Characterization of the Structure and Dynamics of a Near-native Equilibrium Intermediate in the Unfolding Pathway of an All β-Barrel Protein*

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The structure and dynamics of equilibrium intermediate in the unfolding pathway of the human acidic fibroblast growth factor (hFGF-1) are investigated using a variety of biophysical techniques including multidimensional NMR spectroscopy. Guanidinium hydrochloride (GdnHCl)-induced unfolding of hFGF-1 proceeds with the accumulation of a stable intermediate state. The transition from the intermediate state to the unfolded state(s) is cooperative without the accumulation of additional intermediate(s). The intermediate state induced maximally in 0.96 M GdnHCl is found to be obligatory in the folding/unfolding pathway of hFGF-1. Most of the native tertiary structure interactions are preserved in the intermediate state. 1H,15N chemical shift perturbation data suggest that the residues in the C-terminal segment including those located in the β-strands IX, X, and XI undergo the most discernible structural change(s) in the intermediate state in 0.96 M GdnHCl. hFGF-1 in the intermediate state (0.96 M GdnHCl) does not bind to its ligand, sucrose octasulfate. Limited proteolytic digestion experiments and hydrogen-deuterium exchange monitored by 15N heteronuclear single quantum coherence (HSQC) spectra show that the conformational flexibility of the protein in the intermediate state is significantly higher than in the native conformation. 15N spin relaxation experiments show that many residues located in β-strands IX, X, and XI exhibit conformational motions in the micro- to millisecond time scale. Analysis of 15N relaxation data in conjunction with the amide proton exchange kinetics suggests that residues in the β-strands II, VIII, and XII possibly constitute the stability core of the protein in the near-native intermediate state.

Understanding the mechanism by which a disordered polypeptide chain folds to a unique three-dimensional structure is one of the major challenges in molecular structural biology (1–4). It is elucidated that folding of moderate size proteins (>10-kDa) proceeds along well defined intermediates similar to a chemical reaction (5–9). Formation of partially folded intermediate states during the protein-folding process is believed to aid in avoiding search of large conformation space on the energy landscape (10, 11). In addition to their role in protein folding, partially structured intermediates are proposed to be involved in a number of biological processes such as interaction with molecular chaperones, translocation across biological membranes, formation of amyloid, and dissociation of supramolecular complexes (12–14). Thus, investigation of the structural features of equilibrium folding/unfolding intermediates would not only enhance our understanding of the mechanism of protein folding but is also expected to provide strong clues on the interplay of molecular forces in many natural and disease-related processes.

Dynamic information about a protein on different time and length scales is important to gain useful knowledge on the mechanism of the protein folding process (15–17). In addition, investigation of the conformational dynamics in the partially structured state(s) can provide valuable information about the interaction potential energy landscape, which is crucial for understanding protein stability and rationalizing protein design (18). In this context, NMR spectroscopy is an apt technique to probe protein folding landscape, because it provides a unique opportunity to study the conformational dynamics of unfolded, partially folded, and native state(s) at the level of individual amino acids (3, 19–21). Amide proton exchange kinetics and 15N-relaxation measurements (using NMR) have been successfully used to probe the structural and dynamic features of partially structured intermediate state(s) of proteins (17, 22–25).

Acidic human fibroblast growth factor (hFGF-1) is a 16-kDa, all β-sheet protein, devoid of disulfide bonds. The secondary structural elements in the protein include 12 β-strands arranged antiparallel into a β-barrel architecture (Fig. 1 (26–31)). hFGF-1 is a potent mitogen and plays crucial roles in important cellular processes such as morphogenesis, development, angiogenesis, and wound healing (32). hFGF-1 lacks secretion signal sequence (upstream of the N terminus), which is crucial for insertion and translocation across cell membrane(s) (33, 34). Hence, the mechanism by which hFGF-1 (synthesized in the cytosol) transports itself across the cell membrane to bind to its cell surface receptor is still an enigma. Wiedlocha et al. (35) proposed that hFGF-1 under physiological conditions exists in a partially unfolded state, which promotes its facile transport across cell membrane. Middaugh and co-workers (36, 37) showed that hFGF-1 exists in a partially structured state(s) that exhibits high affinity to bind to negatively charged phospholipid vesicles. Recently, Samuel et al. (38) identified and

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characterized an obligatorily partially unfolded intermediate state in the GdnHCl unfolding pathway of hFGF-1. In the present study, we investigate the structure and dynamic features of this obligatory intermediate state of hFGF-1 using a variety of techniques including multidimensional NMR.

MATERIALS AND METHODS

Ingredients for Luria Broth were obtained from AMRESCO. Aprotinin, leupeptin, phenylmethylsulfonyl fluoride, Triton X-100, 1-anilino-8-naphthalene sulfonate (magnesium salt), and β-mercaptoethanol were obtained from Sigma. Guanidine hydrochloride (GdnHCl, ultra pure) was purchased from Merck. Heparin-Sepharose was procured from Amersham Biosciences. All other chemicals used were of high quality analytical grade. Unless specified, all solutions were made in 100 mM phosphate buffer.

