Identification and Characterization of an Equilibrium Intermediate in the Unfolding Pathway of an All β-Barrel Protein*

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The guanidinium hydrochloride (GdnHCl)-induced unfolding of an all β-sheet protein, the human acidic fibroblast growth factor (hFGF-1), is studied using a variety of biophysical techniques including multidimensional NMR spectroscopy. The unfolding of hFGF-1 in GdnHCl is shown to involve the formation of a stable equilibrium intermediate. Size exclusion chromatography using fast protein liquid chromatography shows that the intermediate accumulates maximally at 0.96 M GdnHCl. 1-Anilinonaphthalene 8-sulfonate, FPLC, fast protein liquid chromatography; SEC, size exclusion chromatography; HSQC, heteronuclear single quantum coherence.

There is increasing evidence that the refolding of monomeric proteins in vitro proceeds along pathways involving the formation of partially folded, intermediate states. Characterization of the intermediate states is central to the understanding of protein stability and folding. Such intermediate states will provide useful information on the range of contexts in which the same amino acid sequence will fold into a stable structure and should reveal which interactions are driving the folding process.

In the present study, using various biophysical techniques including multidimensional NMR spectroscopy, we identify and characterize, a stable intermediate state in the GdnHCl-induced unfolding pathway of hFGF-1. To our knowledge, this study represents the first report of a detailed structural characterization of an equilibrium intermediate in the unfolding pathway of an all β-barrel protein.

MATERIALS AND METHODS

Heparin-Sepharose was obtained from Amersham Pharmacia Biotech. Labeled 15NH4Cl was purchased from Cambridge Isotope Laboratories. All other chemicals were of high quality analytical grade. Unless otherwise mentioned, all solutions were made in 100 mM phosphate buffer (pH 7.0) containing 100 mM of ammonium sulfate. All experiments were performed at 20 °C.

Protein Expression and Purification—Residues are numbered as per their position in the primary structure of the human acidic fibroblast growth factor (hFGF-1). Expression vector for the truncated form of the human FGF-1 (hFGF-1, residues 15–154) was constructed and inserted between the NdeI and BamHI restriction sites in pET20b. The plasmid containing the hFGF-1 insert was transformed into Escherichia coli BL21(DE3)pLysS. The expressed protein was purified on heparin-Sepharose column using a NaCl gradient (0–1.5 M). The protein was desalted by ultrafiltration using an Amicon set-up. The homogeneity of the protein was assessed using SDS-PAGE. The authenticity of the sample was further verified.
Operatively in urea.

Protein expression yields were in the range of 25–30 mg/liter of the isotope enriched medium. The extent of 15N labeling was achieved using M9 minimal medium containing 15NH4Cl. To realize maximal expression yields, the composition of the M9 medium was modified by the addition of a mixture mixture of vitamins. The expression host strain E. coli. BL21(DE3)pLysS is a vitamin B1-deficient host, and hence the medium was supplemented with thiamine (vitamin B1). Protein expression yields were in the range of 25–30 mg/liter of the isotopically enriched medium. The extent of 15N labeling was verified by electron spray mass analysis. The concentration of the protein was estimated from the extinction co-efficient value of the protein at 280 nm.

Preparation of Isotope-enriched hFGF-1—Uniform 15N isotope labeling was achieved using M9 minimal medium containing 15NH4Cl. To realize maximal expression yields, the composition of the M9 medium was modified by the addition of a mixture mixture of vitamins. The expression host strain E. coli. BL21(DE3)pLysS is a vitamin B1-deficient host, and hence the medium was supplemented with thiamine (vitamin B1). Protein expression yields were in the range of 25–30 mg/liter of the isotopically enriched medium. The extent of 15N labeling was verified by electron spray mass analysis.

