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Test for cooperativity in the early kinetic intermediate in lysozyme folding

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Abstract

During the folding of many proteins, collapsed globular states are formed prior to the native structure. The role of these states for the folding process has been widely discussed. Comparison with properties of synthetic homo and heteropolymers had suggested that the initial collapse represented a shift of the ensemble of unfolded conformations to more compact states without major energy barriers. We investigated the folding/unfolding transition of a collapsed state, which transiently populates early in lysozyme folding. This state forms within the dead-time of stopped-flow mixing and it has been shown to be significantly more compact and globular than the denaturant-induced unfolded state. We used the GdmCl-dependence of the dead-time signal change to characterize the unfolding transition of the burst phase intermediate. Fluorescence and far-UV CD give identical unfolding curves, arguing for a cooperative two-state folding/unfolding transition between unfolded and collapsed lysozyme. These results show that collapse leads to a distinct state in the folding process, which is separated from the ensemble of unfolded molecules by a significant energy barrier. NMR, fluorescence and small angle X-ray scattering data further show that some local interactions in unfolded lysozyme exist at denaturant concentrations above the coil-collapse transition. These interactions might play a crucial role in the kinetic partitioning between fast and slow folding pathways.

1. Introduction

During protein folding, a polypeptide chain starts from an ensemble of unfolded states to finally reach a highly cooperative native structure with well-defined side-chain and backbone interactions. For some, mainly small, proteins the acquisition of the native state occurs in a single exponential reaction, indicating a cooperative barrier crossing event and the absence of transiently populated intermediates [1]. Folding of the majority of proteins, however, is more complex, comprising several kinetic steps [2–5]. Starting from the denaturant-unfolded state, the polypeptide chain commonly undergoes very rapid structural changes in the submillisecond time region upon diluting out of the denaturant. As indicated by changes in spectroscopic properties [6] and by a decrease in the radius of gyration [7,8], these burst

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phase reactions lead to compact, but still largely, solvent accessible states, which are commonly termed 'molten globules' [9–12]. In some cases, stable hydrogen bonds were observed in kinetic molten globules, indicating the formation of specific secondary structural elements [13,14]. In other proteins, the earliest steps were proposed to lead to a non-specific collapse without a stable secondary structure [15].

From the viewpoint of polymer theory, a rapid molecular collapse at the beginning of refolding is not surprising. The unfolded polypeptide chain is dissolved in high concentrations of chemical denaturants like urea or GdmCl, which are good solvents for all parts of the polypeptide chain. Under these conditions, the favorable interactions with the solvent dominate over the intramolecular interactions, leading to an ensemble of random coil conformations, as predicted for polymers above the Θ-temperature [16]. Upon initiation of refolding, the denaturant is removed and the polypeptide chain is transferred to water, which is a bad solvent with mainly unfavorable chain-solvent interactions. This corresponds to conditions below the Θ-temperature and favors the formation of compact globular conformations. Coil-globule transitions at the Θ-temperature were shown to be rather sharp in many polymers, with an increase in steepness with increasing chain length [17,18]. However, when the same transition is monitored with different probes, the curves often do not superimpose [19] and it is still an unresolved question as to whether the process is a first order (two-state) or a more gradual transition [18]. From theoretical studies, it was concluded that polymer collapse is a gradual non two-state transition without major energy barriers separating the different states [18]. Based on the similarities of the earliest intermediates in protein folding with collapsed polymers, it was proposed that the burst phase folding reactions correspond to the coil-globule transition in other polymers and that the process is a gradual higher order transition in accordance with theoretical models for polymer collapse [20].

Due to the transient nature of kinetic intermediates, they are difficult to characterize. However, in several proteins partially folded states could be populated under equilibrium conditions, which resemble kinetic intermediates in many properties [11]. These intermediates are readily accessible for a detailed characterization. They often unfold in cooperative transitions and a surprising finding was the presence of a partially native-like topology and native side-chain interactions in the equilibrium molten globule states of α-lactalbumin [21–23] and apo-myoglobin [24]. For the apo-myoglobin molten globule, it was further shown that urea-induced transition curves monitored by intrinsic tryptophan fluorescence and by far-UV CD superimpose, indicating a cooperative unfolding transition [24]. It is still open, however, as to whether the properties found for equilibrium intermediates also apply to their kinetic counterparts.