Purification procedure was the same as that explained earlier (27). The expression host strain Escherichia coli BL21(DE3)pLys reconstituting the recombinant plasmid pET20a. Recombinant truncated human acidic fibroblast growth factor (with 21 residues at the N terminus deleted) expressed in E. coli was purified on a heparin-Sepharose affinity column using a stepwise sodium chloride gradient (0–0.85–1.5 M). Desalting of the purified protein was achieved by ultra-filtration using an Amicon (Amersham Biosciences) set-up. The purity of the protein was assessed using SDS-PAGE. The homogeneity was confirmed by electron spray mass analysis.

Preparation of Isootope-enriched FGF-1—

15N isotope labeling was achieved using M9 minimal medium containing 100 mg/liter 15NH4Cl. The expression host strain E. coli BL21(DE3)pLys is a vitamin B12-deficient host, and hence the medium was supplemented with thiamin (vitamin B1). The protein yields were in the range of 20–25 mg/liter. Purification procedure was the same as that explained earlier (27). The extent of labeling was verified by electron spray mass analysis, and the isotope incorporation was found to be more than 95%.

GdnHCl-induced Denaturation—Equilibrium unfolding of hFGF-1 was monitored by fluorescence and CD measurements as a function of GdnHCl concentration. Fluorescence spectra were measured on a Hitachi F-2500 fluorimeter 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 or at different temperatures using a Neslab RTE-110 circulating water bath.

CD spectra were measured using a Jasco J720 spectropolarimeter. CD spectra were collected with the slit width set to 1 nm, a response time of 1 s, and a scan speed of 20 nm/min. Each spectrum was an average of at least five scans. All far UV CD measurements were made using a 0.2-cm path length quartz cuvette. The protein concentration used for the far-UV CD measurements was 50 μM.

Limited Proteolytic Digestion—These experiments on the native and the intermediate (in 0.96 μM GdnHCl) states were carried out at (35 ± 2 °C) using trypsin. The proteolytic digestions were performed at an enzyme (trypsin) to substrate (hFGF-1) molar ratio of 1:100. The proteolytic activity was stopped by desired time interval by the addition of the gel loading dye (Bio-Rad) at 80 ± 2 °C. The degree of proteolytic cleavage was measured from the intensity of the ~16-kDa band corresponding to the uncleaved protein (hFGF-1) using a densitometer.

NMR Spectroscopy—All NMR experiments were carried out on a Bruker DMX 600 MHz spectrometer at 25 °C. An inverse probe with a self-shielded z-gradient was used to obtain all gradient-enhanced 1H-15N HSQC spectra (39). 15N decoupling during acquisition was accomplished using the GARP sequence (40). 2048 complex data points were collected in the 1H dimension of the 1H-15N HSQC experiments. In the indirect 15N dimension of the spectra, 512 complex data points were collected. The HSQC spectra were recorded at 32 scans at all concentrations of GdnHCl. The concentration of the protein sample used was 0.2 mM in 95% H2O and 5% D2O containing 100 mM phosphate and 100 mM ammonium sulfate. 15N chemical shifts were referenced using the consensus ratio of 0.0101329118 (41). All spectra were processed on a Silicon Graphics work station using XNMR and Aurelia software. The GdnHCl-induced unfolding was monitored by the disappearance of the cross-peaks (that solely correspond to the native state of the protein) using the procedure reported by Van Mieroel et al. (42). Cross-peak volumes instead of cross-peak intensities were determined to avoid artifacts arising (due to line broadening effect with increase in the viscosity of the solvent) upon addition of GdnHCl.

15N Relaxation Measurements—15N longitudinal transverse relaxation and NOE data were collected on Varian Inova 500 MHz and Bruker DMX 600 MHz NMR spectrometers. Protein samples for relaxation measurements were dissolved at a concentration of ~1.5 mM in 100 mM phosphate buffer at pH 6.0 (containing 100 mM ammonium sulfate) prepared using 10% D2O and 90% H2O. The intermediate state samples were prepared by rapidly exchanging hFGF-1 with 100 mM phosphate buffer containing 100 mM ammonium sulfate and 0.96 μM GdnHCl, pH 6.0, using a Centriplus ultra-filtration set-up.