Steady State Fluorescence Measurements—All fluorescence spectra were collected on a Hitachi F-2500 spectrofluorimeter at 2.5- or 10-nm resolution, using an excitation wavelength of 280 nm. Intrinsic fluorescence measurements were made at a protein concentration of 100 μg/ml. ANS binding affinity to hFGF-1 at various concentrations of the denaturant was monitored with the excitation wavelength set at 390 nm. The emission was monitored between 450 and 600 nm. The excitation and emission bandwidths were set at 5 nm. The concentration of the dye (ANS) and the protein used were 200 and 1 μM, respectively.

Circular Dichroism—All CD measurements were made on a Jasco J-720 spectropolarimeter using a 0.1-cm-pathlength quartz cell. Each spectrum was an average of five scans. The concentration of the protein used was 0.5 mg/ml. Necessary background corrections were made in all spectra.

Equilibrium Unfolding and Data Analysis—Equilibrium unfolding data obtained were converted to plots of F_u, fractions of the protein in the unfolded state, versus denaturant concentration using the following equation.

\[
P_u = (X_D - (X_U + m_U[D]))/[X_U + m_U[D]] - (X_U + m_U[D]) \quad (\text{Eq. 1})
\]

where \(X_u\) is the value of the spectroscopic property measured at denaturant concentration ([D]), \(X_D\) and \(X_U\) represent intercepts, and \(m_U\) and \(m_D\) are the slopes of the folded and unfolded base lines of the data, respectively, and were obtained from the linear least square fits of the base lines. In the case of urea-induced unfolding, a two-state (folded \(\leftrightarrow\) unfolded) transition was assumed, and the free energy of unfolding by the denaturant (\(G_u\)) at concentration ([D]) is related to \(F_u\), by transformation of the Gibbs-Helmholtz equation. It is assumed that the free energy of unfolding, \(\Delta G_u\), has a linear dependence on the concentration of the denaturant ([D]).

\[
\Delta G_u = \Delta G(H_2O) + m[D] \quad (\text{Eq. 2})
\]

where \(\Delta G(H_2O)\) and \(m\) are the intercept and slope, respectively, in the plot of \(\Delta G_u\) versus concentration of the denaturant. \(m\) is the measure of the co-operativity of the unfolding reaction and \(\Delta G(H_2O)\) is the difference in the free energy between the folded and unfolded states of the protein in the absence of any denaturant.

Size Exclusion Chromatography—All gel filtration experiments were carried out at 25 °C on a superdex-100 column using a AKTA FPLC device (Amersham Pharmacia Biotech). The column was equilibrated with 2 bed volumes of the buffer (10 mM phosphate buffer (pH 7.2) containing 100 mM of ammonium sulfate) containing appropriate concentrations of GdnHCl. The flow rate of the eluent was set at 1 ml/min. Protein peaks were detected by their 280 nm absorbance. The concentration of the protein used was about 1 mg/ml.

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Characterization of an Equilibrium Intermediate

Stopped Flow Fluorescence—Kinetic measurements of protein refolding or unfolding were performed using a SF-61 stopped flow spectrophotometer (Hi-Tech Scientific Co). For measuring changes in the intrinsic tryptophan fluorescence of Trp at different concentrations of GdnHCl, an excitation wavelength of 280 nm was routinely used with a monochromator slit width of 4 nm. All folding and unfolding reactions were performed at 25 °C. Unfolding reactions involved mixing of hFGF-1 with 10-fold excess of GdnHCl to yield a final protein concentration of about 1.0 μM. The kinetics associated with refolding involved mixing one volume of unfolded protein with 10 volumes of the refolding buffer (100 mM phosphate buffer (pH 7.2) containing 100 mM of ammonium sulfate). The kinetics data were analyzed by plotting the refolding and unfolding rates as a function of denaturant concentration in semilogarithmic plots (Chevron plots) as per the following equations.

\[
\ln k_u = \ln k_{uw} + m_u[D] \quad \text{(Eq. 3)}
\]

\[
\ln k_f = \ln k_{fw} + m_f[D] \quad \text{(Eq. 4)}
\]

where \( k_u \) and \( k_f \) represent the observed rate constants for the unfolding and refolding reactions in various concentrations of the denaturant ([D]), respectively. \( k_{uw} \) and \( k_{fw} \) are the rate constants of unfolding and refolding extrapolated to zero denaturant concentration. \( m_u \) and \( m_f \) are slopes of the unfolding and refolding reactions.