2. Chain collapse during lysozyme folding

Here, we investigate whether hydrophobic collapse during lysozyme folding represents a cooperative two-state process. Formation of native lysozyme starting from GdmCl-unfolded protein is known to occur in several steps [6,15,25–27]. Within the first millisecond after the start of refolding, major signal changes are observed with several probes. Fig. 1 compares the burst phase reaction monitored by the changes in intrinsic tryptophan fluorescence (panel a), far-UV CD (panel b), ANS fluorescence (panel c) and in the radius of gyration (R_G; panel d). The applied probes are sensitive for different properties of the polypeptide chain. Far-UV CD detects changes in the polypeptide backbone and tryptophan fluorescence monitors the molecular environment of the six tryptophan residues, ANS-binding is sensitive for the formation of patches of hydrophobic regions and the radius of gyration is a direct measure for the chain dimensions. All four probes detect major signal changes in the dead-time of stopped-flow mixing, as indicated by the comparison of the initial signal with the respective signal of GdmCl-unfolded lysozyme. This burst phase reaction detected by four independent probes reveal rapid global structural changes, which lead to a collapsed state with a R_G half way between a completely unfolded and native protein (Fig. 1d). Construction of the scattering profile of the col-

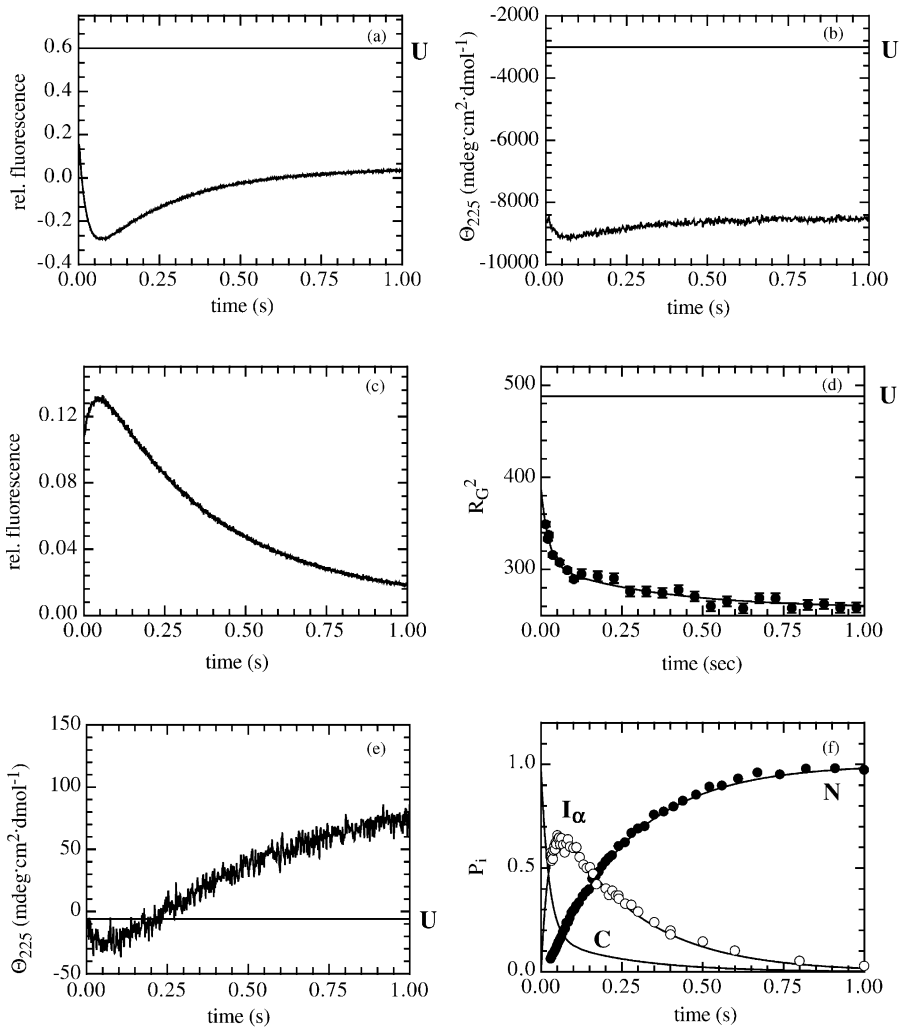
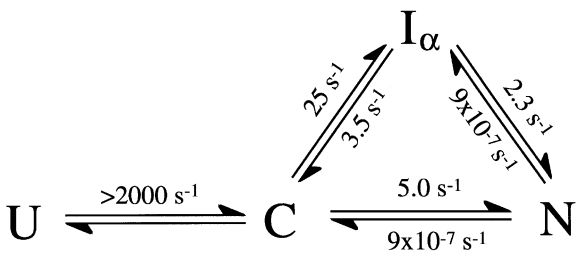