15N longitudinal (R1) and transverse (R2) relaxation rates and heteronuclear 1H-15N NOE were measured using two-dimensional 1H-15N HSQC spectra (39). The HSQC spectra were recorded at 32 scans at all concentrations of GdnHCl. The concentration of the protein sample used was 0.2 mM in 95% H2O and 5% D2O containing 100 mM phosphate and 100 mM ammonium sulfate. 15N chemical shifts were referenced using the consensus ratio of 0.0101329118 (41). All spectra were processed on a Silicon Graphics work station using XNMR and Aurelia software. The GdnHCl-induced unfolding was monitored by the disappearance of the cross-peaks (that solely correspond to the native state of the protein) using the procedure reported by Van Mieroel et al. (42). Cross-peak volumes instead of cross-peak intensities were determined to avoid artifacts arising (due to line broadening effect with increase in the viscosity of the solvent) upon addition of GdnHCl.

Data Processing and Analysis—All spectra were processed identically on a Silicon Graphics work station using the AURELIA and UXNMR software. The peak intensities in the two-dimensions of the spectra with and without proton presaturation were determined from the peak picking subroutines. The R1 and R2 values were determined by fitting the peak intensities as a function of the relaxation interval (t) to a two-parameter (I0 and R1) exponential decay function, I = I0 exp (–R1t), using a non-linear least squares analysis (44). The average uncertainty values of R1 were determined from several data sets. The steady-state NOE values at 500 MHz were determined form the ratios of the peak intensities with and without presaturation. The standard deviation of the NOE was determined from the root mean square value of the background noise in the spectra. In all, three separate NOE data sets were collected at both 500 and 600 MHz.
MHz. Analysis of R1, R2, and NOE data followed the procedure outlined by Mandel et al. (45).

Hydrogen-Deuterium (H/D) Exchange—The native hFGF-1 was lyophilized (1.5 mM in 0.5 ml of the buffer containing 100 mM phosphate, 100 mM ammonium sulfate, pH 6.0) and dissolved in 0.5 ml of D2O, immediately prior to data collection. Sample (in the intermediate state) for the H/D exchange experiment was prepared by repeatedly exchanging the protein with buffer containing 100 mM phosphate, 100 mM ammonium sulfate, 0.96 M GdnHCl (pH 6.0), using an Amicon set-up. The sample volume was reduced to 0.5 ml, lyophilized, and redissolved in 0.5 ml of D2O just before data acquisition.

1H-15N HSQC at different time points were collected on Varian Inova 500 MHz spectrometer. The time needed to dissolve the sample and collect the first spectrum was 40 min. For the native hFGF-1, 1H-15N HSQC spectra were collected continuously for 3 days for every 40 min. Since the exchange was almost complete in 540 min, in the intermediate state, data collection was stopped after 600 min of exchange. Amide proton delays were followed by measuring peak intensities in 1H-15N HSQC spectra. The intensities of the cross-peaks in the HSQC spectra collected at various refolding times were normalized based on the peak height of the Ile-70 methyl protons (at 0.2 ppm) in the one-dimensional 1H NMR spectra collected each time prior to acquiring the HSQC spectra. The time courses of change in proton occupancies were fitted to a single exponential decay (y = A exp(-kt) + C, where A is the amplitude of the phase, k is the apparent exchange rate constant, and C is the final amplitude) using the Levenberg-Marquardt non-linear least squares method. All data analysis was performed using Kaleidagraph software (Synergy software).

RESULTS AND DISCUSSION

Accumulation of an Equilibrium Unfolding Intermediate State(s)

The GdnHCl-induced unfolding of hFGF-1 is non-cooperative (38). GdnHCl-induced unfolding of hFGF-1 was monitored by steady-state fluorescence (based on the 350/308 nm fluorescence changes) and far-UV CD (using ellipticity changes at 228 nm). These spectral probes have been shown to reliably report the gross conformational changes occurring during the unfolding of hFGF-1 (26, 27, 34–38). The GdnHCl-induced unfolding curves monitored by fluorescence and far-UV CD spectroscopy do not superimpose, implying that the unfolding process is non-cooperative, involving the accumulation of equilibrium intermediate state(s) (Fig. 2) (38, 46–50).
ANS is a popular hydrophobic dye to detect folding/unfolding intermediates with solvent-exposed hydrophobic surfaces (51, 52). GdnHCl-induced unfolding of hFGF-1 monitored by changes in ANS fluorescence revealed that a stable equilibrium intermediate state(s) accumulates maximally at 0.96 M GdnHCl (Fig. 3). The ANS emission intensity (at 520 nm) of the protein (hFGF-1) in the presence of 0.96 M GdnHCl is at least twice that observed upon binding to the protein in the native conformation (in the absence of GdnHCl). In addition, the dye (ANS) upon binding to the protein (hFGF-1) in the intermediate state shows a 30 nm blue shift in the emission maxima (Fig. 3, 520 to 490 nm). These results clearly suggest that a stable equilibrium intermediate with non-polar solvent-exposed hydrophobic surfaces accumulates maximally in 0.96 M GdnHCl.

