Time-dependent Changes in the Denatured State(s) Influence the Folding Mechanism of an All β-Sheet Protein* §

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Newt fibroblast growth factor (nFGF-1) is an ~15-kDa all β-sheet protein devoid of disulfide bonds. Urea-induced equilibrium unfolding of nFGF-1, monitored by steady state fluorescence and far-UV circular dichroism spectroscopy, is cooperative with no detectable intermediate(s). Urea-induced unfolding of nFGF-1 is irreversible, but the percentage of the protein recovered in the native state depends on the time of incubation of the protein in the denaturant. The yield of the protein in the native state decreases with the increase in time of incubation in the denaturant. The failure of the protein to refold to its native state is not due to trivial chemical reactions that could possibly occur upon prolonged incubation in the denaturant. H15N heteronuclear single quantum coherence (HSQC) spectra, limited proteolytic digestion, and fluorescence data suggest that the misfolded state(s) of nFGF-1 has structural features resembling that of the denatured state(s). GroEL, by the presence of ATP, is observed to rescue the protein from being trapped in the misfolded state(s). H15N HSQC data of nFGF-1, acquired in the denatured state(s) (in 8 M urea), suggest that the protein undergoes subtle time-dependent structural changes in the denaturant. To our knowledge, this report for the first time demonstrates that the commitment to adapt unproductive pathways leading to protein misfolding/aggregation occurs in the denatured state ensemble.

The mechanism by which a protein refolds to its unique native conformation remained shrouded in mystery until recently. It is no longer believed that protein folding/unfolding occurs via a specific channel of mandatory intermediate states. The “new view” of protein folding envisages folding as a stochastic search of many conformations available to a polypeptide chain (1–3). In principle, the conformational search process is guided by the quest of the polypeptide chain to find the lowest energy structure (2). Although protein folding is a very efficient process, the folding reactions are known to go awry, leading to misfolding and aggregation of proteins (4). Previously it was believed that denatured states of proteins are random coil ensembles where the conformational averaging is independent of the neighboring environment (5, 6). However, it is now increasingly clear that denatured states are distinct from unstructured random coils (6). High-resolution NMR studies have provided a vast wealth of information on the ensemble-averaged structural properties of unfolded proteins (5, 6). Hydrophobic clusters mostly formed by local side-chain interactions were observed to be populated in a number of proteins (7, 8). It is proposed that transient interactions between hydrophobic clusters that persist in the unfolded states are responsible for the initial collapse of the polypeptide chain that occurs during protein refolding (7). Similarly, the folding of barnase is believed to be initiated from the native-like local structures that persist in the unfolding state(s) (9). In proteins like drkN, multiple types of structures ranging from conformers with non-native structure(s) possessing long range contacts to those with compact structures maintaining native-like secondary structures are found to be persistent in the denatured state(s) (10, 11). Consequently, these proteins adapt multiple pathways of refolding to their native state. Conformers with non-native hydrophobic contacts refold via a hydrophobic collapse mechanism, and those with a residual native-like secondary structure refold via a hierarchical condensation mechanism (10, 11). On the other hand, unfolded states of proteins, such as the chymotrypsin inhibitor with no detectable residual structures, refold cooperatively by a two-state mechanism (9). The results of these studies clearly demonstrate that the refolding mechanism adapted by a protein is strongly influenced by the nature of residual structures that persist in the ensemble of denatured states.

In general, protein folding involves a kinetic competition between on-pathway reactions that result in the formation of the native state and non-productive pathways leading to misfolded states and, subsequently, aggregation (4, 13). Precursors for protein aggregation are often suggested to be folding intermediates such as the molten globule states (14–17), largely because such partially folded intermediates accumulate in connection with aggregation (17, 18). Aggregation is primarily attributed to the attraction between interchain hydrophobic surfaces, which are transiently solvent-exposed in the folding intermediates (16). It is generally believed that proteins in their unfolded states are not susceptible to aggregation because of the absence of native-like hydrophobic surfaces (19). This notion was proved incorrect by Silow et al. (20), who showed that, in proteins such as U1A, aggregation occurs directly through coalescence of the denatured states (20). However, the formation of these aggregates is transient, and they dissociate so that individual chains fold. Chiti et al. (21) reported that amyloid-like fibril formation is prevented in acyl phosphatase

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§ The on-line version of this article (available at http://www.jbc.org) contains supplemental material presenting nFGF-1 fluorescence spectra (Fig. S1) and electrospray-mass spectra (Fig. S2).

