Identification of Rare Partially Unfolded States in Equilibrium with the Native Conformation in an All β-Barrel Protein*

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Human acidic fibroblast growth factor 1 (hFGF-1) is an all β-barrel protein, and the secondary structural elements in the protein include 12 antiparallel β-strands arranged into a β-trefoil fold. In the present study, we investigate the stability of hFGF-1 by hydrogen-deuterium exchange as a function of urea concentration. Urea-induced equilibrium unfolding of hFGF-1 monitored by fluorescence and CD spectroscopy suggests that the protein unfolds by a two-state (native to denatured) mechanism. Hydrogen exchange in hFGF-1, under the experimental conditions used, occurs by the EX2 mechanism. In contrast to the equilibrium unfolding events monitored by optical probes, native state hydrogen exchange data show that the β-trefoil architecture of hFGF-1 does not behave as a single cooperative unit. There are at least two structurally independent units with differing stabilities in hFGF-1. β-Strands I, II, III, VI, VII, X, XI, and XII fit into the global unfolding isotherm. By contrast, residues in β-strands IV, V, VIII, and IX exchange by the subfolding isotherm and could be responsible for the occurrence of high-energy partially unfolded state(s) in hFGF-1. There appears to be a broad continuum of stabilities among the four β-strands (β-strands IV, V, VIII, and IX) constituting the subglobular folding unit. The slow exchanging residues in hFGF-1 do not represent the folding nucleus of the protein.

The mechanism by which an unstructured polypeptide chain regains its unique three-dimensional structure remains one of the main puzzles in modern biology (1–6). The transient nature of the kinetic intermediates in the folding reaction eludes their detection by many techniques (7–10). Nevertheless, it is becoming increasingly evident that structural information is a prerequisite to formulate mechanisms of protein folding. One approach to obtain such information would be characterizing the structures of intermediate states populated in the equilibrium unfolding pathways of proteins (11–17). This exercise appears meaningful because residual structures in equilibrium intermediates are often related to that observed in kinetic intermediates (18–20).

The lack of evidence for a detectable intermediate in the folding/unfolding process is generally assumed to imply that the process is cooperative or two-state (native ↔ unfolded, Refs. 21–23). Observation of same single exponential kinetics using two different spectroscopic probes, such as CD and fluorescence, is the usual criteria for a two-state process (24–26). However, it is well known that the conventional optical probes used are inadequate to detect small populations of intermediate states that exist at higher energy levels, which are swamped by signals from the abundant native state (27–29). In contrast, hydrogen-deuterium (H/D)1 exchange measurements can identify some of those native-like conformers because it has the advantage that native state does not always contribute to the measurement because it is a H/D exchange-incompetent state with regard to the majority of the slowly exchanging amide protons. Hence, H/D exchange experiments could provide valuable clues about the ruggedness of the energy landscape and may be useful to characterize partially unfolded states (30–36). Native state H/D exchange has been successfully used to detect partially unfolded states within the native state of several proteins including cytochrome c (27), RNase H (37), and T4 lysozyme (38).

Human acidic fibroblast growth factor 1 (hFGF-1) is a potent mitogen and is involved in the regulation of key cellular processes such as angiogenesis, morphogenesis, and differentiation (39, 40). hFGF-1 is a ~16-kDa, all β-sheet protein devoid of disulfide bonds (41–43). The secondary structural elements in hFGF-1 include 12 β-strands arranged into a β-trefoil architecture (Fig. 1). The β-trefoil structure of hFGF-1 consists of three sequential trefoil units, each comprising four β-strands (Fig. 1; Refs. 40 and 44–46). Interestingly, the β-trefoil structure is one of the fundamental protein folds adopted by several protein families including Kunitz soybean trypsin inhibitors, ricin-like toxins, plant agglutinins, and histactophilin-like actin-binding proteins (47–49). Therefore, a detailed understanding of the stability and folding of hFGF-1 may help gain insights into the structural determinants in the folding of all β-trefoil proteins.

