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ORIGINAL PAPER

Refolding of the hyperthermophilic protein Ssh10b involves a kinetic dimeric intermediate

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Abstract The α/β-mixed dimeric protein Ssh10b from the hyperthermophile *Sulfolobus shibatae* is a member of the Sac10b family that is thought to be involved in chromosomal organization or DNA repair/recombination. The equilibrium unfolding/refolding of Ssh10b induced by denaturants and heat was fully reversible, suggesting that Ssh10b could serve as a good model for folding/unfolding studies of protein dimers. Here, we investigate the folding/unfolding kinetics of Ssh10b in detail by stopped-flow circular dichroism (SF-CD) and using GdnHCl as denaturant. In unfolding reactions, the native Ssh10b turned rapidly into fully unfolded monomers within the stopped-flow dead time with no detectable kinetic intermediate, agreeing well with the results of equilibrium unfolding experiments. In refolding reactions, two unfolded monomers associate in the burst phase to form a dimeric intermediate that undergoes a further, slower, first-order folding process to form the native dimer. Our results demonstrate that the dimerization is essential for maintaining the native tertiary interactions of the protein Ssh10b. In addition, folding mechanisms of Ssh10b and several other α/β-mixed or pure β-sheet proteins are compared.

Keywords Protein dimer · Folding kinetics · Dimeric intermediate · α/β-Mixed protein · Stopped-flow circular dichroism

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Introduction

Compared with monomeric proteins, multimeric proteins are under-represented in folding studies despite the fact that oligomeric proteins are prevalent in biological systems. The folding of oligomers requires the coordination of secondary and tertiary structures as well as the docking and assembly of quaternary structures involving multiple polypeptide chains, processes which are absent in the folding of monomers. Studies have shown that lack of reversibility, often due to aggregation, is a frequent complication in the folding of many larger, multimeric proteins in vitro (Jaenicke and Seckler 1997). Therefore recent research on the folding of multimeric proteins has mainly focused on smaller dimeric proteins. The folding pathways of a few dimeric proteins have been reported and reveal a variety of mechanisms. Some small dimeric proteins, e.g., the P22 Arc repressor (Milla and Sauer 1994; Srivastava and Sauer 2000), ORF56 from *Sulfolobus islandicus* (Zeeb et al. 2004), and GCN-4-derived leucine zipper peptides (Zitzewitz et al. 1995, 2000), follow a two-state mechanism in which folding and dimerization of two subunits occur simultaneously and no intermediates are detected. Some larger dimers containing subdomains, e.g., ketosteroid isomerases (Kim et al. 2001a, b), *Escherichia coli Trp* repressor (Gittelman and Matthews 1990; Mann et al.

1995; Gloss et al. 2001), creatine kinase (Zhu et al. 2001a, b; Kuznetsova et al. 2002), factor for inversion stimulation (FIS) (Topping et al. 2004), archael histones (Topping and Gloss 2004), glutathione transferases (Wallace et al. 1998; Wallace and Dirr 1999), the ATPase SecA (Doyle et al. 2000, 2004), R67 dihydrofolate reductase (Bodenreider et al. 2002), and bacterial luciferase (Clark et al. 1997; Noland et al. 1999; Inlow and Baldwin 2002) tend to have more complex folding mechanisms involving monomeric or dimeric transient kinetic intermediates and even parallel pathways. However, no clear rules for the prediction of features of folding mechanisms of dimeric proteins have yet been discerned.

It is noteworthy that, heretofore, most detailed investigations of protein folding have focused on predominately α-helical proteins. This bias in protein selection may give rise to weaknesses in our understanding of protein folding. In contrast to interactions that stabilize α-helices, those that stabilize β-sheets are predominantly nonlocal in nature; thus the folding properties of predominately β-sheet or α/β-mixed proteins may differ from those of α-helical proteins.