Fig. 1. Refolding of lysozyme at 0.6 M GdmCl, pH 5.2, 23 °C monitored by changes in (a) intrinsic tryptophan fluorescence (b) far-UV CD, (c) ANS fluorescence, (d) radius of gyration and (e) near UV CD after stopped-flow mixing. Panel f shows the time course of formation and decay of the different kinetic species during lysozyme folding under the same conditions. The radius of gyration was measured by a combination of continuous-flow and stopped-flow small-angle X-ray scattering experiments. These data are taken from [8]. The population of the different kinetic species was measured in interrupted refolding experiments and are also taken from [8].

lapsed state from time-resolved small angle X-ray scattering (SAXS) experiments further reveals a globular shape [8]. Despite the observed global changes in the polypeptide conformations, there is no evidence for specific side chain interactions being formed within the first millisecond. Monitoring the near-UV CD signal, which is sensitive for the immobilization of aromatic amino acid side chains, does not show any dead-time changes (Fig. 1e). In addition, no major protection from hydrogen deuterium exchange was observed [15], indicating no or only weakly stable hydrogen bonds. These results were interpreted as evidence for a rather non-specifically collapsed state, consisting of a large ensemble of compact globular conformations. Time resolved fluorescence spectra fur-



Scheme 1.

ther suggested that hydrophobic residues are still partially solvent exposed at this stage, as judged by the emission maximum of tryptophan fluorescence at 339 nm [8].

After the rapid burst phase reaction, two parallel folding pathways lead to the native state, giving rise to two kinetic phases with time constants of ~30 ms and ~400 ms at 20 °C [28,29]. In the faster reaction, approximately 20% of the refolding molecules reach the native state directly from the burst phase intermediate (C) and the remaining 80% of the molecules fold through a second partially folded state (I_1; Fig. 1f). This intermediate is converted to the native state with a time constant of ~400 ms (Scheme 1).

I_1 and R_G have native-like shapes [8], and a native-like secondary structure in the α-helices of the α-domain of the protein, but no evidence for stable hydrogen bonds in the β-sheets [15]. The tryptophan fluorescence emission spectrum of I_1 shows a maximum at 324 nm, indicating a very hydrophobic and solvent shielded environment of all six tryptophan residues [8]. The major decrease in fluorescence intensity compared to both native and unfolded lysozyme, further suggests the presence of side-chain interactions of tryptophan residues with groups capable of fluorescence quenching (Fig. 1a). These results indicate that the helical intermediate has a tight, but non-native, packing. Folding kinetics measured by near-UV CD detect a slight decrease in the signal intensity at 289 nm, upon formation of the helical intermediate (Fig. 1e). Obviously, I_1 has a distinct near UV CD spectrum, which exhibits less ellipticity at 289 nm than both unfolded and native lysozyme, indicating some specific side chain interactions involving aromatic residues.

3. Global and local interactions in collapsed lysozyme

The structural changes during lysozyme folding have been studied in great detail, but much less is known about the development of stability and cooperativity during the folding process. A quantitative treatment of folding kinetics shows that the helical intermediate is separated by from the collapsed state by a significant free energy barrier. It is formed with a rate constant (k_{CI}) of 25 s^{-1} [29]. Assuming a maximum rate of protein folding of ~10^8 s^{-1} [30], this corresponds to a free energy barrier of ~42 kJ/mol. Despite its native-like structural properties, the helical intermediate is still much less stable than the native state ($\Delta\Delta G^0 = 36$ kJ/mol) and is only approximately 4 kJ/mol more stable than collapsed lysozyme at 20 °C [8], which is surprising considering the native-like size and shape and partially native secondary structure of I_1. This indicates that tight but non-native hydrophobic packing is not sufficient to gain stability.

The burst phase intermediate (C) is much more difficult to characterize, since it is short lived and its formation escapes stopped-flow detection. It is formed at least 100 times faster than any subsequent kinetic reaction under all experimental conditions, which uncouples it kinetically from the following steps. Consequently, the dead-time signal changes reflect the rapid pre-equilibrium between collapsed lysozyme and the random coil conformations [31]. This allows us to characterize the coil-globule transition of this kinetic molten globule, by measuring the response of the burst phase signal to changes in the solvent conditions. We can then apply the superposition assay, to check for a cooperative two-state unfolding transition between unfolded and collapsed lysozyme. In these assays, the unfolding transition is monitored with different spectroscopic probes, which monitor different properties of the polypeptide chain. All probes will give the same normalized transition curve for a two-state transition [32,33]. In contrast, if there is a gradual shift of populations