In the present study, we investigate the stability of hFGF-1 by native state H/D exchange as a function of denaturant. The data obtained have allowed the local, subglobal, and global structures of hFGF-1 to be dissected. In addition, the results obtained indicate that the β-trefoil structure of hFGF-1 does not behave as a single cooperative unit.

MATERIALS AND METHODS

Heparin-Sepharose was obtained from Amersham Biosciences. Labeled [13]NH4Cl and 99.9% D2O were purchased from Cambridge Isotope Laboratories. d4-urea was purchased from Sigma. All other chemicals used were of high quality analytical grade. All experiments were performed at 25 °C.

Expression and Purification of [15]N-Labeled hFGF-1—Residues are numbered as per their position in the primary structure of the 154-amino acid hFGF-1. The expression vector for the truncated form of the

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1 The abbreviations used are: H/D, hydrogen-deuterium; hFGF-1, human acidic fibroblast growth factor 1; HSQC, heteronuclear single quantum coherence.
FIG. 1. MOLSCRIPT representation of the backbone folding of hFGF-1. The secondary structural elements in hFGF-1 include 12 β-strands arranged into a β-trefoil architecture. The β-strands in the protein are labeled with Roman numerals.

hFGF-1 (residues 15–154) was constructed and inserted between the NdeI and BamHI restriction sites in pET20b (+). Escherichia coli BL21(DE3)pLysS harboring pET20b (+)-hFGF-1 was cultured in minimal medium containing 15NH4Cl. Recombinant protein was purified on heparin-Sepharose using gradient (0–1.5 mM NaCl). Protein expression yields were in the range of 25–30 mg/L. The extent of 15N labeling was verified by electron spray mass analysis.

Equilibrium Unfolding—Urea-induced unfolding of hFGF-1 was performed by fluorescence spectroscopy at 2.5 nm resolution using an excitation wavelength of 280 nm (using a Hitachi F-2500 spectrofluorometer) and far-UV CD (at 228 nm) spectroscopy (using a Jasco J720 spectropolarimeter). All CD measurements were carried out using a quartz cell of 2 mm pathlength. 25 and 250 μg/ml hFGF-1 in 100 mM phosphate buffer (pD 6.1) in D2O containing 200 mM ammonium sulfate were used for the unfolding experiments monitored by fluorescence and far-UV CD, respectively.

NMR Spectroscopy of hFGF-1 at Varying Concentrations of Urea—NMR sample of hFGF-1 contained 1.5 mM protein in 100 mM phosphate buffer and 200 mM ammonium sulfate in 90% H2O/10% D2O at pH 6.1. 15N HSQC spectra of hFGF-1 in 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.5, 3.0, 3.5, and 4.0 M urea were collected on a Bruker DMX600 spectrometer. Each spectrum was collected with 8 transients of 2,048 complex points and 64 t1 increments.

Measuring Amide Proton Exchange Rates—Protein solution was prepared in 100 mM phosphate buffer and 200 mM ammonium sulfate at pH 6.0 and pH 7.0. The sample was concentrated to ~1.5 mM by ultrafiltration (Millipore) and dried by lyophilization. H/D exchange was initiated by dissolving dry protein in 10 mM phosphate-buffered D2O containing appropriate concentrations of d1-urea (0–1 mM d1-urea at 0.1 mM interval). The maximum shift observed in pD values upon addition of d1-urea is an indication of the accumulation of intermediate species at the exchange reaction. The dependence of unfolding free energy on urea concentration is expressed as the equation ΔGex(den) = ΔGex(0) – m[urea], where ΔGex(0) is the stabilization free energy at zero denaturant concentration, and the slope, m, depends on the denaturant binding surface newly exposed in the unfolding reaction. The m value for each residue was obtained by fitting the linear part of the plot of ΔG(den) as a function of the urea concentration.