The DNA binding protein Ssh10b from the hyperthermophilic archaeon *Sulfolobus shibatae*, is a member of the Sac10b family that is thought to be involved in chromosomal organization or DNA repair/recombination (Xue et al. 2000). Ssh10b is a highly thermostable dimeric protein composed of two identical subunits (Xu et al. 2004). The crystal structure of Ssh10b reveals that the monomer is α/β-mixed structure comprising four β-strands and two α-helices (Wardleworth et al. 2002). A previous work of our laboratory reported the temperature-dependent equilibrium denaturation of Ssh10b in the presence of guanidine hydrochloride (GdnHCl) monitored by circular dichroism (CD) (Xu et al. 2004). Both GdnHCl- and heat-induced unfolding of Ssh10b are fully reversible and follow a two-state mechanism involving a native dimer and two denatured monomers (Xu et al. 2004).

In the present work we investigate the folding/unfolding kinetics of Ssh10b in detail using stopped-flow circular dichroism (SF-CD). In unfolding reactions, the native Ssh10b turned rapidly into fully unfolded monomers within the stopped-flow dead time with no detectable kinetic intermediate, agreeing well with the results of equilibrium unfolding experiments. In refolding reactions, kinetic data are described well by a two-step folding mechanism where two unfolded monomers associate to form a dimeric intermediate within the stopped-flow dead time, and then follow a slower, subsequent folding of the dimeric intermediate to the native dimer. Our results demonstrate that the dimerization is essential for maintaining the native tertiary interactions of the protein Ssh10b. In addition, folding mechanisms of Ssh10b and several other α/β-mixed or pure β-sheet proteins are compared.

Materials and methods

Protein expression and purification

The Ssh10b protein was expressed in *E. coli* and purified as previously described (Xu et al. 2004). The protein samples were dialyzed against 50 mM NH_4HCO_3 then lyophilized and stored at −20°C.

Analytical ultracentrifugation

Ssh10b was analyzed at monomer protein concentrations of 20 μM–0.2 mM in 20 mM potassium phosphate (pH 6.8) in a Beckman-Coulter XL-A analytical ultracentrifuge with an An60Ti rotor. The protein concentration was determined by the Lowry method using bovine serum albumin (BSA) as the standard. Sedimentation velocity was performed in the presence or absence of 2.8 M GdnHCl at 25°C and 60,000 rpm with standard double sector aluminum centerpieces. UV absorption was scanned every 5 min for 6 h. Data were analyzed with software provided by Beckman Instruments (Palo Alto, CA, USA).

SF-CD

All stopped-flow measurements were performed on a PiStar-180 ($\pi^* - 180$) stopped-flow CD apparatus (Applied Photophysics Ltd, UK) using entrance and exit slit widths of 2 mm at 25°C. The dead time of the stopped-flow instrument was about 20 ms. Unfolding measurements were carried out following 1:10 dilutions from native Ssh10b in 20 mM potassium phosphate (pH 6.8) with 6.6 M GdnHCl, 20 mM potassium phosphate (pH 6.8). Refolding measurements were carried out following 1:10 dilutions from fully unfolded Ssh10b in 6 M GdnHCl, 20 mM potassium phosphate (pH 6.8) (Xu et al. 2004) with different refolding buffer. SF-CD data were collected at 222 nm, and typically ten to fifteen shots were averaged for each sample to enhance the signal-to-noise ratio on the observed kinetics traces. Controls of refolding buffer and GdnHCl solutions were recorded separately to correct kinetic traces for any instrument offsets. To study dependence of the final GdnHCl concentrations on refolding velocity, refolding measurements were taken between 0.6 and 2.8 M post-mix GdnHCl concentrations. Dependence of final protein concentration on refolding velocity was studied by recording refolding measurements between 20 μM and 0.15 mM post-mix protein concentrations.

Kinetic data analysis

Individual kinetic traces were locally fitted with Sigmaplot 9.0 to an equation in the exponential form:

$$A(t) = \sum^{i} A_i \exp(-t/\tau_i) + A_\infty \quad (1)$$

where $A(t)$ is the value of the CD signal at a given time t, A_i is the amplitude corresponding to each individual phase (i), τ_i is the fitted relaxation time for each kinetic phase (i), and A_∞ is the final signal amplitude of the sample at equilibrium.