**Co-operativity of the Intermediate State to the Unfolded State(s) Transition State**

It would be interesting to examine if the transition from the intermediate state (populated maximally in 0.96 M GdnHCl) to the denatured state(s) involves accumulation of any other equilibrium intermediates. In this context, we studied the unfolding of the 0.96 M intermediate state to the denatured state(s) using fluorescence (the 350/308 nm emission) and far-UV CD (228 nm ellipticity changes) spectroscopy. The GdnHCl-induced unfolding curves of the protein (in the intermediate state) monitored by these spectroscopic probes are superimposable implying that the transition from the intermediate state (accumulated at 0.96 M GdnHCl) to the denatured state(s) is cooperative, and no additional intermediates populate between these two states of the protein (Fig. 4, panel A).

Thermal denaturation of native hFGF-1 probed by fluorescence and far-UV CD reveals that the unfolding profiles obtained (using these two spectroscopic techniques) are non-superimposable (Fig. 4, panel B), suggesting that the unfolding of the protein proceeds via the accumulation of stable intermediate(s). The apparent $T_m$ (temperature at which 50% of the protein molecules exist in the unfolded state) values estimated from the unfolding profiles monitored by the changes in the 350/308 nm fluorescence and 228 nm ellipticity are 51 ± 0.6 and 56 ± 0.8 °C, respectively. Interestingly, thermal unfolding curves of hFGF-1 in 0.96 M GdnHCl (monitored by steady-state fluorescence and far UV CD) are nearly superimposable, indicating that no stable intermediate(s) accumulate in the temperature-induced unfolding pathway of hFGF-1 in 0.96 M GdnHCl (Fig. 4, panel C). Similar observations were made recently by Blaber et al. (53), who showed that the thermal denaturation of hFGF-1 is reversible and cooperative only in the presence of low concentrations (<1 M) of GdnHCl. The irreversibility of the thermal unfolding process (in the absence of low concentrations of GdnHCl) is proposed to primarily stem from the accumulation and subsequent aggregation caused due to the coalescence of the stable intermediate (53). Although it is difficult to judge if the intermediate realized in 0.96 M GdnHCl shares common structural features with the one(s) accumulated in the thermal-induced unfolding pathway, it may not be far-fetched to believe that the GdnHCl and temperature-induced unfolding of hFGF-1 mandatory proceeds via the formation of intermediate state(s) possessing conformational properties resembling the native state.

**Structural Changes Induced by GdnHCl**

Although fluorescence and CD spectroscopy provide valuable data on the structural transitions occurring during the unfolding of the protein, they yield limited information on the local folding/unfolding of a protein. NMR spectroscopy on the other hand enables the study of the conformational changes in the

![FIG. 4. Unfolding profiles of hFGF-1 monitored by steady-state fluorescence (open squares) and far-UV CD (closed circles) spectroscopy. Panel A depicts the GdnHCl-induced conformational transitions (unfolding) of hFGF-1 from the intermediate state (in 0.96 M GdnHCl) to the unfolded state(s). The fraction-unfolded species was estimated by assuming the fraction of unfolded species in 0.96 M GdnHCl as zero. Panels B and C show the thermal unfolding profiles of hFGF-1 in the presence and absence of 0.96 M GdnHCl, respectively. It appears that the intermediate state induced maximally in 0.96 M GdnHCl is an obligatory intermediate in the unfolding/folding pathway of hFGF-1. Thermal unfolding experiments were carried out in 10 mM phosphate buffer (pH 7) containing 100 mM ammonium sulfate.](https://example.com/fig4.png)
to the depletion of the population of the native species (as the concentration of the denaturant increases). It is pertinent to mention that all the $^{1}$H-$^{15}$N HSQC spectra are corrected for the GdnHCl concentration-dependent reduction in the NMR receiver coil quality factor (42). The spectra acquired at and beyond 1.5 M GdnHCl show limited chemical shift dispersion in

![Fig. 5. $^{1}$H-$^{15}$N HSQC spectra of hFGF-1 at various concentrations of GdnHCl (panel A). The cross-peaks, which undergo maximal chemical shift perturbation, are indicated in red. The lower sub-panel in panel A shows the $^{1}$H-$^{15}$N chemical shift perturbation of hFGF-1 in the intermediate state (in 0.96 M GdnHCl). Most prominent chemical shift perturbation occurs in the C-terminal heparin binding region, such as the $\beta$-strands VIII, IX, and XI, respectively. Panel B depicts the GdnHCl induced unfolding profiles of hFGF-1 (at the individual amino acid level) monitored by changes in the $^{1}$H-$^{15}$N cross-peak volume. Unfolding profiles of residues such as Gly-33 and Ile-130 show a clear three-stage transition with a plateau between 0.5 and 1.0. In contrast, the unfolding curves of residues such as Ile-56 and Gln-63 appear to represent a two-state (native state to unfolded state) transition.](image-url)
the 1H dimension and is typical for an unfolded protein (Fig. 5, panel A).