RESULTS AND DISCUSSION

Equilibrium Unfolding of hFGF-1—The thermodynamic stability of hFGF-1 (in D2O) using urea denaturation curves is monitored by steady state fluorescence (based on 350/308 nm fluorescence changes) and far-UV circular dichroism (using ellipticity changes at 228 nm) spectroscopy. The urea-induced unfolding of hFGF-1 at pH 6.0 is completely reversible. The unfolding of hFGF-1 monitored by fluorescence spectroscopy shows that the protein unfolds completely beyond 4.0 M urea (Fig. 2). The Cm (concentration of urea at which 50% of the molecules exist in the unfolded state(s)) and m (a measure of the cooperativity of the unfolding reaction) are estimated to be 2.79 ± 0.07 M and 1.80 ± 0.02 kcal/mol·deg⁻¹, respectively. The free energy difference (ΔG(D2O)) between the folded and unfolded states of hFGF-1 in D2O is 5.00 ± 0.09 kcal/mol⁻¹. Within experimental error(s), the unfolding profile of hFGF-1 monitored by fluorescence and the ellipticity change at 228 nm superimpose quite well, implying that the denaturation of the protein is a cooperative process without the accumulation of stable equilibrium intermediate(s) (Fig. 2). We recently investigated the chronology of events in the refolding pathway of hFGF-1 from its urea-unfolded state(s) (50). The Chevron plot (natural logarithm of the unfolding/refolding rate constant) versus the denaturant concentration shows a prominent curvature at low concentrations of urea (Fig. 3). In general, a deviation from linearity (“roll over”) is an indication of the accumulation of intermediate species at low denaturant concentrations. These results suggest that although intermediates are not detected under equilibrium conditions, the refolding of hFGF-1 appears to proceed via the formation of kinetic intermediates.
Native state H/D exchange has been successfully used to identify rare partially unfolded conformations of six proteins, namely, RNase A (51), RNase II (37), barstar (52), cytochrome c (27), apocytochrome b$_{562}$ (53), and histactophilin (54). Native state H/D exchange monitored by NMR spectroscopy could help in distinguishing regions of differing stability (in proteins) and consequently yield information about the structural transitions occurring within the population of protein molecules (34, 35). It is in this context that we monitored the H/D exchange of hFGF-1 by acquiring a series of $^1$H-$^1$H HSQC spectra (at various time periods) in the absence and presence of subdenaturing amounts of urea.

**Dynamics of the Native State—**The $^1$H-$^1$N HSQC spectrum of hFGF-1 is well dispersed, and all the cross-peaks in the spectrum have been unambiguously assigned (Fig. 4A). The H/D exchange rates of 74 of 120 amide protons in hFGF-1 could be measured at pH 6.0 at 25 °C. 46 main-chain amide protons exchange out within dead time of measurement (20 min), and most of them correspond to residues located in the unstructured regions of the hFGF-1 molecule (Table I). In all, 11 residues exchange very fast, with rate constants ($k_{ex}$) greater than $1 \times 10^{-2}$ s$^{-1}$. 37 residues mostly belonging to $\beta$-strands III, VII, VIII, IX, X, and XI exhibit moderately fast exchange rates ($2 \times 10^{-4}$ min$^{-1} < k_{ex} < 1 \times 10^{-2}$ min$^{-1}$). Eight residues including Arg$^{38}$, Ile$^{39}$, and Leu$^{40}$ (located in $\beta$-strand II), Tyr$^{78}$, Leu$^{79}$, and Ala$^{80}$ (constituting $\beta$-strand VI), Leu$^{106}$ (in $\beta$-strand VII), and Leu$^{160}$ (in $\beta$-strand VIII) are strongly protected ($k_{ex} < 7 \times 10^{-5}$ min$^{-1}$) from exchange (Table I). In particular, Ala$^{80}$ shows the largest protection factor (Fig. 4B, $P = 1.45 \times 10^6$). The amide proton of Ala$^{80}$ is involved in a hydrogen bond with Tyr$^{38}$ located in $\beta$-strand VII. This hydrogen bond favors the development of a hydrophobic cluster comprised of residues in $\beta$-strands VI and VII. The amide proton of Ala$^{80}$ is located in the middle of the non-polar cluster and hence is protected from solvent exchange.