The program Savuka 5.1 was used to fit the GdnHCl dependence of the folding and unfolding relaxation times of multiple SF-CD data sets to the following equation:

$$1/\tau_i = k(\mathrm{H_2O})\exp\left[\frac{\mathrm{m[GdnHCl]}}{\mathrm{RT}}\right] \quad (2)$$

where [GdnHCl] is the concentration of GdnHCl, $k(\mathrm{H_2O})$ is the rate constant in the absence of GdnHCl and m reflects the sensitivity of the reaction to [GdnHCl].

Results

Ssh10b exists as a dimer under refolding conditions

The association state of Ssh10b in refolding condition experiments was analyzed by analytical ultracentrifugation. As Fig. 1 shows the apparent molecular mass of Ssh10b was determined to be Mr = 20,000 Da (in the presence or absence of 2.8 M GdnHCl when protein concentrations were in the range 20 μM–0.2 mM). This value was close to the expected value (21,000 Da), indicating that Ssh10b existed as a stable dimer in solution under all the refolding conditions used in this study.

Kinetic folding/unfolding of Ssh10b

In unfolding reactions, most of the total ellipticity amplitude was lost by the end of the stopped-flow dead time, and the CD signal was found to undergo no obvious change after the dead time (data not shown). These results indicate that there is no detectable kinetics intermediates during unfolding and there is residual structure in the fully unfolded state, agreeing well with the equilibrium unfolding results (Xu et al. 2004). In refolding reactions, as expected from spectra of the unfolded monomer and native dimer, the refolding was reflected in a gain of ellipticity amplitude. A typical refolding SF-CD kinetic trace is shown in Fig. 2. About 55% of the total ellipticity amplitude was reached by the end of the dead time, indicating that a very rapid phase was completed within the first 20 ms, the signal then decreased more slowly to reach the ellipticity of the native dimeric protein. All observed kinetic traces were monophasic, and well described by a fit to a single, first-order exponential equation. Thus, the refolding reaction appears to follow a complex kinetic model that may consist of at least two distinct phases: a burst phase and a slow phase.

Protein concentration dependence of Ssh10b refolding kinetics

Since Ssh10b existed as a native dimer under all the final refolding conditions used in this study, its folding must include a bimolecular reaction, and hence a step with a rate constant dependent on protein concentration. To find out which of the two phases corresponded to the dimerization step, refolding experiments were carried out at various protein concentrations using a final GdnHCl concentration of 2.5 M. The relaxation time (τ) of the observed folding kinetic phase was independent of the final monomer concentration (Fig. 3), suggesting that the kinetic folding phase observed on the SF time-scale is a first-order reaction, and that dimerization may have occurred during the stopped-flow dead time.

Final GdnHCl concentration dependence of the refolding kinetics of Ssh10b

GdnHCl-dependent folding reactions were carried out at a final concentration of 50 μM monomer. The relaxation times (τ) of the observed folding kinetic phase exhibited a significant GdnHCl dependence, increasing with increasing GdnHCl concentrations, as is typical for a protein folding reaction. The kinetic data for 0.6–2.8 M GdnHCl concentrations were globally fitted to Eq. 2 to determine the GdnHCl dependence of the folding kinetics. Values obtained were $k(\mathrm{H_2O}) = 30.0\ \mathrm{S}^{-1}$ and $m = -4.87\ \mathrm{kJ\ mol}^{-1}\ \mathrm{M}^{-1}$. Figure 4 shows there is good agreement between local fits and global fits: filled circles represent locally fitted relaxation times, and the continuous line represents the results of globally fitting the kinetic traces to Eq. 2. The quality of the global fit is shown for a representative trace at final 2.5 M GdnHCl concentration by the fitted line in Fig. 2.