Proteolytic Digestion Assay—Digestion experiments were carried out by incubating hFGF-1 under appropriate buffer conditions with trypsin in a 1:50 ratio. The protease action was stopped by heating the mixture (protein + trypsin) at 90 °C for 10 min. The products of the protease reaction were analyzed by SDS-PAGE. The degree of cleavage was monitored by the intensity of the band corresponding to hFGF-1 (remaining after trypsin digestion) using a scanning densitometer. The intensity of the band corresponding to hFGF-1 was measured by estimating the intensity of the band (on SDS-PAGE) using a scanning densitometer. The intensity of the band corresponding to hFGF-1 was analyzed by SDS-PAGE. The degree of cleavage was analyzed by estimating the intensity of the band (on SDS-PAGE) using a scanning densitometer.

NMR Experiments—All NMR experiments were carried out on a Bruker DMX 600 MHz spectrometer at 20 °C. A 5 mm inverse probe with a self-shielded z-gradient was used to obtain all gradient-enhanced 1H-15N HSQC spectra (23, 24). 15N decoupling during acquisition was accomplished using the GARP sequence (25). 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 was 0.5 mM in 95% H2O and 5% D2O (containing 100 mM phosphate and 100 mM of ammonium sulfate). 15N chemical shifts were referenced using the consensus ratio of 0.0101329118 (26). All spectra were processed on a Silicon Graphics workstation using UXNMR and Aurelia software.

RESULTS AND DISCUSSION

GdnHCl-induced Unfolding of hFGF-1 Is Not a Two-state Process—The fluorescence spectrum of hFGF-1 is dominated by tyrosine emission at 308 nm (20). The emission of the lone tryptophan emission at position 121 is quenched by the presence of proximate positive charges in the three-dimensional structure of the protein. This quenching effect is completely relieved in the unfolded state of the protein, and the characteristic tryptophan emission is observed at 350 nm. These spectral features are ideal to monitor the conformational changes induced in the protein during the unfolding process. Unfolding of hFGF-1 monitored by steady state fluorescence reveals that the protein is completely unfolded beyond 1.8 M GdnHCl (Fig. 2). The unfolding process is found to be completely reversible. The GdnHCl-induced unfolding process probed by the ellipticity changes at 228 nm (a composite CD signal contributed by the secondary structural elements and optically active aromatic groups in the protein) shows that hFGF-1 undergoes complete unfolding only beyond 2.3 M GdnHCl (Fig. 2). The Cm (concentration of the denaturant at which 50% of the protein is unfolded) values of unfolding curves obtained from the CD and the fluorescence experiments

FIG. 5. Binding of ANS to hFGF-1 at various concentrations of GdnHCl. It could be observed that the emission changes at 520 nm are maximal at 0.96 M GdnHCl, implying that protein accumulates maximally in the MG-like state at this concentration (0.96 M) of the denaturant. The inset depicts the emission spectra of the protein in the native (N), intermediate (I), and unfolded (U) states. Note that the formation of the I state is accompanied by blue shift in the emission maxima.

| Concentration of GdnHCl [M] | RFI at 520 nm |
|-----------------------------|--------------|
| 0                           | 20           |
| 0.5                         | 30           |
| 1                           | 40           |
| 2                           | 50           |
| 2.3                         | 60           |