There are prominent differences in the average protection factors of the various $\beta$-strands constituting the structure of hFGF-1. Among the 12 $\beta$-strands in hFGF-1, $\beta$-strands IV and XI exhibit the least average protection factor values (Fig. 5). The higher susceptibility of residues in $\beta$-strand XI (to H/D exchange) is consistent with the solution structure of hFGF-1 determined by NMR spectroscopy. The $\beta$-trefoil structure of hFGF-1 in solution is shown to contain only 11 $\beta$-strands (45), instead of the 12 $\beta$-strands depicted in the crystal structure (44). The observed discrepancy between the solution and crystal structures could be attributed to the higher flexibility of residues in $\beta$-strand XI in solution, which precludes the observation of intramolecular nuclear Overhauser effects characterizing $\beta$-strand XI (45). $^{15}$N chemical shift perturbation data obtained upon titration of hFGF-1 with structural analogs of heparin have shown that residues in $\beta$-strand XI are involved in the heparin binding site (46). It appears that the higher flexibility of residues in $\beta$-strand XI possibly favors complex formation with the proteoglycan (heparin sulfate) by lowering the free energy barrier. Similarly, higher flexibility of residues in $\beta$-strand IV (as indicated by the low average protection factor value) also appears to bear physiological relevance. Crystal structure of fibroblast growth factor receptor-ligand (fibroblast growth factor 1) complex reveals that residues in $\beta$-strand IV interact with the D2 extracellular domain of the fibroblast growth factor receptor (55). The higher flexibility of residues in $\beta$-strand IV possibly increases the solvent-accessible surface area (in this portion of hFGF-1 molecule) and consequently enhances the probability of binding and optimization (decrease in the free energy of interaction) of the ligand to the receptor.

$\beta$-Strands II and VI exhibit significantly large average protection factor values (Fig. 5). Residues in $\beta$-strand VI are lodged in a hydrophobic core that is stabilized by a hydrogen bond between Ala$^{80}$NH and Tyr$^{88}$CO. The dense hydrophobic cluster formed by residues in $\beta$-strand VI insulates their amide protons from solvent exchange. Similarly, the high average protection factor of $\beta$-strand II primarily stems from the network of hydrogen bonds between Leu$^{37}$NH and Leu$^{28}$CO and between Leu$^{148}$NH and Leu$^{27}$CO. These hydrogen bonds forge a hydrophobic cluster consisting of Leu$^{27}$, Leu$^{28}$, Tyr$^{29}$, Phe$^{36}$, Leu$^{37}$, Ile$^{39}$, Leu$^{147}$, and Leu$^{149}$. The results discussed so
far clearly suggest that the 12 β-strands do not contribute equally toward the structural stability of hFGF-1. Residues in β-strands II and VI appear to constitute the stable core of the protein.

EX2 Versus EX1 Kinetics—EX2 is generally the dominating exchange mechanism for backbone amide protons in proteins. However, under extreme pH or temperature conditions or in the presence of high denaturant concentrations, the H/D exchange is dominated by the EX1 mechanism (32). A direct test of the EX2 condition is to vary the pH at which exchange is carried out and confirm that the change in observed H/D exchange rate is as predicted by the corresponding change in $k_{ex}$ (34). Such a test was carried in hFGF-1 at two pH conditions (pD 6.0 and 7.0). Logarithms of amide proton exchange rates obtained at pD 6.0 and 7.0 showed a linear correlation (slope, 0.9) with a correlation coefficient of 0.91 (data not shown), thus validating the assumption that H/D exchange in hFGF-1 under the given conditions (between pD 6.0 and 7.0) occurs predominately by the EX2 mechanism.

H/D Exchange in the Presence of Urea—The effect of urea on 1H–15N HSQC spectra of hFGF-1 is monitored at concentrations of the denaturant at or below 2.0 M urea (Fig. 6). However, to avoid errors arising due to viscosity effect(s) on hydrogen exchange rates, $ΔG_{ex}$ (free energy of exchange) of various amide protons was estimated from the H/D exchange data acquired at or below 1.0 M urea. We could unambiguously monitor the H/D exchange of 74 backbone amide protons (of 120 total backbone amide protons) in the protein. We could not reliably monitor the H/D exchange of hFGF-1 beyond 2.0 M urea because the 1H–15N spectra begin to collapse, presumably due to significant interconversion of the folded and unfolded states of the protein.