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because of the stabilization of elements of local secondary structures in the denatured state(s) of the protein (21). Although these studies provide useful clues to the interactions that lead to aggregation, very little information exists on the mechanisms that partition pathways toward productive folding or to misfolding and aggregation.

Newt acidic fibroblast growth factor 1 (nFGF-1) is a 15-kDa, all β-sheet protein devoid of disulfide bonds (22, 23). The secondary structural elements in the protein include 12 anti-parallel β-strands arranged into a β-barrel structure. The folding/unfolding pathway(s) of nFGF-1 have been well characterized (22–25). In the present study we demonstrate that the folding mechanism of nFGF-1 depends on the time of incubation of the protein in the denaturant. nFGF-1 fails to refold to its native state upon prolonged incubation in 8 M urea. However, when the refolding is initiated in the presence of GroEL, the time period of incubation of the protein in the denaturant does not appear to significantly affect the refolding process. To our knowledge, this report for the first time demonstrates that areas of residual structure(s) in denatured state(s) dictate the kinetic partitioning of pathways leading to correct folding or misfolding of the protein.

MATERIALS AND METHODS

Unless stated otherwise, all solutions were prepared in 10 mM phosphate buffer (pH 7.2) containing 100 mM sodium chloride. All experiments were conducted at 25 °C.

Equilibrium Unfolding—Denaturant-induced equilibrium unfolding was initiated by mixing appropriate volumes of 8 M urea (recrystallized from methanol) with fixed volumes of the stock solution of the protein (1 mg/ml). Equilibrium unfolding of nFGF-1 was monitored by steady state fluorescence and circular dichroism measurements as a function of the denaturant concentration. Fluorescence spectra were measured with a Hitachi F-2500 fluorometer at 2.5- or 10-nm resolution, using an excitation wavelength of 280 nm. All fluorescence measurements were made using a protein concentration of 100 μg/ml. The sample temperature was maintained at 25 °C using a Neslab RTE-110 circulating water bath. Circular dichroism spectra were measured using a Jasco J-720 spectropolarimeter. CD spectra were characterized (22–25). In the present study we demonstrate that the folding mechanism of nFGF-1 depends on the time of incubation of the protein in the denaturant. nFGF-1 fails to refold to its native state upon prolonged incubation in 8 M urea. However, when the refolding is initiated in the presence of GroEL, the time period of incubation of the protein in the denaturant does not appear to significantly affect the refolding process. To our knowledge, this report for the first time demonstrates that areas of residual structure(s) in denatured state(s) dictate the kinetic partitioning of pathways leading to correct folding or misfolding of the protein.

RESULTS AND DISCUSSION

Urea-induced Equilibrium Unfolding of nFGF-1—nFGF-1 contains a single, well conserved tryptophan (22). The fluorescence spectrum of nFGF-1 is dominated by tryrosine fluorescence at 308 nm (Fig. 1A, inset) (22). However, in the completely unfolded state nFGF-1 exhibits emission spectra dominated by tryptophan fluorescence at 350 nm (Fig. 1A, inset). These spectral features are ideal for monitoring the denaturant-induced unfolding/refolding of nFGF-1 (22). Urea-induced equilibrium unfolding of nFGF-1 is monitored by changes in the ratio of the fluorescence intensities at 350/308 nm. The ratio of fluorescence intensities at 350/308 nm yields a reliable estimate of the fraction of the native/unfolded species formed at various concentrations of urea. nFGF-1 is observed to unfold completely at urea concentrations beyond 3.8 ± 0.1 M (Fig. 1B). The concentration of urea (Cm) at which 50% of the nFGF-1 are unfolded (when ΔGo = 0) is estimated to be 3.0 ± 0.02 M. The change in free energy of stabilization (ΔG_H2O) is calculated to be 4.12 ± 0.12 kcal/mol. Urea-induced equilibrium unfolding of nFGF-1 monitored by far-UV CD (at 228 nm) yielded nearly identical Cm (3.0 ± 0.08 M) and ΔG_H2O (4.12 ± 0.08 kcal/mol) values, suggesting that the denaturant-induced unfolding of the protein is cooperative without the accumulation of detectable intermediate(s) (data not shown).