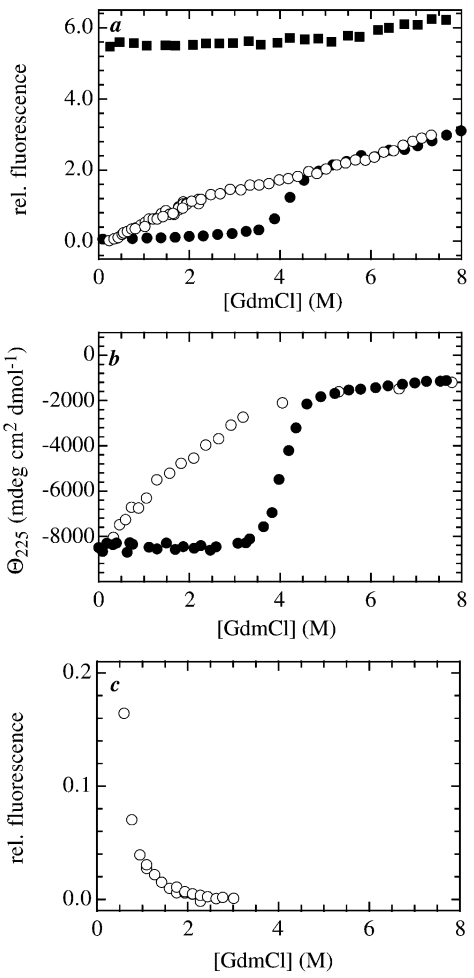


Fig. 2. GdmCl-dependence of the burst phase change (o) in (a) tryptophan fluorescence, (b) far-UV CD and (c) ANS fluorescence during lysozyme refolding at 20 mM NaOAc, pH 5.2, 20 °C. In addition, the final signal after completion of refolding (●) is shown for tryptophan fluorescence and far-UV CD, representing the equilibrium unfolding transition of the native protein. For comparison, the fluorescence signal of a mixture of *N*-acetyl tryptophanamide and *N*-acetly-tyrosineamide in the same molar concentrations as in lysozyme under the experimental conditions is shown in panel a (■). All other experimental conditions were as described in Fig. 1.

to a new ensemble of states, it is expected that different probes will not superimpose, as observed for some coil-globule transitions in synthetic homo and heteropolymers [17].

Fig. 2 shows the GdmCl-dependence of the dead-time changes in far-UV CD, in intrinsic tryptophan fluorescence and ANS fluorescence between 0.3 and 8 M GdmCl. At all denaturant concentrations, collapse was too fast to be resolved by stopped-flow mixing, with a dead-time of approximately 1.2 ms. For CD and tryptophan fluorescence measurements, the final signal of the refolded protein is also displayed, reflecting the equilibrium unfolding transition for the native state. Neither native lysozyme nor the GdmCl-unfolded state exhibit significant ANS binding, and consequently the ANS fluorescence intensity of both the initial state and of the final state are zero. The effect of GdmCl on the pre-equilibrium between collapsed lysozyme and the unfolded state, shows that the transition is complete at approximately 2.5–3.0 M GdmCl for all probes. At higher denaturant concentrations, lysozyme seems to be largely unfolded and no transient ANS binding can be detected. However, intrinsic tryptophan fluorescence intensity increases linearly with denaturant concentration above 3 M GdmCl and shows much less intensity than expected from completely solvent accessible fluorophores (Fig. 2a). Comparison with the signal intensity of the free fluorophores further shows that the increase in slope of this linear increase in signal intensity above 6 M GdmCl is caused by an intrinsic effect of GdmCl on the fluorophores.

The increase in tryptophan fluorescence above 3 M GdmCl shows that conformational changes still occur under conditions where other probes suggest the presence of a random ensemble of unfolded states (Fig. 2). Tryptophan fluorescence is very sensitive to small changes in the environment of the fluorophores, but it is not able to distinguish whether signal changes are due to local interactions around tryptophan residues or whether they are caused by global changes in the protein structure. To discriminate between these possibilities, we used small angle X-ray scattering (SAXS), which allows the determination of the size and the shape of proteins. SAXS experiments were performed under equilibrium conditions over the whole range of GdmCl concentrations. Guinier analysis of the scattering data allows the evaluation of the radius of gyration (R_G; see Section 6). Fig. 3 shows the GdmCl-dependence of the R_G of lysozyme between 0 and 8 M denaturant. The