The framework within which to relate dependence of H/D exchange rates to the nature of physical motions of the protein underlying the H/D exchange event is well established (27, 34, 37). The denaturant exchange rate is proposed to be directly related to the stabilization of the exchange-competent state relative to the native (exchange incompetent) state by increase in the accessible surface area available for binding of the denaturant. The relationship between unfolding free energy

| Rate constant ($k_{ex}$) range | Residues |
|--------------------------------|----------|
| $k_{ex} < 7 \times 10^{-5}$ min$^{-1}$ | R38, I39, L40, Y78, L79, A80, L86, L100 |
| $7 \times 10^{-5}$ min$^{-1} < k_{ex} < 2 \times 10^{-4}$ min$^{-1}$ | L28, L37, L58, V68, Y69, I70, S72, D82, G85, F99, I112, F122, V123, A143, I144, L145, F146, L147 |
| $2 \times 10^{-4}$ min$^{-1} < k_{ex} < 1 \times 10^{-2}$ min$^{-1}$ | K26, C30, N32, F36, G43, T44, V45, D46, G47, T48, I56, Q57, Q59, E67, K71, L87, Y88, G89, S90, C97, L98, E101, R102, N109, T110, Y111, S113, K114, W121, G124, L125, K126, S130, K132, G134, R136, L147 |
| $k_{ex} > 1 \times 10^{-2}$ min$^{-1}$ | H35, D53, H55, Q77, S61, D84, E104, Y108, G129, T137, V151 |
| Residues that disappeared in the first 20 min of exchange | K24, S21, G23, G34, D42, R49, R51, S52, Q54, L60, E63, S64, V65, G66, T73, E74, T75, G76, M81, T83, Q91, T92, N94, E95, E96, L103, E105, N106, H107, K115, H116, A117, E118, K119, N120, K127, N128, C131, R133, H138, Y139, G140, K142, S152, S153, D154 |

**Fig. 5.** Average protection factors of various β-strands in hFGF-1. The exchange rates of β-strands II and VI are relatively higher than those of the other β-strands in the protein.

**Fig. 6.** 1H–15N HSQC spectra of hFGF-1 in the absence (A) and presence of 2.0 M urea (B). The presence of 2.0 M urea does not appear to affect the chemical shifts (1H and 15N) of residues in the protein, implying that no gross conformational changes occur in the protein in the presence of the denaturant (2.0 M urea).
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**TABLE II**

| $m$ value | Residues |
|-----------|----------|
| Local ($m < 0.37$ kcal mol$^{-1}$ M$^{-1}$) | N32, H35, G43, T44, V45, D53, H55, I56, Q57, S61, E67, Q77, D84, L87, C97, E101, R102, E104, G129, S130, R139, T137, L149, V151 |
| Subglobal ($0.37 < m < 1.7$ kcal mol$^{-1}$ M$^{-1}$) | L68, Q59, V68, Y69, K71, D82, G89, S90, L98, F99, L100, Y108, N109, T110, K114, W121, K132, G134, L147 |
| Global ($m > 1.7$ kcal mol$^{-1}$ M$^{-1}$) | K26, L28, C30, F36, L37, R38, I39, L40, D46, G47, T48, I70, S72, Y73, L79, A80, G85, L86, Y88, Y111, I112, S113, F122, V123, G124, L125, K126, A143, I144, L145, F146 |

**FIG. 7**. Urea concentration dependence of free energy of H/D exchange ($\Delta G_{ex}$) illustrated for selected amide protons. $\Delta G_{ex}$ of Leu$^{97}$ (○) and Glu$^{101}$ (●) shows a linear relationship with the denaturant concentration, implying that these residues exchange by the global unfolding isotherm. Leu$^{87}$ (○) and Glu$^{101}$ (●) show very little change in the $\Delta G_{ex}$ values up to 1.0 M urea, indicating that these residues exchange by local unfolding. By contrast, the $m$ (the slope) values of residues Leu$^{147}$ (■) and Lys$^{114}$ (▲) are similar (0.78 ± 0.08 kcal mol$^{-1}$ M$^{-1}$) and are intermediate to those of residues that exchange by global and local unfolding mechanisms. These residues represent the subglobal unfolding isotherm and suggest the presence of partially unfolded states in the equilibrium unfolding pathway of hFGF-1.