The GdnHCl-induced unfolding at the individual amino acid residue level was monitored by the change in the 1H-15N cross-peak volume. Site-specific change(s) in cross-peak volume could be monitored for 72 of 126 HSQC cross-peaks. These cross-peaks are isolated and mostly distributed uniformly all over the 1H-15N HSQC spectrum. The profiles obtained by monitoring the change(s) in the cross-peak volume are sigmoidal and resemble typical denaturant-induced protein unfolding curves (Fig. 5). About 44 residues located in the protein show curves reminiscent of a three-state unfolding process (Fig. 5, panel B, and Table I). These residues show a prominent plateau in the GdnHCl concentration range of 0.8 to 1.0 M indicative of the accumulation of a stable intermediate species (Fig. 5, panel A). About 28 residues show a profile which resembles a two-state transition (Fig. 5, panel B, and Table I). Interestingly, several other residues in the protein show unfolding curves resembling a two-state transition (Fig. 5, panel B, and Table I). The non-uniform unfolding patterns of various residues suggest that hFGF-1 undergoes non-cooperative unfolding in GdnHCl.

Most of the cross-peaks in the 1H-15N HSQC spectrum at 0.96 M GdnHCl remain unperturbed. Many residues show composite 1H-15N chemical shift perturbation of less than 0.1 ppm. The majority of the residues that show chemical shift perturbation exceeding 0.1 ppm are located in the C-terminal heparin binding region spanning β-strands IX–XI. In addition, some other residues such as Lys-9, Lys-10, His-21, Phe-22, Tyr-64, Gly-75, Glu-90, and His-93 also show chemical shift perturbation greater than 0.1 ppm. These results reveal that the inter-

### Table I

| Classification of residue level unfolding profiles monitored by changes in the 1H-15N cross-peak volumes at various concentrations of GdnHCl |
|----------------------------------------------------------|
| Residues showing two-state (one step) unfolding curves   |
| H14, S17, I25, V31, H41, I42, G52, Y55, T66, Q63, L65, A66, T69, S76, L84, L86, E87, E91, Y97, H98, K105, G110, G115, T123, L133, S138 |
| Residues showing three-state (two steps) unfolding curves |
| K10, K12, Y15, C16, N18, G20, H21, R24, L26, G29, D32, G33, T34, Q40, S47, G62, G75, T78, E90, N95, T96, S99, K100, H102, W107, P108, V109, K112, S116, C117, G120, R122, Y125, G126, Q127, K128, A129, I130, L131, F132, L135, V137, S139, D140 |

**Fig. 6.** Time-dependent limited trypsin digestion of hFGF-1 in the native (closed circles) state and the intermediate (open squares) state (in 0.96 M GdnHCl). The inset shows the SDS-PAGE of molecular weight markers (lane M); hFGF-1 not treated with trypsin (lane 1, control); lanes 2 and 3 depict hFGF-1 incubated with trypsin (for 10 min) in the absence and presence of 0.96 M GdnHCl, respectively. The percentage of cleavage was estimated from the intensity of the ~16-kDa band representing uncleaved hFGF-1. Control experiments with bovine serum albumin revealed that the higher susceptibility of the protein (hFGF-1) to trypsin cleavage in 0.96 M GdnHCl is not a general denaturant effect.

**Fig. 7.** The time-dependent change(s) in the number of cross-peaks detected in the 1H-15N HSQC spectra of the hFGF-1 in the presence (open circles) and absence (closed circles) of 0.96 M GdnHCl. The conformational flexibility of the protein in the intermediate state is significantly higher than in the native state.

#### Conformational Flexibility of the Near-native Intermediate State

**Limited Proteolytic Digestion**—Limited proteolytic digestion is a popular technique to probe the gross conformational flexibility of proteins (54). Proteolytic digestion, in general, is governed not only by stereochemistry and accessibility of the protein substrate but also by the specificity of the proteolytic enzyme. Hence, subtle conformational changes could be easily detected by the limited proteolytic digestion technique. As hFGF-1 is rich in arginine and lysine residues, we opted to perform a limited trypsin digestion to probe the conformational flexibility of the protein in the native and the intermediate state induced in 0.96 M GdnHCl. Time-dependent trypsin digestion of the protein (in the native and intermediate state(s)) was monitored by SDS-PAGE analysis. The degree of digestion was measured based on the intensity (after Coomassie Blue staining) of the ~16-kDa band (on the polyacrylamide gel) corresponding to the undigested protein (hFGF-1). The curve depicting the degree of proteolytic cleavage clearly shows that the protein in the intermediate state is highly susceptible to the enzymatic cleavage (Fig. 6). In the intermediate state, nearly 25% of the protein (16-kDa band) is cleaved within a time span of 3 min (Fig. 6). In contrast, the protein in the native state is relatively more resistant to trypsin cleavage, and less than 2% of the protein (in the native state) is cleaved after 3 min of incubation with the enzyme (Fig. 6). These results suggest that although the gross structures of the native and intermediate (induced in 0.96 M GdnHCl) states are similar, the conformational flexibility of the protein in the intermediate state is significantly higher than in the native state.