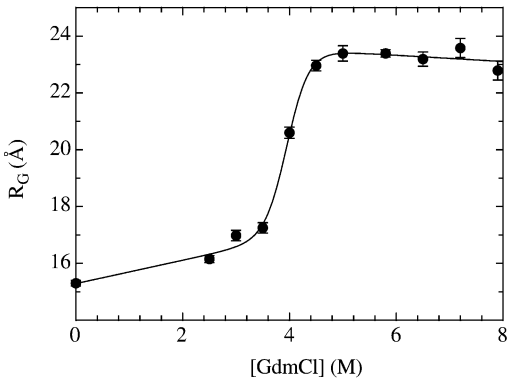


Fig. 3. Equilibrium unfolding transition of lysozyme at 20 mM NaOAc, pH 5.2, 23 °C, measured by the changes in radius of gyration (●). Fitting the transition to the two-state model gives values of ΔG^0 of -46.8 ± 6.8 kJ/mol ($m = d\Delta G^0/d[\text{GdmCl}] = 11.8 \pm 1.7$ [kJ/mol]/M).

unfolding transition occurs between 3 and 5 M GdmCl, with a change in R_G from 15.3 Å for the native protein to 23.5 Å for unfolded lysozyme. This transition is identical to the transition monitored by spectroscopic probes under the same conditions. Above 5 M GdmCl, where major changes in tryptophan fluorescence were detected, no changes in R_G occur, arguing against global structural changes in unfolded lysozyme. The presence of specific local interactions in the unfolded protein is, however, supported by Kratky plots of the scattering profiles [34], which allow the determination of the shape of a polymer (Fig. 4). Between 0 and 3 M GdmCl Kratky, plots of folded lysozyme give profiles typical for globular proteins, with a distinct maximum at $S = 0.02$ Å^{-1}. At GdmCl-concentrations above the unfolding transition (> 5 M GdmCl), the shape of the Kratky plots still changes significantly in the high angle region, indicating local intrachain interactions [34]. Even at 7.2 M GdmCl, unfolded lysozyme still displays some non-random local structure.

These results suggest the presence of local interactions in unfolded lysozyme, under conditions where no global changes in the ensemble of unfolded molecules occur. The presence of specific interactions in the unfolded state is also detected by 1-D ^{1}H NMR measurements in the tryptophan region (Fig. 5). Between 3 and 5 M GdmCl, the characteristic chemical shifts of the native state disappear (Fig. 5a). However, between 5 and 8 M GdmCl, two of the six tryptophan residues still exhibit significant chemical shift changes of approximately 0.1 ppm, suggesting the presence of specific interactions involving tryptophan residues in the unfolded state. A similar observation was reported for thermally unfolded [35] and urea-unfolded lysozyme [36,37], using ^{1}H and ^{15}N NMR spectroscopy, which showed the presence of local interactions involving mainly aromatic residues, but gave no evidence for long range interactions. The change in the Kratky plots of unfolded lysozyme between 5.2 and at 7.2 M GdmCl (Fig. 5b) suggests that these local interactions are weakened at high denaturant concentrations.

4. Cooperativity in collapsed lysozyme

Fig. 2 shows that the transition between unfolded and collapsed lysozyme occurs between 0 and 3 M GdmCl. However, no baseline for the collapsed state is observed at low denaturant concentrations, which makes a normalization and superposition of the curves impossible. The absence of a baseline could be due to the low stability of the collapsed state. However, the observed GdmCl-dependent signal changes in tryp-

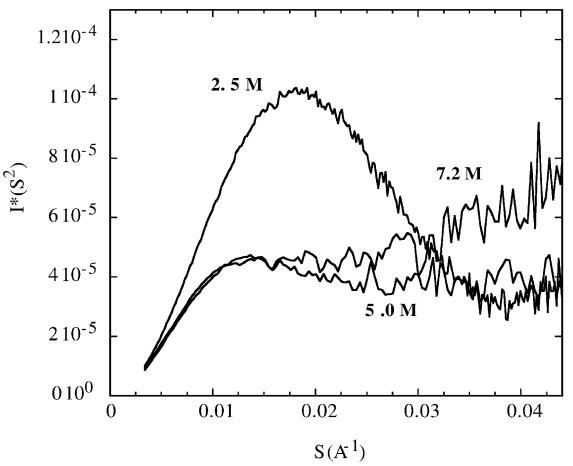


Fig. 4. Comparison of Kratky plots of native lysozyme in 2.5 M GdmCl with unfolded lysozyme in 5.0 7.2 M GdmCl. All scattering curves were recorded at 20 mM NaOAc, pH 5.2, 23 °C.