change and the denaturant concentration is linear ($\Delta G_{ex}(den) = \Delta G_{ex}(0) - m den$) (56, 57), and the slope $m$ depends on the denaturant binding surface newly exposed to the unfolding reaction and is reflected in the exchange behavior of the slowly exchanging hydrogen. Thus, different physical motions leading to H/D exchange-competent states would lead to states that result in different associated changes in the accessible surface area. Because the $m$ value is a measure of the degree of denaturant-accessible surface area, an estimation of this parameter is expected to provide information on the nature of physical motions in a particular state(s) of the protein (22, 23). In principle, amide protons participating in a common motion will exhibit $m$ values in a common range. Residues that expose little new accessible surface area (upon unfolding) predominately exchange by various local structural fluctuations and exhibit near zero or smaller $m$ values. These amide protons can be categorized to local unfolding. By contrast, amide protons that show strong dependence on denaturant concentration and exhibit large $m$ values undergo cooperative unfolding. In general, all the faster-exchanging amide protons are controlled by the global unfolding reaction as denaturant concentration is increased. If the unfolding of the protein is noncooperative involving the accumulation of partially unfolded state(s), residues with $m$ values greater than these of local openings but smaller than the $m$ values dictated by the global unfolding can be detected (27). The residues that exhibit $m$ values in the intermediate range (between local and global isotherms) fit into the subglobal isotherm of exchange. Amide protons that converge to form each isotherm help to identify the cooperative unfolding unit(s) that exposes those residues to exchange (27).

Global, Local, and Subglobal Unfolding in hFGF-1—The change in accessible surface area ($\Delta ASA$) is related to the $m$ value by the equation $m = (\text{cal} \cdot \text{mol}^{-1} \cdot \text{m}^{-1}) = 0.11 \Delta ASA (\AA^2) + 374$ (58). Hence, if there is no change in the accessible surface area upon addition of the denaturant, the minimum $m$ value for the protein is expected to be 0.374 kcal mol$^{-1}$ M$^{-1}$. This value serves as a guideline to categorize the $m$ values (estimated from native state exchange experiments) of various amide protons into three groups, such as $m \leq 0.38$ (local unfolding), $0.38 < m < 1.7$ (subglobal unfolding), and $m \geq 1.7$ (global unfolding). 24 of the 74 amide protons whose exchange could be reliably followed at various concentrations of urea were found to exchange by local unfolding (Table II). Most of the residues that follow the local unfolding isotherm are those involved in local hydrogen bonding and are located in the unstructured loop regions of the molecule. In addition, several residues located in β-strands such as His$^{35}$ (β-strand II), Thr$^{44}$ and Val$^{45}$ (β-strand III), Gln$^{57}$ and Ser$^{61}$ (β-strand IV), Glu$^{57}$ (β-strand V), Gln$^{77}$ (β-strand VI), Leu$^{86}$ (β-strand VII), Cys$^{97}$, Glu$^{101}$, and Arg$^{102}$ (β-strand VIII), Ser$^{130}$ (β-strand X), and Leu$^{147}$ and Leu$^{149}$ (β-strand XII) show $m$ values <0.38 kcal mol$^{-1}$ M$^{-1}$ at low concentration of urea (<0.4 M), implying that local motions in these regions leading to H/D exchange do not produce significant exposure of the buried surface. Local fluctuations exhibited by some of these residues (His$^{35}$, His$^{45}$, Ile$^{56}$, Leu$^{87}$, Cys$^{97}$, Arg$^{102}$, and Ser$^{130}$) involve large $\Delta G_{ex}$ values for produc-
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**Fig. 9.** Schematic representation of the arrangement of the 12 β-strands in the three limbs of the β-trefoil structure of hFGF-1. The boxed triangular region represents β-strands (β-strands IV, V, VIII, and IX) in which residues predominately exchange by the subglobal isotherm and represent an independent cooperative unit.