Time-dependent Reversibility of the Denaturant-induced Unfolding of nFGF-1—Urea-induced unfolding of nFGF-1 at 25 °C is completely reversible (Fig. 1B). The urea-induced unfolding and refolding profiles of nFGF-1, obtained by monitoring tryptophan fluorescence, are completely superimposable (Fig. 1B). However, the reversibility of the urea-induced unfolding of nFGF-1 is observed to be dependent on the time of incubation of the protein in the denaturant (8 M urea). Refolding initiated (by 20-fold dilution of the protein in the denaturant) within ~60 min of incubation in the denaturant (8 M urea) results in nearly complete (>85%) recovery of the protein in the native state conformation (Fig. 1A). However, the percentage of protein molecules regaining the native state progressively decreases with the increase in the time of incubation of the protein in the denaturant (8 M urea; Fig. 1A). The percentage of protein regaining the native state almost reaches zero when the time of incubation in 8 M urea exceeds 100 h.

Failure of the Protein to Refold Is Independent of the Nature of the Denaturant Used—Time-dependent irreversibility of unfolding is also observed when an ionic denaturant such as...
GdnHCl is used. GdnHCl-induced unfolding of nFGF-1 is completely reversible when the refolding process is initiated within 3 h of incubation of the protein in 6 M GdnHCl (supplementary Fig. S1 in the on-line version of this article). The unfolding process is almost completely irreversible after >200 h of incubation of the protein in the denaturant (GdnHCl). The structural characteristics (as probed by fluorescence and CD spectroscopy) of the non-native, misfolded state(s) obtained by “refolding” of the protein (nFGF-1) after prolonged hours of incubation in 8 M urea (>100 h) and 6 M GdnHCl (>200 h) are similar (data not shown). These results clearly suggest that nFGF-1 fails to refold to its native conformation after prolonged incubation in the denaturant and that this phenomenon is independent of the chemical nature of the denaturant used.

Inability of the Protein to Refold Is Not Due to Trivial Chemical Reactions Induced by the Denaturant—It is well known that chemical modification of side-chain groups during the refolding process often results in misfolding or aggregation of proteins (26). Comparison of the electrospray ionization mass spectra of nFGF-1 incubated for 100 h in the absence of urea in 10 mM phosphate buffer (pH 7.2) containing 100 mM NaCl (wherein nFGF-1 is in the native conformation) and in the presence of 8 M urea (supplementary Fig. S2, available in the on-line version of this article) yielded the same molecular mass (15,147 Da), suggesting that the failure of the protein to refold (observed in nFGF-1) is not due to urea-induced chemical modification of the protein. Temperature is one of the physical parameters that has a profound influence on the protein folding reaction (27). Proteins subjected to refolding are often trapped as non-productive off-pathway intermediates. In some cases, increasing the temperature of the folding reaction results in the channelization of off-pathway intermediates to productive “folding tracks” to yield the native state (27). In this context, the refolding of nFGF-1 from its urea-denatured state was initiated (after 100 h of incubation in the denaturant) under various temperature conditions ranging from 15 to 35 °C. At a fixed protein concentration (100 μg/ml) and in the range of temperature used, the percentage of the protein molecules refolded to the native conformation remains <2% (data not shown).

Folding of proteins often involves the formation of non-native oligomeric intermediate states (28). In most cases, these non-native oligomeric species are off-pathway, dead-end intermediates (28). Because oligomerization is a multimeric reaction, the formation of the perceived oligomeric intermediate(s) is expected to increase with increase in protein concentration. In this context, the influence of protein concentration was examined by initiating the refolding of nFGF-1 after 100 h of incubation in 8 M urea. The percentage yield of the protein recovered in the native state did not show any significant variation with the increase in the protein concentration (in the concentration range of 25–500 μg/ml) used for the refolding reaction (Fig. 2). These results possibly suggest that the inability of the protein to refold to its native state, upon prolonged incubation in the denaturant, is not primarily due to formation of off-pathway oligomeric intermediate state(s). The possibility of misfolding/aggregation caused by the formation of non-native intramolecular or intermolecular disulfide bonds can also be discounted, because “refolding” initiated in the presence and absence of a thiol reagent (such as β-mercaptoethanol) results in similar yields of the protein in the native state (data not shown). These results clearly suggest that the inability of the protein to refold to its native state (after prolonged incubation in the denaturant) is not due to trivial chemical reactions in the denatured state(s) or the inappropriate physical conditions used for refolding of the protein.