The graph shows the binding of ANS to hFGF-1 at various concentrations of GdnHCl. The RFI at 520 nm increases with increasing concentration of GdnHCl, indicating the binding of ANS to the protein. The inset depicts the emission spectra of the protein in the native (N), intermediate (I), and unfolded (U) states. Note that the formation of the I state is accompanied by blue shift in the emission maxima.
do not match, implying that the GdnHCl-induced unfolding of hFGF-1 is not cooperative and involves the formation of equilibrium intermediate(s). We investigated the urea-induced unfolding of hFGF-1 to examine whether the accumulation of the equilibrium intermediate(s) is dependent on the nature of the denaturant (Fig. 3). Surprisingly, unlike GdnHCl, the unfolding of hFGF-1 in urea is a two-state process without the accumulation of intermediates. The $DG(H_2O)$ and $C_m$ values estimated from the urea unfolding curves monitored by the steady state fluorescence and CD techniques are nearly identical ($C_m = 2.3$ M and $DG(H_2O) = 3.25$ kcal/mol). These results could either be attributed to different unfolding pathways of hFGF-1 in urea and GdnHCl or to specific stabilization of the intermediate(s) in GdnHCl.

Unfolding of hFGF-1 Occurs in Two Stages—Size exclusion chromatography (SEC) is an useful technique to obtain information of integral changes of molecule dimensions under denaturant effect (27, 28). This technique has been successfully used to identify and obtain hydrodynamic data on stable intermediates in the folding/unfolding pathway(s) of proteins (27).

hFGF-1, in its native state, in 100 mM phosphate buffer (pH 7.2) containing 100 mM each of sodium chloride and ammonium sulfate, elutes as a single peak (retention time $5\sim 95.7$ min) on the superdex-100 SEC-FPLC column. The area under the peak corresponding to the native state of the protein shows a progressive decrease with the increase in the GdnHCl concentration (Fig. 4). In addition to the native peak, two new peaks with retention times around 91 and 87 min are observed in the FPLC profiles of hFGF-1 collected in the GdnHCl concentration range between 0.2 and 2 M (data not shown). The population of the protein molecules representing the intermediate peak (retention time $= 91$ min) is maximum ($\sim 30\%$) at a denaturant concentration of 1 M. A single prominent peak (retention time $= 87$ min) representing the protein in its unfolded state could be observed in the FPLC profile obtained beyond 1.5 M GdnHCl. The results of the SEC-FPLC experiment clearly show that the GdnHCl unfolding of hFGF-1 proceeds via the accumulation of a stable intermediate at around 1.0 M GdnHCl. The GdnHCl unfolding profile of hFGF-1 monitored by the changes in the area under the native peak (retention time $= 95$ min) is noncooperative and is found to occur in two stages. This is evident from the biphasic nature of the denaturation curve (Fig. 4). Multiphasic equilibrium unfolding profiles, monitored by FPLC, have been reported in several proteins and are attributed to the formation of intermediates in the time scales of the FPLC experiments (27, 28). The first phase of unfolding (0–1.0 M GdnHCl) of hFGF-1 appears to represent an equilibrium transition between the native and the intermediate state(s). The transition between the intermediate and the unfolded state that occurs in the second phase of unfolding (1.0–2.0 M GdnHCl) appears to be co-operative. This aspect is evident from the steep slope and lesser number of data points in the second phase of unfolding (Fig. 4). To judge the cooperativity of the transition from the intermediate to the unfolded state, we investigated the thermal unfolding of hFGF-1 in 0.96 M GdnHCl using the fluorescence and CD spectroscopy (data not shown). The thermal unfolding curves obtained using both the techniques were superimposable, implying that the intermediate 219 transition is co-operative and involves no intermediate(s). It is pertinent to mention that because of the overlap of the FPLC peaks representing the native, intermediate, and unfolded states of hFGF-1, the hydrodynamic data pertaining to these conformational states could not be quantitatively estimated accurately.

**FIG. 6.** One-dimensional $^1$H NMR spectra of hFGF-1 in 0, 0.96, and 2 M GdnHCl. It could be observed that the spectrum of the protein in 0.96 M GdnHCl shows prominent line broadening effects in some of the resonances corresponding to the amide, aromatic (A), and aliphatic (B) protons, which are characteristic of an MG-like state. The concentration of the protein used was 0.5 mM.