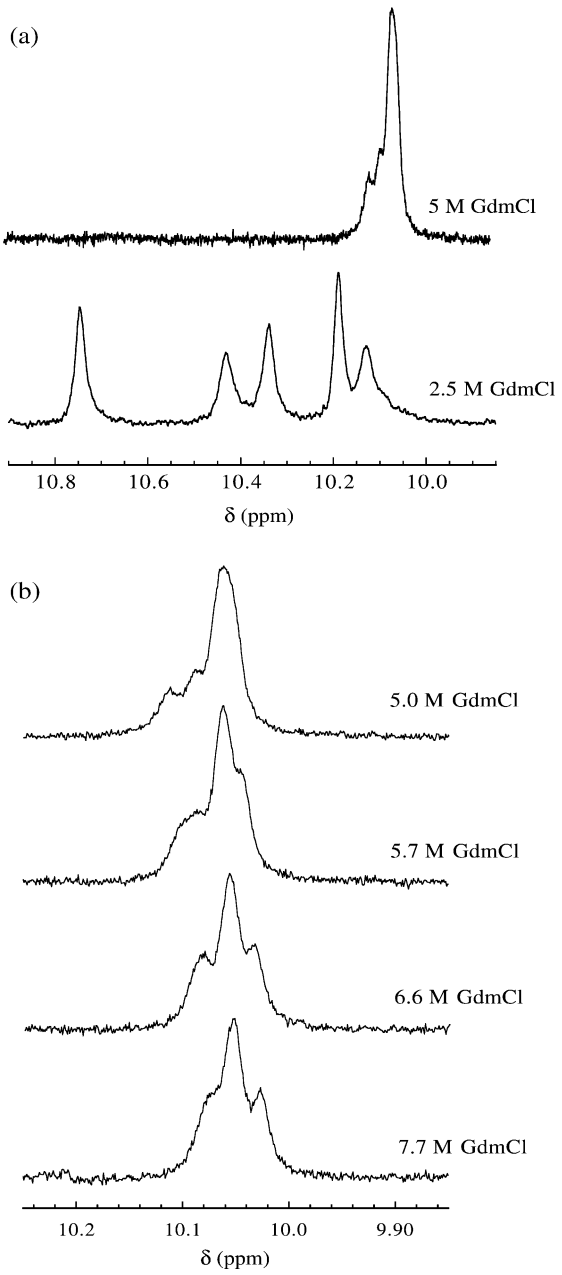


Fig. 5. (a) Comparison of the 10 1H-NMR spectra in the tryptophan NH region of native lysozyme in 2.5 M GdmCl, with unfolded lysozyme in 5.0 M GdmCl. (b) Effect of increasing GdmCl concentrations on the ^{1}H tryptophan NH resonances in unfolded lysozyme. All spectra were taken in 20 mM NaOAc, pH 5.2 at 20 °C. Each spectrum represents the average of 512 scans.

tophan fluorescence and CD could also reflect a gradual shift to more extended conformations, in a non-two state transition. We thus performed refolding experiments in the presence of Na_2SO_4,

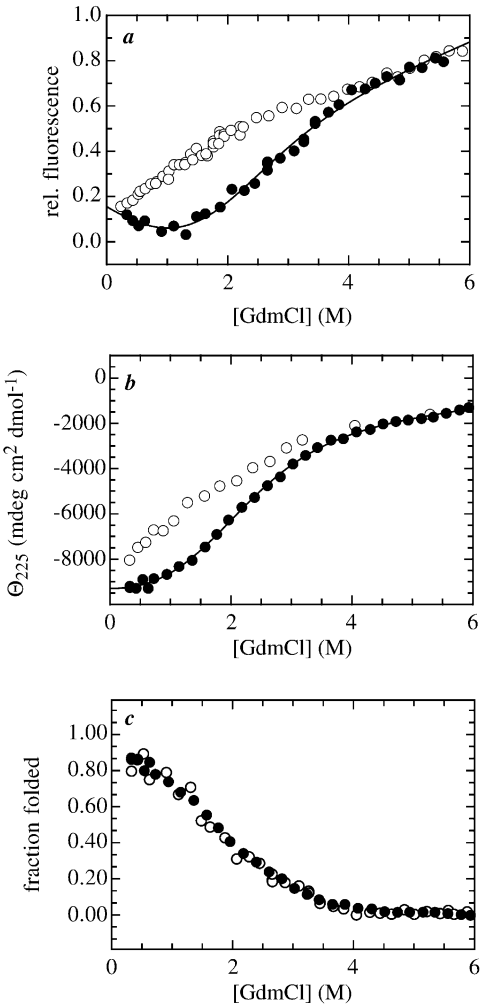


Fig. 6. Effect of the presence of 0.5 M Na_2SO_4 (●) on the burst phase change in tryptophan fluorescence (a) and far-UV CD during lysozyme refolding under same conditions shown in Fig. 2. For comparison, the respective signal in the absence of Na_2SO_4 is shown (o). The transitions in the presence of the Na_2SO_4 can be fit to the two-state model [38] with values for ΔG^0 of -5.60 ± 1.55 kJ/mol ($m = 3.34 \pm 0.54$ (kJ/mol)/M) and -5.64 ± 1.49 kJ/mol ($m = 3.28 \pm 0.42$ (kJ/mol)/M) for fluorescence and far-UV CD transitions, respectively. This allowed normalization of the data to obtain the GdmCl-dependence of the fractional populations of the collapsed state (panel c).