**Amide Proton Exchange**—Measurement of the rates of H/D exchange...
exchange of backbone amide protons in proteins is a powerful tool for investigating protein folding, stability, and dynamics at atomic resolution (55, 56). In this context, we measured the amide proton exchange kinetics of hFGF-1 using NMR spectroscopy in the native and 0.96 M GdnHCl-induced intermediate states based on a series of $^{1}$$H-^{15}N$ HSQC collected for a period of 72 h (at 40 min interval). 71 of the expected 126 cross-peaks survive exchange in the first $^{1}$$H-^{15}N$ HSQC spectrum of the native state acquired after 40 min of initiation of exchange (Fig. 7). In contrast, only 62 cross-peaks are observed (after 40 min of exchange) in the HSQC spectrum of the intermediate state (Fig. 7). The intensities of the cross-peaks in the intermediate are significantly lower than those observed in the $^{1}$$H-^{15}N$ HSQC spectra of the native state. In the intermediate state, except for the five cross-peaks corresponding to Val-54, Leu-26, Gly-126, Ile-130, and Leu-131, amide protons of all other residues are completely exchanged out within 10 h. In comparison, 56 intense cross-peaks could be noticed in the $^{1}$$H-^{15}N$ HSQC spectrum of the native state collected after 10 h of the exchange (Fig. 7). These results strongly suggest that the conformational flexibilities of the hFGF-1 in the native and near-native intermediate (induced in 0.96 M GdnHCl) state are significantly different.

The degree of protection of residues in the various $\beta$-strands in the native and intermediate states is distinctly different (Fig. 8). For example, residues in the $\beta$ strands IV, IX, X, and XI, which are strongly protected in the native state, show high degree of exchange in the intermediate state. This observation is quite consistent with the $^{15}N$ chemical shift perturbation data, which show that residues in the C-terminal segments (in $\beta$ strands IX–XI) undergo most prominent structural changes in the intermediate state of the protein (Fig. 8) (38). In contrast, the average proton occupancy of residues in $\beta$-strands, I, II, V, VIII, and XII are relatively higher than the other $\beta$-strands in the protein in the intermediate state (Fig. 8, panel B). In addition, three residues (Lys-128, Ala-129, and Ile-130), which are located in the C-terminal region in the loop region connecting $\beta$-strands XI and XII (and involved in hydrogen bonding with residues in the N-terminal), show strong protection against exchange in the intermediate state. Thus, it appears that the hydrogen bonds linking the residues in the N and C termini, which are crucial for the maintenance of the $\beta$-barrel architecture of hFGF-1, are preserved in the intermediate state.

Backbone Dynamics in the Intermediate State

Heteronuclear NMR is a powerful tool to understand the intramolecular conformational dynamics (3, 43, 57). In particular, slow protein dynamics on the microsecond or millisecond time scale can be quantified explicitly by $^{15}N$ transverse relaxation rate ($R_2$) experiments using the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (58–61). In general, quantitative measurement of millisecond time scale dynamics of equilibrium folding intermediates is difficult due to overlap in the $^{1}$$H-^{15}N$ HSQC spectra. However, as the $^{1}$$H-^{15}N$ HSQC spectrum of hFGF-1 in the near-native intermediate state is well dispersed with minimal reduction in the signal sensitivity, it is feasible to understand the slow dynamics (microsecond to milliseconds regime) in the intermediate state (induced in 0.96 M GdnHCl).

Relaxation data, $R_1$, $R_2$, and heteronuclear NOE were obtained for 110 and 100 $^{15}N-^{1}H$ resonances (out of the expected 126 $^{15}N-^{1}H$ resonances) in the native and intermediate state(s) of hFGF-1, respectively. $^{15}N$ relaxation data were collected at 25 °C at 500 and 600 MHz field strengths. In all cases, the decay intensity for all residues is found to be strictly exponential for both the $R_1$ and $R_2$ data.

The backbone dynamics of hFGF-1 in the native state has been investigated previously (58). In this study, we were particularly interested in understanding the trends in the transverse relaxation rates ($R_2$) in the native and intermediate states, as they are sensitive to the dynamics on the microsecond-millisecond time scale. The $R_2$ values in the native state of hFGF-1 are uniform for most of the molecule, with a mean value of 15.06 ± 2.9 s$^{-1}$ at 600 MHz (Fig. 9, panel A). There are at least 20 residues in native state with $R_2$ relaxation values greater than the trimmed mean average. The most prominent among them are Gly-20, Gln-40, His-41, His-93, Lys-101, Glu-90, Trp-107 and Leu-133. In contrast, several other residues at the N and C termini show $R_2$ values lower than the trimmed average value (Fig. 9, panel A).