Characterization of an Equilibrium Intermediate 34971
Characterization of an Equilibrium Intermediate

The Equilibrium Intermediate Resembles a Molten Globule-like State—ANS is a fluorescent dye that binds to hydrophobic regions of proteins (19). This fluorescent probe has been immensely useful in the identification of equilibrium intermediates such as the MGs. Molten globule intermediates usually display a significant exposure of hydrophobic cores to the solvent. Hence, ANS binds strongly to MG and fluoresces intensely (29, 30). The dye generally exhibits weak binding affinity to the native and unfolded states of proteins (29). The binding affinity of hFGF-1 to ANS at various concentrations of GdnHCl was monitored by the changes in the emission intensity at 520 nm (Fig. 5). The emission intensity of ANS upon binding to the protein (hFGF-1) in 0.96 M GdnHCl is more than twice that observed with the protein in its native state (Fig. 5). Fluorescence spectra of ANS in the presence of the protein in 0.96 M GdnHCl reveals that the emission maxima of the dye blue shifts by about 30 nm (from 520 to 490 nm). Further increase in the concentration of GdnHCl (to 0.96 M) results not only in the progressive decrease in the emission intensity but also is accompanied by a continuous red shift in the emission maxima. The protein in its unfolded conformation(s) at and beyond 2 M GdnHCl exhibits weak binding to ANS (Fig. 5). Thus, results of the ANS binding and the SEC-FPLC experiments analyzed in conjunction, clearly suggest a MG-like intermediate state maximally accumulates at 0.96 M GdnHCl.

The Intermediate State Has Higher Conformational Flexibility—Proteins in the MG state are proposed to have considerable native secondary structural interactions and greater flexibility of the side chains because of loss in some tertiary structural interactions (19). In this context, one-dimensional proton NMR spectra is expected to provide useful gross information on the conformational status of the equilibrium intermediate of hFGF-1 accumulated at 0.96 M GdnHCl. One-dimensional 1H NMR spectra of hFGF-1 (in its native state) is well dispersed, which reflects the highly specific inter-residue interactions within the compact folded state(s) of the protein (Fig. 6). These features are notably evident in the fairly narrow line widths of resonances in the amide, aromatic (10.0–7.0 ppm) and aliphatic (−0.5 to 3.0 ppm) regions of the one-dimensional 1H NMR spectrum of the protein in its native state. The one-dimensional 1H NMR spectra of the protein in 0 and 0.96 M GdnHCl states show an overall similarity (Fig. 6). This appears reasonable as the concentration of the native species (−70%) in 0.96 M predominates over that of the intermediate state (−30%). However, a critical comparison of the two spectra reveals that the chemical shift dispersion of some of the resonances in the amide, aromatic and aliphatic regions of the spectrum of the protein in 0.96 M GdnHCl is noticeably reduced (Fig. 6). These spectral features either imply a conformational exchange between the native and intermediate species or a free motion of some of the segments of the protein in the interme-

Fig. 7. Densitometry profile of the intensities of the 16-kDa band (in SDS-PAGE) upon treatment with trypsin (bottom panel). P, E, and D in the bottom panel represent the protein (hFGF-1), enzyme (trypsin), and 0.96 M denaturant (GdnHCl), respectively. The concentration of the protein used was 0.5 mg/ml. The intensities of the bands in the densitometric scan were normalized to the 16-kDa band of the untreated hFGF-1 (lane 1). Lanes 2 and 3 in the SDS-PAGE (in the top panel) represent the trypsin treated hFGF-1 in 0 and 0.96 M GdnHCl, respectively. The higher susceptibility of the protein in 0.96 M GdnHCl (lane 3) to trypsinic cleavage is indicative of increased conformational flexibility of the protein in the intermediate state.