which is known to stabilize compact folded states of proteins. If collapsed lysozyme represents a distinct state, then the addition of SO_4^{2-} should shift its unfolding transition to higher denaturant concentrations. Fig. 6 shows the GdmCl-dependence of the burst phase signal the presence of 0.5 M SO_4^{2-}. Both far-UV CD and intrinsic tryptophan fluorescence show a significant shift in the unfolding transitions to higher denaturant concentrations. In the presence of SO_4^{2-}, both probes give sigmoidal transitions with linear baselines for collapsed and unfolded protein. The fluorescence and CD signals at low concentrations of denaturant are nearly identical to the respective signals in the absence of SO_4^2, indicating little effect of the stabilizing anion on the structure of the collapsed state. Fitting both transitions independently to the two-state model [38] gives values of -5.6 ± 1.5 kJ/mol ($m = d\Delta G^0/d$ [GdmCl] $= 3.3 \pm 0.5$ [kJ/mol]/M)) for ΔG^0 of both the fluorescence and the CD-detected curves (Fig. 6 a,b). The good agreement between these values is confirmed by the coincidence of the normalized transition curves monitored with the two different probes (Fig. 6c). The equilibrium unfolding transition for the native protein in the presence of 0.5 M Na_2SO_4 gives a ΔG^0 of -84.5 kJ/mol ($m = 17.3$ (kJ/mol)/M)). Since the *m*-values were shown to be proportional to changes in the accessible surface area [39,40], our data indicate that only approximately 20% of the total change in accessible surface area upon folding occurs during collapse. This is in agreement with the fluorescence spectrum of the collapsed state, with an emission maximum at 339 nm, indicating partially solvent accessible tryptophan residues [8]. Furthermore, SAXS measurements had shown that a major decrease in solvent accessibility occurs between the collapsed state and I_1, as judged by the forward scattering amplitude [8].

The perfect superposition of the normalized transition curves monitored by probes, which detect largely different properties of the polypeptide chain, indicates a cooperative two-state transition between unfolded and collapsed lysozyme and strongly argues against a gradual unfolding reaction through an ensemble of different states. These results show that the initial collapse during lysozyme folding represents a folding reaction between two distinct states, separated by an energy barrier. Baldwin et al. pointed out that a positive superposition test indicates a cooperative transition, but it is no prove for a two-state state transition [24,41]. In the case of lysozyme, the coincidence of fluorescence and far-UV CD transition curves would be surprising if the transition would involve partially folded intermediates. The six tryptophan residues, which give rise to the fluorescence changes, are spread throughout the entire molecule. Tryptophan residues 28, 108, 111 and 123 are located in the α-domain and tryptophans 62 and 63 are in the β-domain. Far-UV CD in contrast monitors the overall change in secondary structure. A coincidence of these probes in a non-two state transition would require all putative intermediates to show the same fractional changes in the interactions of the tryptophan residues, as in secondary structure content. Since this is very unlikely, especially with the given diversity of structural environments of the tryptophan residues, the coincidence of the unfolding curves strongly points at a two-state transition.

Anions like SO_4^{2-} and TCA were shown to increase the cooperativity in the equilibrium molten globule state of apo-myoglobin [41] and might also effect the coil-globule transition of the early kinetic intermediate in lysozyme folding. However, comparison of the transitions measured by different probes in the absence of SO_4^{2-} suggests that the global unfolding transition occur in the same region of GdmCl concentrations, independent of the probe. This suggests that the cooperativity is not induced by the anions. Also, the steepness of the transition does not seem to be changed significantly by the anions, as judged by a comparison of the transitions in the absence and presence of SO_4^{2-}.

5. Conclusions

The results on lysozyme folding show that the initial stages of protein folding share many properties with heteropolymers collapse below the Θ temperature. However, molecular collapse during lysozyme folding represents a cooperative two-

state transition in contrast to current models for polymer collapse [18,19], arguing for a significant energy barrier for the coil-globule transition. This finding is in agreement with rapid kinetic measurements on cytochrome *c* folding, which were able to kinetically resolve initial collapse. As inferred from the temperature-dependence of this reaction and assuming a two-state transition, an activation energy of approximately 30 kJ/mol was found. This shows that collapse in cytochrome *c* encounters significant energy barriers [42]. Obviously, the folding polypeptide chain encounters barriers very early in the folding process. Crossing these barriers leads to cooperative structures, which seem to be distinct from the collapsed states found in many synthetic homo and heteropolymers [17,18]. The properties of collapsed lysozyme are comparable to those of the equilibrium intermediates of apo-myoglobin [24] and a disulfide-free α-lactalbumin [43], which also gave positive superposition tests.

