobic core but lack of the stable secondary structure is presence in the folding of lysozyme. From the analysis of the folding rate constants of lysozyme folding in the absence and presence of glycerol, the conversion of the intermediate to the native state is decelerated due to the reorganization of non-native interactions. Therefore, the occurrence of the intermediate state is a rate-limiting step as lysozyme folds in aqueous-glycerol solutions.

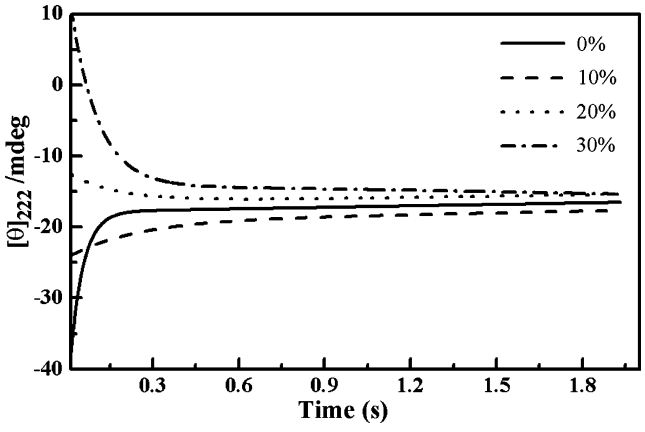

Fig. 6. The time course refolding of lysozyme monitored by far-UV CD spectra at pH 4 in the presence of glycerol (from 0% to 30%). The reaction is initiated by a GdnCl concentration jump from 6 to 1.2 M/L. The CD spectra are monitored at 222 nm.

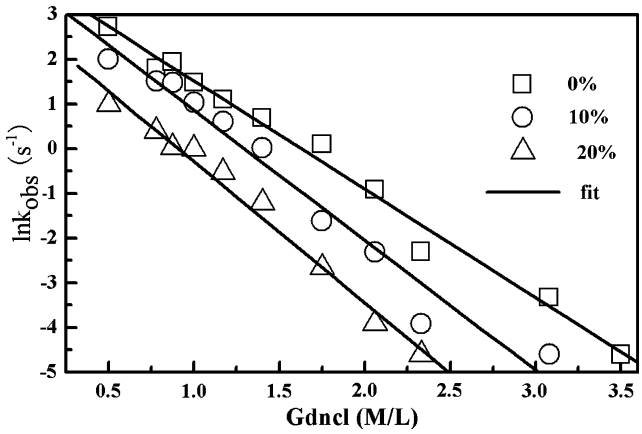

Fig. 7. The natural logarithm of the observed folding rate constants of lysozyme monitored by far-UV CD spectra in the slow phase at pH 4 in the presence of glycerol (0%, 10% and 20%). The folding rate constants per second are obtained from the time course of the folding curves by Eq. (1), labeled as k_{obs} (s^{-1}).

The folding of proteins is driven by different types of local and long-range interactions, e.g., local hydrogen bonding and hydrophobic interactions [6]. The conformation space accessible to the peptide chain is restricted by the hydrophobic interactions while the secondary structure is companied with the rapid formation of local hydrogen bonding. Glycerol as a stabilizer of proteins is known to enhance the hydrophobic interactions within proteins, but dose not contributes to the peptide–peptide hydrogen bonds [20,21]. In the case of lysozyme, the hydrophobic core collapses in α-domain, and the formation of excessively stabilizing interactions in the hydrophobic core will generate a compact intermediate conformation, which can cause an increase of the energy barrier for the secondary structure assembly. Therefore, the recovery of the ellipticity of lysozyme is retarded by glycerol, and the whole folding of lysozyme is decelerated.

Finally, glycerol, as an important stabilizer of protein, alters the solvent property and protein folding. The addition of glycerol converts the two-state folding of lysozyme into a three-state process. On kinetic folding, a new intermediate structure is formed by the non-native hydrophobic interactions, which will be a rate-limiting step in the folding process of lysozyme. These results are available for the studies of protein folding in the organic solvent.

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