Fig. 8. A, 1H-15N HSQC spectra of the hFGF-1 in 0, 0.96, and 2 M GdnHCl. The cross-peaks that show a pronounced change in the chemical shift in 0.96 M GdnHCl have been boxed. B, weighted average of 1H and 15N chemical shift differences. Profound changes in the chemical shift values could be observed for residues located in the C-terminal segment (residues 105–140) of the hFGF-1 molecule.
FIG. 9. $^1$H-$^1$N HSQC spectra of hFGF-1 in D$_2$O at various concentrations of GdnHCl. The additional cross-peaks that exchange out in 0.96 M GdnHCl mostly correspond to the residues located in the $\beta$-strands VIII, IX, and X. Most of the other secondary structural interactions in the protein appear to be unaffected in the intermediate state.
Characterization of an Equilibrium Intermediate

Limited proteolytic digestion has been applied to investigate the conformational flexibility of proteins (31, 32). 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. hFGF-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. Because the cleavage sites for trypsin correspond to the carboxyl ends of lysine and arginine residues, trypsin is an apt choice to monitor the conformational differences that possibly exist between the native and intermediate (identified at 0.96 M GdnHCl) states of hFGF-1. In addition, the optimal pH for the proteolytic action of trypsin (pH ~8.0) is closer to the pH at which the intermediate state is realized (pH 7.2). Undigested hFGF-1 yields a band on SDS-PAGE, which corresponds to a molecular mass of about 16 kDa (Fig. 7). The intensity of this band (after Coomassie Blue staining) is used as a control to monitor the degree of action of trypsin on the native and the intermediate states of hFGF-1. After 20 min of incubation, hFGF-1 (in its native state) with trypsin leaves about 45% of the protein uncleaved. However, treatment of hFGF-1 in the intermediate (in 0.96 M GdnHCl) state with trypsin shows that more than 75% of the 16-kDa band is cleaved (Fig. 7). These results reveal that the protein in the intermediate state as compared with the native state is more susceptible to proteolysis implying increased conformational flexibility of the protein in the intermediate state. It should be mentioned that control experiments with bovine serum albumin revealed that the presence of the denaturant (0.96 M GdnHCl) does not have significant effect(s) on the cleavage efficiency of trypsin (data not shown). In addition, comparison with the results of the control experiments with bovine serum albumin showed that the higher susceptibility of the protein (hFGF-1) to trypsin cleavage in 0.96 M GdnHCl is not a general denaturant effect.

Structural Changes in the Intermediate State of the Protein—NMR spectroscopy enables the study at the level of individual amino acid residues during the folding/unfolding of a protein (4, 33). Especially, the heteronuclear correlation experiments have been shown to be very sensitive because of high magnetization transfer between directly bonded nuclei (4). The 1H and 15N backbone chemical shifts of hFGF-1 have been recently determined (34). This aspect enables us to use the 1H-15N heteronuclear single quantum coherence (HSQC) technique to investigate the GdnHCl-induced unfolding of the protein at high resolution.

The 1H-15N HSQC spectrum serves as a fingerprint of the conformation state of a protein at each concentration of GdnHCl. The HSQC spectrum of hFGF-1 in its native state is well dispersed, which is characteristic for a folded protein (Fig. 8). Increasing the GdnHCl concentration below 0.6 M does not cause appreciable chemical shift changes in most of the cross-peaks in the HSQC spectra. However, the cross-peaks corresponding to the residues belonging to the N-terminal end of the hFGF-1 molecule exhibit significant chemical shift perturbation (Fig. 8). At 0.96 M GdnHCl, at which the intermediate state is maximally accumulated, the residues in the C-terminal segment (spanning residues 95–140) are most prominently perturbed (Fig. 8). Most of the residues that show significant chemical shift changes or that are broadened appear to be those that are not involved in the secondary structure formation. Majority of the residues involved in the secondary structural interactions in the native state do not show appreciable chemical shift changes or broadening (Fig. 8) in 0.96 M GdnHCl. Among the 12 β-strands comprising the β-barrel structure, the residues located in β-strand IX and X (residues 95–112) appear to be maximally affected in 0.96 M GdnHCl. The cross-peaks of most of these residues show an average chemical shift difference exceeding 0.1 ppm. The cross-peaks of some of the residues belonging to the C-terminal domain (residues 105–140) are broadened because of exchange (between the native and intermediate species) or increased conformational fluctuations in the intermediate state (Fig. 8). The HSQC spectra of hFGF-1 acquired beyond 1.3 M GdnHCl show limited chemical shift dispersion in the 1H dimension and are typical for an unfolded protein (Fig. 8). Because the concentration of the native species predominates at 0.96 M GdnHCl, it is not possible to obtain a complete description of the intermediate species. However, from the results discussed above, it is reasonably clear that the β-strands located in the C-terminal segment are maximally perturbed in the intermediate state (realized in 0.96 M GdnHCl).