Characterization of the Misfolded State—Size exclusion chromatography of the conformational states formed upon refolding (of the protein incubated for 100 h in 8 M urea) showed a single peak corresponding to the molecular mass of the monomeric form of nFGF-1 (~15.2 kDa). UV light measurements at 350 nm reveal that the solution containing the protein in the misfolded state(s) remains clear even up to 48 h. Mild aggregation is noticed only after ~60 h of incubation of the protein at room temperature.

1-Anilino-8-naphthalene-sulfonate (ANS) is a fluorescent dye that binds to hydrophobic regions of proteins (29). ANS has been used to probe solvent-exposed, non-polar surfaces in proteins. The dye generally exhibits weak binding affinity to the native and completely unfolded states of proteins (29). ANS binds strongly to the misfolded state(s), and the emission intensity of ANS upon binding to the protein (nFGF-1) is more than twice that observed with the protein in its native and completely unfolded state(s) in 8 M urea (Fig. 3A). Fluorescence spectra of ANS (Fig. 3A) upon binding to the misfolded state(s) reveal that the emission maxima of the dye blue shifts by ~30 nm (from 520 to 490 nm). These results suggest that the protein in misfolded state(s) has solvent-exposed, non-polar surfaces.
Limited proteolytic digestion has been applied to investigate the conformational flexibility of proteins (30). The basic assumption underlying this technique is that the proteolysis event is governed by the stereochemistry and accessibility of the protein substrate as well as the specificity of the proteolytic enzyme. Hence, even subtle conformational changes in the protein could be successfully detected using the limited proteolytic digestion technique. nFGF-1 possesses many lysine and arginine residues in its sequence, and most of them are concentrated in the C-terminal segment (spanning residues 105–140), which constitutes the putative heparin binding site. As the cleavage sites for trypsin correspond to the carboxyl ends of lysine and arginine residues, trypsin is an apt choice to monitor the conformation of the misfolded state(s) of nFGF-1. Undigested nFGF-1 yields a band on SDS-PAGE, which corresponds to a molecular mass of ~15 kDa (Fig. 3B). After 20 min of incubation, nFGF-1 (in its native state) with trypsin leaves ~90% of the protein uncleaved. However, treatment of nFGF-1 in the misfolded state(s) with trypsin shows that >90% of the15-kDa band is cleaved (Fig. 3B). These results suggest that the protein in the misfolded state(s) has high conformational flexibility.

$^1$H-$^{15}$N HSQC spectrum is a fingerprint of the backbone conformation of a protein (31). $^1$H-$^{15}$N HSQC spectrum of nFGF-1 is well dispersed, and all of the 126 cross-peaks in the protein can be unambiguously identified (Fig. 4A), implying that the protein in its native state is well structured (Fig. 4A). Comparison of the $^1$H-$^{15}$N HSQC spectrum of the native and the misfolded state(s) shows that the misfolded state(s) of the protein is primarily unstructured and that most of the $^1$H-$^{15}$N cross-peaks are located within a very narrow region of the spectrum (Fig. 4, B and C). These results suggest that refolding of nFGF-1 upon prolonged incubation in the denaturant results in entrapment of the protein in a conformational state(s) that has structural characteristics resembling that of the denatured state(s).