Discussion

Further to our previous report on the homodimeric protein, Ssh10b, from the hyperthermophilic archaeon *Sulfolobus shibattae* that focused on thermodynamic investigations by equilibrium denaturation (Xu et al. 2004), the experiments reported here deal with kinetic aspects of the refolding of this protein. Here, our aim was to understand how the development of quaternary interactions is coordinated with the formation of secondary and tertiary structure during the

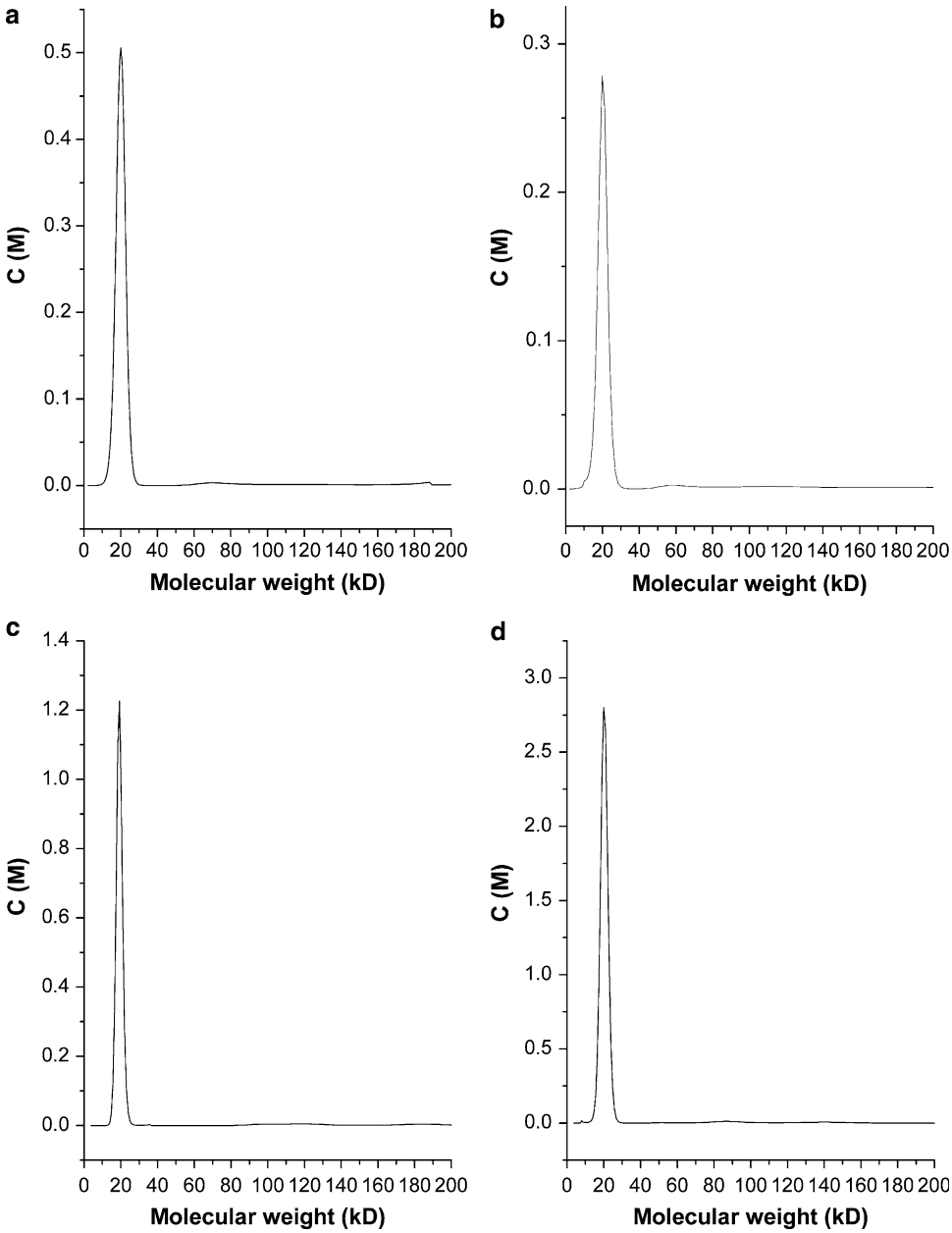

Fig. 1 Sedimentation velocity experiments of Ssh10b: **a** 20 μM in 20 mM potassium phosphate (pH 6.8); **b** 0.2 mM in 20 mM potassium phosphate (pH 6.8); **c** 20 μM in 2.8 M GdnHCl, 20 mM potassium phosphate (pH 6.8); **d** 0.2 mM in 2.8 M GdnHCl, 20 mM potassium phosphate (pH 6.8)

folding reaction and, more specifically, the role of nonlocal interactions in the folding of a α/β-mixed protein.

Folding mechanism of Ssh10b

The experiments reported above show that folding of Ssh10b denaturant-unfolded monomers to native dimers requires at least two steps: a rapid development of secondary structure in a burst phase and a slower folding reaction that leads to the native dimer. The initial CD signal observed after the stopped-flow dead time was substantially greater than that expected for unfolded protein (Fig. 2), indicating