The average $R_2$ relaxation rate of the protein in the intermediate state is 19.4 ± 0.86 s$^{-1}$. In most cases, the $R_2$ values of residues in the intermediate state are higher than in the native state (Fig. 9, panel A). There are several residues, which show $R_2$ relaxation rate values greater than the trimmed average. Most notable among them are Lys-12, Lys-13, Ser-38, Asn-80, His-41, Ser-47, Val-54, Tyr-55, Gln-63, Met-67, Leu-84, Lys-91, His-102, Trp-107, Gly-110, Gly-126, and Ala-129 (Fig. 9, panel B). Trp-107 and Ala-129 show extraordinarily large $R_2$ values of 33.1 ± 0.65 and 27.5 ± 0.15 s$^{-1}$, respectively. Similarly several residues, such as Ile-56 (9.49 ± 0.935 s$^{-1}$), Leu-26 (9.5 ± 0.72 s$^{-1}$), Gly-29 (13.7 ± 0.29 s$^{-1}$), Ile-56 (13.8 ± 0.355 s$^{-1}$), Leu-65 (14.1 ± 0.6 s$^{-1}$), Asn-80 (10.2 ± 0.97 s$^{-1}$), Tyr-125 (12.6 ± 0.27 s$^{-1}$), and Asp-140 (10.3 ± 0.115 s$^{-1}$) in the intermediate state display $R_2$ relaxation rates significantly lower than the average trimmed $R_2$ rate.

In general, residues displaying low $R_2$ values are believed to be involved in internal motions in the nano- to picosecond time scale. In this background, comparison of the residue-wise $R_2$
relaxation rates of hFGF-1 in its native and intermediate states reveals that a number of residues showing internal motions in the nano- to picosecond time scale are common in the two conformational states (native and intermediate states). The notable exceptions are Tyr-64 (in β-strand VI), Thr-96 (in β-strand IX), and Phe-108 (in β-strand X) and Ser-138 (in β-strand XII), which exhibit dynamics in the nano- to picosecond time scale in the native state, which show $R_2$ rates higher than the trimmed average in the intermediate state. A similar analysis of the residues exhibiting exceptionally large $R_2$ could not be provided because anomalously large $R_2$ rates could either due to large contributions from conformational exchange dynamics ($R_{ex}$) on the micro- to millisecond time scale or due to anisotropic rotational diffusion of the protein. It should be mentioned that the heteronuclear NOE and $R_1$ relaxation rates almost show similar trends (in the native and intermediate states) as the $R_2$ relaxation rates (data not shown).

As neither the dimensions nor the global topology of the intermediate state (in hFGF-1) have been established, the orientation of the NH vector relative to the principal axes of motion of the structure cannot be defined. Under these circumstances, as a first approximation, rotational tumbling was assumed to be effectively isotropic. Van Mierlo et al. (59) and Alexanderscu and Shortle (60) optimized the correlation times both globally and locally to indirectly test this assumption for isotropic tumbling and demonstrated that this approximation is reasonable. The overall correlation time ($\tau_w$) calculated in the present study for each individual $^{15}$N nucleus from $R_2/R_1$.

**Fig. 9.** $^{15}$N spin relaxation measurements ($R_x$ and $R_{xy}$) in the native (N) and the intermediate (I) states of hFGF-1. Panel A shows the transverse relaxation rates ($R_x$) of residues in hFGF-1 in the native and intermediate state(s). The conformational exchange factor ($R_{ex}$) values of various residues in hFGF-1 in the native (N) and intermediate (I) state are plotted in panel B. Many residues in β-strands IX, X, and XI in the intermediate state are observed to undergo motions in the micro- to millisecond time regimen.
ratios are in the similar range throughout the protein backbone (in both the native and the intermediate states), suggesting that the overall tumbling of the molecule is isotropic. The residues contributing to the anisotropic motion could be easily recognized because any deviation from the average $R_2/R_1$ ratio caused by motional anisotropy is expected to affect both $R_1$ and $R_2$ in opposite directions. Hence, for calculation of the optimized $r_m$ we excluded all residues wherein the $R_2$ value exceeds the average $R_2$ by 1 S.D. The optimized $r_m$ estimated for the native and intermediate state are $10.4 \pm 1.0$ and $11.7 \pm 0.71$ ns, respectively. The marginal increase in the $r_m$ value of the intermediate state could be attributed to the increase in viscosity of the solvent due to the presence of $0.96 \text{ M GdnHCl}$.