GroEL Prevents the Formation of the Misfolded State(s)—Molecular chaperones such as GroEL are known to aid proteins to fold to their native conformations by suppressing the accumulation of unproductive, off-pathway intermediates that lead to misfolding and aggregation of proteins (32–34). In this context, we were curious to investigate whether GroEL can rescue the protein (nFGF-1) from being trapped in the misfolded state(s) formed upon refolding the protein that was subjected to prolonged incubation in 8 M urea. Fluorescence and far-UV CD spectra of nFGF-1 refolded after 30 min of incubation in 8 M urea overlap well with those obtained for the native state of the protein (Fig. 5, A and B, curves a and c). However, the spectral features (far-UV CD and fluorescence) of the protein refolded after a prolonged incubation in the denaturant (>100 h) resembles that of the denatured state(s) (Fig. 5, A and B, curves b and d). These results corroborate well with those of the limited trypsin cleavage and NMR data, which suggest that the misfolded state has structural features resembling those of the denatured state(s). Interestingly, refolding nFGF-1 (incubated in 8 M urea for 100 h) in the presence of a 10-fold excess of GroEL and ATP (at 100-fold excess) resulted in the regaining of the native conformation by a majority (>85%) of protein molecules. Attainment of the native conformation of nFGF-1 is exemplified by the steady state fluorescence spectrum of the protein with an emission maximum at 308 nm (Fig. 5A, curve e) and a far-UV CD spectrum with a positive ellipticity band centered at 228 nm (Fig. 5B, curve e), reminiscent of the native β-barrel structure in the protein. It should be mentioned that refolding initiated in the presence of 10-fold excess of GroEL (but in the absence of ATP) yielded only 10% of the protein in the native state (data not shown). These results suggest that GroEL can successfully rescue nFGF-1 from being entrapped in the misfolded state(s) in an energy-dependent manner. Steady state fluorescence and circular dichroism data only provide a gross picture of the conformational state(s) of a protein. There-
FIG. 4. $^1$H-$^15$N HSQC spectra of nFGF-1 (at 25 °C). A, native state of nFGF-1 (single letter amino acid abbreviations are used with position numbers). B, misfolded state of nFGF-1. C, denatured state of nFGF-1 in 8 M urea. D, nFGF-1 refolded from the 8 M urea-denatured state in the presence of a 10-fold excess of GroEL and ATP (at 100-fold excess). The concentration of the nFGF-1 used was 0.1 mM. All solutions were prepared in 10 mM phosphate buffer (pH 7.2) containing 100 mM NaCl.

FIG. 5. A, fluorescence spectra of nFGF-1 (at 25 °C) in its native conformation (curve a) and denatured state(s) (curve b) in 8 M urea. Curves c and d represent the fluorescence spectra of nFGF-1 refolded after 30 min of incubation (curve c) and 100 h of incubation (curve d) of the protein in 8 M urea. Curve e shows the emission spectrum of nFGF-1 refolded in the presence of a 10-fold excess of GroEL and ATP (at 100-fold excess) after 100 h of incubation in 8 M urea. The final concentration of protein used is 25 µg/ml. B, far-UV CD spectra of nFGF-1 (at 25 °C) in its native conformation (curve a) and its denatured state in 8 M urea (curve b); curves c and d show far-UV CD spectra of nFGF-1 refolded after 30 min (curve c) and 100 h (curve d) of incubation in 8 M urea, respectively. Curve e shows the emission spectrum of nFGF-1 refolded in the presence of a 10-fold excess of GroEL and a 100-fold excess of ATP after 100 h of incubation in 8 M urea. The final concentration of protein used was 100 µg/ml. All spectra are an average of 10 scans. All far-UV spectra were acquired using a 0.1-cm pathlength cell.
fore, it is important to verify whether the conformational state of nFGF-1 attained in the presence of GroEL is indeed the native conformation of the protein. In this context we monitored the GroEL-mediated refolding (in the presence of ATP) of nFGF-1 using two-dimensional $^1$H-$^{15}$N HSQC spectroscopy. The $^1$H-$^{15}$N HSQC spectrum of nFGF-1, refolded in the presence of 10-fold excess of GroEL and ATP (at 100-fold excess), is well dispersed and matches quite well with that obtained for the native protein (Fig. 4, A and D). These results unambiguously show that GroEL thwarts the formation of the misfolded protein and consequently guides the protein to the native conformation. The exact mechanism by which GroEL prevents the formation of misfolded state(s) is not clear. It appears that GroEL binds to one of the intermediates populated in the very early stages of the non-productive folding pathway(s) and re-directs the protein to the folding track to yield the native state.

Possible Mechanism(s) Underlying the Misfolding of nFGF-1—The non-productive pathway(s) leading to the formation of the misfolded state(s) appears to be the predominant pathway for the protein (nFGF-1) refolded after prolonged incubation in 8 mM urea. The commitment to adapt the non-productive folding pathway(s) appears to occur in the denatured state ensemble. The $^1$H-$^{15}$N HSQC spectrum of nFGF-1 acquired after 30 min of incubation in 8 mM urea resembles that of a typical denatured state(s) (Fig. 6A). All of the cross-peaks are distributed in a very narrow region of the $^1$H-$^{15}$N HSQC spectrum (8.0 to 7.0 ppm). Several new cross-peaks begin to appear (in the $^1$H-$^{15}$N HSQC spectra) when the protein is incubated in 8 M urea beyond 40 h (Fig. 6A). The intensities of the new cross-peaks are observed to increase with time, suggesting that subtle conformational change(s) occur in the protein upon prolonged incubation in the denaturant (Fig. 6B) (35). These results suggest that nFGF-1 exists in at least two conformational states (D1 state and D2 state) in 8 mM urea (Fig. 7). Prolonged incubation of the protein in 8 mM urea appears to cause an irreversible