tive H/D exchange because in the transition region of global unfolding (beyond ~2 M urea), the slope of the ΔGex curves for these amide protons matches the global m value (Fig. 7). These results suggest that the protein surface exposed associated with equilibrium global unfolding. The free energy of exchange of many amide protons shows a linear dependence on the denaturant concentration (Fig. 8). Within experimental uncertainties, the m value of these amide protons agrees well with the value (~1.8 kcal mol⁻¹ M⁻¹) estimated based on global equilibrium unfolding experiments using steady state fluorescence and CD (Fig. 8). Most of the amide protons of residues located in β-strands I, II, III, VI, VII, X, XI, and XII exchange by the global unfolding isotherm (Fig. 7; Table II). Interestingly, β-strands I, II, III, and XII together constitute one limb (limb A) of the β-trefoil structure of hFGF-1 (Fig. 9). Except for two weak hydrogen bonds between Val123NH/Tyr111CO and Trp121NH/Ser113CO, amide protons of residues in β-strand X (which fit into the global unfolding isotherm) have hydrogen partners only in β-strand XI (Fig. 9). Residues in β-strand XI have no hydrogen bond interactions with the other two β-strands (β-strands VIII and IX) in limb C. Therefore, β-strands X and XI mostly exist as an independent structural unit within limb C of the β-trefoil structure (Fig. 9).

Several H/D exchange isotherms in hFGF-1 mirror the behavior observed for the global unfolding isoform but exhibit serially decreasing values for ΔGex and m (Fig. 8). This aspect reflects the presence of partially unfolded states. Critical analysis of the residues depicting m values in the range of 0.38–1.7 shows that several residues in β-strand IV, β-strand V, β-strand VIII, and β-strand IX fit into the subglobal isotherm (Figs. 7 and 8). The four β-strands (β-strands IV, V, VIII, and IX) do not behave as a single cooperative unit. Although residues within an individual β-strand show common m values, the average m value of each of these four β-strands (β-strands IV, V, VIII, and IX) falls within a broad range (m = 0.511–1.42 kcal mol⁻¹ M⁻¹). Similar variability in individual elements of secondary structure has been reported for RNase H (37) and lysozyme T4 (38). Therefore, the distributions of ΔGex and m values in hFGF-1 are consistent with a large manifold of energy levels, or an energy landscape (4). In such a framework, ΔGex and m parameters represent averages, weighted over the population of excited states. Although an energy landscape could be either rugged or smooth and featureless, the nonuniform distribution of ΔGex and m values as a function of the amino acid sequence of the protein (hFGF-1) suggests that the energy landscape of hFGF-1 is rugged. The observed continuum of m values in hFGF-1 suggests that assignment of discrete partially unfolding forms may be an overly simplistic interpretation of the data (Fig. 8). In summary, the results discussed so far clearly show that β-strand IV, β-strand V, β-strand VIII, and β-strand IX behave as independent cooperative units and thus represent a broad continuum of high energy states.