the existence of a burst phase. The structure detected in this burst phase represents at least one transient kinetic intermediate that is competent for conversion to the native dimer by the subsequent, slower folding process.

In general, our results demonstrate that there is an obligatory kinetic intermediate, I_2, in which the two monomer chains are associated and partially folded, formed in the burst phase. Firstly, the observed folding traces were well described by a single exponential fit and exhibited a relaxation time that is totally independent of the final monomer concentration, consistent with a first-order reaction rather than a process involving monomer association, and suggesting that the observed kinetic phase is a unimolecular process. Secondly, ultracentrifugation analysis demonstrated that Ssh10b in its final refolded state

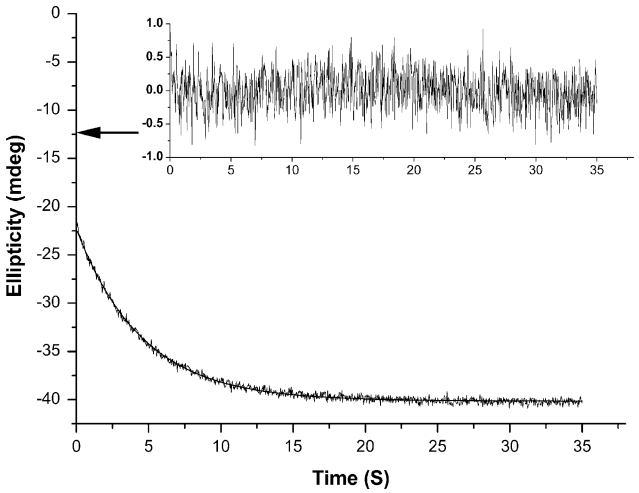

Fig. 2 Representative SF-CD refolding kinetic trace at final 50 μM monomer, 2.5 M GdnHCl in 20 mM potassium phosphate (pH 6.8). The *arrow* indicates the expected ellipticity for the unfolded protein at this final [GdnHCl], extrapolated from the linear unfolded baseline observed in equilibrium experiments. The *thick line* represents a global fit of the data to a single first-order exponential (Eqs. 1, 2). *Inset* residuals of the global fit of the data