FIG. 6. A, $^1$H-$^{15}$N HSQC spectra of nFGF-1 in 8 mM urea after 30 min (cross-peaks shown in green) and 100 h of incubation (cross-peaks shown in red) in 8 mM urea at 25 °C. The non-overlapped cross peaks in the $^1$H-$^{15}$N HSQC spectrum acquired after 100 h of incubation in 8 mM urea are boxed. The concentration of protein used was 0.1 mM. Denatured protein solutions were prepared in 10 mM phosphate buffer (pH 7.2) containing 8 mM urea-d$_4$ and 100 mM NaCl prepared in 90% H$_2$O and 10% D$_2$O. B, time-dependent increase in the intensity of a $^1$H-$^{15}$N cross-peak (boxed in blue) in the $^1$H-$^{15}$N HSQC spectrum shown in panel A) that appears upon prolonged incubation of the protein in 8 mM urea (at 25 °C).

FIG. 7. Schematic representation of the conformational transitions that occur in the urea-induced equilibrium unfolding of nFGF-1. Urea-induced unfolding is completely reversible if the refolding from the denatured (D$_1$) state to the native (N) state is initiated within 60 min of incubation in 8 mM urea. The D$_2$ state is the product of prolonged incubation (>100 h) of the protein in 8 mM urea. It appears that the denatured state(s) ensemble (D$_1$) converts irreversibly to the D$_2$ state(s), which is committed to the unproductive pathway(s) leading to the formation of the misfolded state(s). The appearance of new $^1$H-$^{15}$N cross-peaks observed in the HSQC spectrum (shown in Fig. 6A), acquired after 100 h of incubation of the protein in 8 mM urea, possibly suggest a subtle conformational transition(s) in the denatured (D$_1$ state to D$_2$ state) ensemble. GroEL (in the presence of ATP) appears to bind to one of the early intermediates in the off-pathway (originating from D$_2$ state) and re-channelize the protein to refold to its native state.
conversion from the denatured state ensemble (D1) to another ensemble of denatured states (D2). Such multiple conformational states in the denatured state ensemble have also been reported in Staphylococcal nuclease (12). Refolding initiated from one of these conformational states (D1 state) in nFGF is productive and results in the formation of the native state (Fig. 7). On the other hand, the other conformational state (D2 state) appears to be a committed step for the unproductive pathway(s) leading to the formation of the misfolded state(s) (Fig. 7). The conversion of the D1 state to the D2 state appears to be irreversible and under a kinetic control. The fraction of protein molecules adapting the D2 conformation appears to increase with the increase in the time of incubation of the protein in the denaturant (Fig. 7).

It is reasonable to expect that the residual structure(s) constituting the D1 and D2 states are different. The D1 to D2 conformational transition could either represent a trans-to-cis isomerization of one or more proline residues in the protein or represent a stabilization of new hydrophobic cores formed upon prolonged incubation in the denaturant. The possibility of the existence of prolines in the cis configuration can be ruled out, as refolding initiated in the presence of cis-trans proline isomerase did not have a significant effect on the percentage of recovery of the native state (data not shown). Therefore, it appears that the D1 to D2 state transition is due to time-dependent restructuring of residual hydrophobic cores in the denatured state(s) of the protein. Once formed, the D2 state probably directs the protein to follow the unproductive pathway(s) leading to the formation of the misfolded state. GroEL appears to prevent the formation of the misfolded state by binding to early intermediates in the off-pathway (originating from the D2 state) and re-channelizing the protein to the folding track(s) to yield the native state (Fig. 7). At the present juncture, the mechanism proposed is purely speculative, and more experimental evidence such as the detailed structural characterization of the denatured state(s) is required to confirm the proposal.

To our knowledge, this study is the first report of time-dependent conformational changes in the denatured state(s). We believe that this unusual folding phenomenon observed in nFGF-1 may also be found in other proteins. The results of the study unambiguously suggest that the commitment to unproductive folding pathways leading to aggregation/misfolding occurs in the denatured state(s) ensemble.

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