The collapsed state of lysozyme is of special interest for the folding mechanism of this protein, since kinetic partitioning occurs at this stage (scheme 1). Our results suggest that local interactions in the unfolded chain are present even at 8 M GdmCl. They may play a crucial role in the partitioning into a slow and a fast folding pathway. Possible candidates for these non-native contacts are interactions involving tryptophan residues, as indicated by the large changes in tryptophan fluorescence intensity (Fig. 2) and in the chemical shift changes of two tryptophan residues in unfolded lysozyme, at high denaturant concentrations (Fig. 5). This model is supported by mutational studies, which showed that replacement of tryptophan 63 by tyrosine accelerates the slow folding pathway [44]. Furthermore, the fluorescence properties of the helical intermediate suggest that tryptophan 62, which is solvent exposed in the native state, is forced inside the hydrophobic part of the molecule by non-native interactions [8]. Our results suggest that these interactions are local and may involve the neighboring residue tryptophan 63. This might then explain the inability of the β-domain to form at the stage of the I_1 intermediate.

6. Materials and methods

6.1. Materials

Hen egg white lysozyme was purchased from Sigma Chemical Company, St. Louis, MO and was used without further purification. Ultrapure GdmCl (AA grade) was from Nigu (Waldkraiburg, Germany). All other chemicals were reagent grade and were purchased from Merck (Darmstadt, Germany).

6.2. Methods

Refolding was initiated by diluting completely unfolded lysozyme (20 mM glycine/HCl pH 1.5; 3.6 M GdmCl) to a final concentration of 20 mM sodium acetate, pH 5.2. The intrinsic tryptophan fluorescence above 320 nm was detected after excitation at 280 nm. Far-UV CD was recorded at 225 nm, near-UV CD at 289 nm; ANS fluorescence was recorded above 420 nm, after excitation at 380 nm. The final protein concentration was 3.5 μM for intrinsic tryptophan fluorescence, 30 μM for far UV CD, 120 μM for near UV CD and 60 μM for ANS binding (ANS concentration 20 μM). All measurements were performed with Applied Photophysics SV18 or Pi-Star stopped-flow instruments, with a dead-time of ~1.2 ms. The reference signal of the unfolded state was obtained from a linear extrapolation of the signal of the unfolded baseline to the GdmCl concentration, where the experiments were carried out (0.6 M GdmCl).

6.3. Small Angle X-Ray Scattering (SAXS)

Measurements were made using the SAXS instrument on Beam Line 4-2, at Stanford Synchrotron Radiation Laboratory [45]. X-Ray energy was selected at 8980 eV (Cu edge), using a pair of Mo/B_4C multilayer monochromator crystals [46]. Scattering patterns were recorded by a linear position-sensitive proportional counter. Scattering patterns were normalized by incident X-ray flux, measured with an ionization chamber. The sample-to-detector distance was calibrated to be 218 cm, using a cholesterol myristate sample. To avoid radiation, a flow cell was used [47]. Data were

typically collected for 10 min for the protein sample and 10 min for the buffer (background measurements).

Samples for the SAXS measurements contained 7–11 mg/ml (0.5–0.75 mM) lysozyme and varying GdmCl concentrations. They were incubated >12 h at 20 °C prior to the measurements. After the SAXS measurements, each sample was checked for possible aggregates by absorption. For none of the samples aggregation was observed.

Radii of gyration were calculated according to the Guinier approximation [34]:

$$\ln(I[S]) = \ln(I[0]) - \frac{4\pi^2 R g^2}{3} \cdot S^2 \quad (1)$$

where R_g = radius of gyration, $S = (2\sin\theta)/\lambda$, 2θ = scattering angle and λ = X-ray wavelength. The fitting range used was 0.0045–0.0095 $Å^{-1}$ in S.

6.4. NMR-measurements

Protein concentration for NMR measurements was 1 mM. The buffer contained 20 mM sodium acetate pH 5.2, 5% deuterium oxide and different GdmCl concentrations. Samples were incubated > 12 h at 20 °C before recording the 1D-NMR spectrum. The measurements were performed at 20 °C at a Bruker ARX500 NMR-spectrometer operating at a proton frequency of 500.13 MHz. For each sample, 512 scans were performed.

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