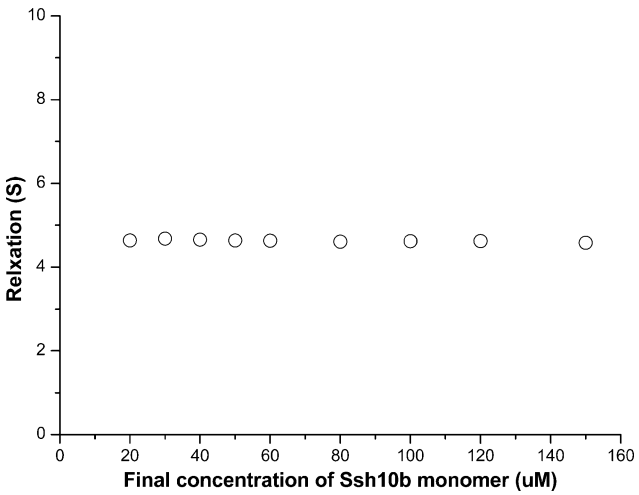

Fig. 3 Monomer concentration dependence of the Ssh10b folding reaction at final [GdnHCl] 2.5 M in 20 mM potassium phosphate (pH 6.8): the observed relaxation time (*open circle*) is independent of the final protein concentration

exists as a stable native dimer. Together with the results discussed above, we conclude that the observed kinetic phase represents a unimolecular process of folding from the dimeric intermediate, I_2, to the native dimer, N_2. Thus we can see that dimerization of the two monomers must occur during the burst phase. Furthermore, Ssh10b loses approximately 80% of its CD signal in 6 M GdnHCl, 20 mM potassium phosphate (pH 6.8) at 25°C and its CD spectrum was found to undergo no further change when the concentration of GdnHCl was increased to 8 M (Xu et al. 2004), suggesting the existence of residual structure in its

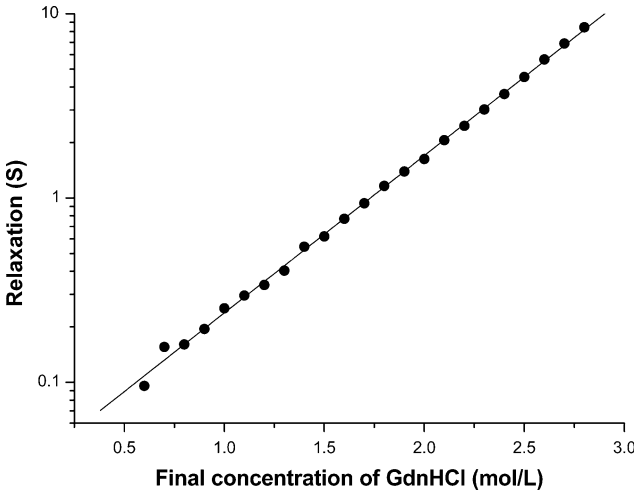

Fig. 4 [GdnHCl] dependence of the folding reactions of Ssh10b monitored by SF-CD at final 50 μM monomer in 20 mM potassium phosphate (pH 6.8). The individual data points (*filled circle*) represent the relaxation times determined from local fits of the data. The *solid line* represents the global fits of the kinetic traces of Ssh10b as a function of GdnHCl, for comparison

fully unfolded state. This residual structure may help the two unfolded monomers recognize each other and associate to form a dimer rapidly at the beginning of folding.

Therefore, we propose the following mechanism to describe the folding kinetics of Ssh10b:

$$2U \xrightarrow{\text{burst phase}} I_2 \xrightarrow{\text{slow phase}} N_2$$

Scheme 1 Kinetic mechanism for the folding of Ssh10b

Two unfolded monomers (U) associate to form a dimeric intermediate (I_2) during the burst phase and then I_2 folds to form the native dimer (N_2) during the following slower phase.

The equilibrium denaturation of protein Ssh10b is characterized by a two-state mechanism, agreeing well with the kinetic unfolding results that there is no detectable kinetic unfolding intermediate was found. Refolding reactions are described well by a two-step folding mechanism involving a kinetic dimeric intermediate. Our results indicate that the dimerization is essential for maintaining the native tertiary interactions of protein Ssh10b.