In general, higher $R_{1\alpha}$ values are indicative of line broadening due to conformational exchange in the micro- to millisecond time regime. A rigorous interpretation of the $R_{1\alpha}$ is complicated as this parameter is a function of not only the rate constant for exchange but also the chemical shift differences and fractional population at the exchanging sites (61). As a rule of thumb, the residues undergoing conformational exchange averaging ($R_{1\alpha}$) are expected to exhibit larger $R_1$ values without concomitant increase in the $R_1$ values. In the native state, 21 residues fit into this criteria and could be attributed to slow motions on the micro- to millisecond time scale. Of these only 8 residues display an $R_{1\alpha}$ value greater than 4.0. These residues are spread throughout the hFGF-1 molecule. The residues exhibiting high $R_{1\alpha}$ values possibly reflect slow global interconversions between folded conformations and not local order/disorder transitions. In contrast, in the intermediate state 51 residues exhibit $R_{1\alpha}$ values greater than 4.0. The majority of the residues depicting high $R_{1\alpha}$ ($R_{1\alpha} > 6.0$) in the intermediate state are located in the unstructured portions of the molecule. In addition, most of the residues located in $\beta$-strands IX–XI also display significantly large $R_{1\alpha}$ values implying that they undergo slow micro- to millisecond time scale of these only 8 residues.

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**Ligand Binding Affinity in the Intermediate State**

hFGF-1, as mentioned earlier, is a potent mitogen and requires heparin for its cell proliferation activity (35–37). The stability of hFGF-1 is known to increase significantly upon binding to heparin or its structural analog, SOS (62). We compared the binding affinities of hFGF-1 in its native and the intermediate state, induced in $0.96 \text{ M GdnHCl}$ by equilibrium thermal unfolding experiments monitored by changes in the tryptophan fluorescence. hFGF-1 in the absence of SOS unfolds completely with a $T_m$ of $41 \pm 2$ °C (Fig. 10, panel A). However, in the presence of SOS, the protein is stabilized significantly, and $T_m$ increases by about $15$ °C (Fig. 10, panel A). In contrast, in the intermediate state (in $0.96 \text{ M GdnHCl}$), there is no appreciable difference in the $T_m$ value in the presence and absence of SOS (Fig. 10, panel B). These results suggest that hFGF-1 in the intermediate state (in $0.96 \text{ M GdnHCl}$) has weak or no affinity to bind to the ligand (SOS). It appears that the heparin-binding site located at the C-terminal end (of the

**Relevance of the Near-native Intermediate**

The results of the present study clearly demonstrate that hFGF-1 exists in a near-native obligatory intermediate state. Although the overall tertiary structure of the protein in the native and the intermediate states are quite similar, the conformational dynamics in these two states are observed to be significantly different. We realize that the condition under which the near-native intermediate is realized is far from those prevailing in the cell. However, based on the knowledge gained on the structural and dynamical features of the near-native intermediate, we are tempted to suggest a tentative model to explain the translocation of hFGF-1 across the cell membrane.

As mentioned earlier, hFGF-1 synthesized in the cell cytosol lacks a conventional signal sequence crucial for translocation across the cell membrane to reach its cell-surface receptor (34–36). In this background, it is possible that hFGF-1 synthesized on the ribosome upon reaching the cell membrane partially unfolds and adopts a structure similar to the near-native intermediate state described in this study. This proposal appears reasonable because the microenvironment in the vicinity of cell membranes is shown to be acidic and hence conducive for
inducing partial unfolding of the protein (63, 64). In the partially unfolded state, the protein is expected to possess solvent-exposed hydrophobic sites, which would provide a thermodynamically favorable environment for the protein to move across the cell membrane. It has been shown that partially unfolded state(s), such as the molten globule-like states, are involved in protein translocation across biomembranes (13, 48). Upon reaching the cell surface, the proteoglycans (like heparin/heparin sulfate) present abundantly on the cell membrane aid the protein (hFGF-1) to regain its native structure and consequently facilitate binding to its receptor. The proposal of translocation of hFGF-1 from the translocation-competent partially unfolded state to the native conformation upon binding to proteoglycans is supported by the work of Wiedlocha et al. (35), who demonstrated that binding of hFGF-1 to heparin or poly-anions inhibits membrane translocation of hFGF-1 fused to diphtheria toxin. Future work focused on the membrane binding affinity and membrane translocation properties of the near-native intermediate of hFGF-1 would help in validating our proposal. However, a more detailed study focused on the membrane binding affinity and membrane translocation properties of the near-native intermediate is required to validate our model for the membrane translocation of hFGF-1.

We have investigated recently (27) the chronology of kinetic events in the refolding pathway of hFGF-1. Refolding of hFGF-1 is observed to be non-cooperative involving the formation of transient kinetic intermediates. Refolding of hFGF-1 involves the formation of a late intermediate with high ANS binding affinity similar to the near-native equilibrium intermediate characterized in the present study. Thus, it appears that the formation of the near-native intermediate is an obligatory event in the folding/unfolding pathway(s) of hFGF-1. In this background, further characterization of the structure of the near-native intermediate using appropriate site-specific mutants would provide useful information on the folding landscape of hFGF-1.

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