**Discrepancies between Free Energy Change of H/D Exchange (ΔGex) and Free Energy Change of Unfolding (ΔGf)—** The ΔGex values obtained from fluorescence and CD are about 2.7–3.0 kcal mol⁻¹ lower than the largest ΔGex values in hFGF-1. Such large discrepancies have also been reported for many other proteins. In RNase A, the ΔGex is 1.6 kcal mol⁻¹ larger than the ΔGf measured by fluorescence and calorimetry (51). Similarly, in peptostreptococcal protein L (22) and cytochrome c (27) and CI2 (30), the ΔGex values are about 2 kcal mol⁻¹ higher than the corresponding values from fluorescence. A number of factors could account for the observed differences (59, 34). ΔGex can be enhanced if the residual structure persists in the denatured state protects the amide protons from exchange. The existence of residual structures in denatured state(s) has been demonstrated in hFGF-1 (42). Another factor that could account for the discrepancy between ΔGex and ΔGf is the occurrence of cis-trans proline isomerization (34). The slow cis-trans isomerization of the prolyl peptide bond in the un-
folded state(s) will lead to a discrepancy between $\Delta G_{\text{unf}}$ and $\Delta G_{\text{a}}$ because an equilibrium between cis and trans forms is reached in the calorimetric or thermal/denaturant-induced unfolding measurements (using optical probes), but not in the H/D exchange experiment (34, 59). Assuming that the cis-trans ratio is 1:4 in the unfolded state, the $\Delta G_{\text{unf}}$ should be reduced by 0.5 kcal mol$^{-1}$ for each of the six trans prolines in the native structured state of hFGF-1. This gives a correlation of 3 kcal mol$^{-1}$ to the estimated $\Delta G_{\text{unf}}$ and hence could significantly account for the observed discrepancy between the estimated $\Delta G_{\text{unf}}$ and $\Delta G_{\text{a}}$ values. In addition, the nonlinear dependence of $\Delta G_{\text{a}}$ on denaturant as denaturant concentration approaches zero is likely to be underestimated by extrapolation from data obtained at higher denaturant concentration near the transition zone (22). Lastly, the dissimilarity of the $\Delta G_{\text{unf}}$ and $\Delta G_{\text{a}}$ could also be due to the overestimation of the $k_{\text{on}}$ value based on poly-$\beta$-alanine. In this background, the difference(s) between $\Delta G_{\text{unf}}$ and $\Delta G_{\text{a}}$ values observed in hFGF-1 could arise due to any one or more of the reasons discussed above.

**Relationship with Folding Events**—The order of sequential unfolding events from the native to the unfolded state should be matched in the reverse refolding process from the unfolded to the native state. This logic, when extended to the native state H/D exchange experiments, essentially means that the unfolding pathway of proteins approximates the reverse order of the native state H/D exchange rates, i.e. the most slowly exchanging proton might identify the first part of the protein to fold—“last out, first in” (60). This correlation is found to be valid to a large extent in several proteins such as bovine pancreatic trypsin inhibitor (60), lysozyme (61), cytochrome c (62), RNase T1 (63), and cardiotinin analog III (64). However, there are examples in which there is no obvious relationship between H/D exchange at equilibrium and the chronology of events in the kinetic refolding pathway(s) (65). In this context, we examined the correlation between the degrees of protection of amide protons against hydrogen and the sequence of kinetic events in hFGF-1 detected by quenched-flow H/D exchange (Fig. 10).

The refolding kinetics of hFGF-1 has been recently studied using a variety of biophysical techniques including quenched-flow H/D exchange monitored by NMR spectroscopy (50). hFGF-1, like other $\beta$-trefoil proteins, folds slowly, and complete refolding of the proteins takes about 100 s. Refolding of hFGF-1 proceeds via the formation of transient kinetic intermediates. Quenched-flow H/D exchange experiments reveal that the earliest detectable event is the formation of hydrogen bonds among residues at the NH$_2$- and COOH-terminal ends of the molecule (50). Among the 12 $\beta$-strands in hFGF-1, $\beta$-strands I, IV, IX, and X form very early during the refolding of the protein and provide the basic $\beta$-trefoil framework. There is no obvious correlation between the average $\Delta G_{\text{a}}$ of various $\beta$-strands (derived from the native state hydrogen-deuterium exchange data) and the corresponding average refolding time constants (of the $\beta$-strands) estimated from the quenched-flow data (Fig. 10). $\beta$-Strand VI, which has the greatest average $\Delta G_{\text{a}}$ value, is formed late during the refolding of the protein. Similarly, $\beta$-strands I, IV, IX, and X, which form very early in the kinetic refolding pathway, exhibit low average $\Delta G_{\text{a}}$ values. In addition, amide protons of many residues that are protected rapidly (within 200 ms of refolding) in the quenched-flow H/D exchange show weak protection against exchange. These results suggest that at least in hFGF-1, the protein folding core does not appear to be a subset of the slow-exchange core.

**Folding Cooperativity in Other $\beta$-Trefoil Proteins**—It is generally believed that proteins with $\beta$-trefoil architecture unfold/ refold cooperatively without intermediates (54). However, available experimental data on $\beta$-trefoil proteins invalidate such cooperativity.
Identification of Partially Unfolded States

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