However, it must be pointed out that Scheme 1 only represents a minimal mechanism and it is possible that other steps may exist that are not detected by SF-CD. Firstly, the dimerization reaction was not detected directly as it occurred faster than the time resolution of SF-CD. Therefore it is not known whether the monomers were unfolded or had undergone a partial folding reaction before associating to form I_2. Secondly, SF-CD only assesses the

formation of secondary structure. Development of tertiary structure can be monitored directly by stopped-flow fluorescence, which relies on the intrinsic fluorescence of aromatic amino acid residues. However, Ssh10b monomers only contain one Tyr, one Phe, and their intrinsic fluorescence is very poor. A more sensitive fluorophore is required to assess the kinetics of the development of tertiary structure. Experiments to introduce unique Trp residues that will exhibit a large fluorescence difference between folded and unfolded states are currently in progress.

Comparison with other α/β-mixed or pure β-sheet structure proteins

Recent research on folding of multimeric proteins, especially smaller dimeric proteins, has revealed a variety of mechanisms. However, most of these reports have focused on the predominately α-helical proteins. We have only limited insights into the folding pathway of predominately β-sheet or α/β-mixed dimeric proteins (Kim et al. 2001a, b; Bodenreider et al. 2002).

It is of great interest to compare the folding of Ssh10b to other α/β-mixed dimeric proteins such as ketosteroid isomerases from *Comamonas testosterone* and *Pseudomonas putida* (Kim et al. 2001a; b), and pure β-sheet proteins such as R67 dihydrofolate reductase (Bodenreider et al. 2002). Comparing our results with those of these three proteins reveals several interesting phenomena. Firstly, the folding processes of these proteins are all consistent with multiple-step folding reactions, including at least one dimeric intermediate and maybe several monomeric intermediates. Due to the high proportion of β-sheet in these proteins and the fact that, in contrast to α-helical proteins, interactions that stabilize β-sheets are predominantly nonlocal in nature, many more nonlocal interactions need to form in a concerted manner during the folding of these proteins; the simultaneous coordination of the formation of these interactions could result in a higher energy barrier which has a low probability of being overcome. Fortunately, this problem can be circumvented by breaking the concerted folding reaction into the formation of smaller, sub-global units of structure (potential intermediates), which coalesce into the native state as folding progresses. Therefore, it is conceivable that intermediates are especially important in the folding of α/β-mixed or pure β-sheet proteins. Secondly, the folding of all these proteins includes a burst phase when a significant amount of secondary structure is developed into an intermediate structure. Compared with the other three proteins, the very rapid formation of secondary structure in the pure β-sheet protein R67 DHFR seems surprising since most β-sheet proteins fold more slowly than α-proteins or α/β-mixed proteins because of the greater numbers of nonlocal stabilizing interactions involved. Finally, though the folding processes of these four proteins all comprise a dimeric intermediate, their association rates are quite different. For Ssh10b, as dimerization is completed within the dead time, the association rate constant should approach the diffusion limit, and is about two orders of magnitude higher than those of the other three proteins. In R67 DHFR, the contact area between the two monomers forming the native dimer, which consists of an intersubunit β-barrel formed by the interaction between the β-sheets from each monomer, is only about 535 $Å^2$. As with R67 DHFR, the monomers of KSI from *C. testosterone* and *P. putida* both interact with each other over a narrow and long patch of β-sheet, e.g., only about 670 $Å^2$ of the solvent-accessible area is buried during dimerization of KSI from *C. testosterone*. In the crystal structure of Ssh10b, the two contact areas (690 and 460 $Å^2$), involving one helix and two strands of each monomer, are much larger than those in the other three proteins. Moreover, in Ssh10b, more than ten hydrophobic residues are involved in the interface, much greater than in the other three proteins, e.g., in R67 DHFR, only three hydrophobic residues constitute the intersubunit hydrophobic core. Taken together, these arguments may explain why the dimerization rate constant is much greater for Ssh10b than for the other three proteins.

This comparison of the folding mechanisms of these four α/β-mixed or pure β-sheet proteins has revealed some common features and some differences. However, further experimental investigation is required to obtain more valuable and conclusive information about the folding of α/β-mixed or pure β-sheet proteins.

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