Hydrogen-deuterium exchange studies provide useful information on the relative solvent accessibility of various amide protons in a protein (35). These experiments derive advantage from the fact that the hydrogen bonded amide protons exchange with solvent at several orders of magnitude slower than
those which are non-hydrogen-bonded. These differences are a direct measure of the local rigidity or flexibility in the protein molecule. In this context, we performed the hydrogen-deuterium exchange experiments on the native, intermediate, and GdnHCl unfolded states of hFGF-1. Ninety cross-peaks could be observed in the HSQC spectra of hFGF-1 obtained after 30 min of equilibration of the protein in D$_2$O (Fig. 9). Except for five residues (which are located in the loop regions), most of the residues protected from exchange are involved in secondary structure formation. In 0.96 M GdnHCl about 60 cross-peaks are protected (Fig. 9). The additional amide protons exchanged out in the intermediate state mostly correspond to the residues in the b-strands IX, X, and XII located in the C-terminal segment (residues spanning 95–140) of the hFGF-1 molecule. Interestingly, there is nearly perfect correlation between the chemical shift perturbation data and the hydrogen-deuterium exchange results; the residues that show significant chemical shift perturbation correspond with those that get exchanged out in the intermediate state at 0.96 M GdnHCl.

Accumulation of Kinetic Intermediates—It would be interesting to understand whether the intermediate identified in the GdnHCl-induced equilibrium unfolding process of hFGF-1 also exists in the kinetic refolding pathway of the protein. In this context, the kinetics of the refolding and unfolding process of hFGF-1 were investigated. Complete refolding of hFGF-1 from its denatured state in 4 M GdnHCl (monitored by the changes in the tryptophan emission at 350 nm) occurs in a time span of 60 s (Fig. 10). Interestingly, the unfolding of the protein in 2 M GdnHCl is very slow, and it takes more than 20 min for complete unfolding of the protein. The unfolding and refolding rates were measured as a function of the denaturant (GdnHCl) concentration to produce a classical Chevron plot (36, 37). The transition midpoint is observed around 1.3 M and agrees closely with that estimated from the equilibrium unfolding data (Fig. 10). The refolding limb (between 0.2 and 1.4 M GdnHCl) exhibits a prominent curvature below 1.0 M GdnHCl (Fig. 10). Such a type of “roll-over” in the Chevron plot is a clear evidence that kinetic intermediate(s) accumulates at lower concentration of GdnHCl (<1.2 M). These results suggest that the equilibrium intermediate accumulated in 0.96 M GdnHCl probably also exists in the kinetic refolding pathway of hFGF-1.

Significance of the Molten Globule-like Intermediate—Molten globules have been implicated in several physiological processes (19). Proteins have been proposed to translocate across bio-membranes in their molten globule states (38). In this background, identification and characterization of a molten globule-like state in the unfolding pathway of hFGF-1 bears significance. hFGF-1 lacks a conventional signal sequence, and its denatured state in 4 M GdnHCl is a prominent curvature below 1.0 M GdnHCl (Fig. 10). Such a type of “roll-over” in the Chevron plot is a clear evidence that kinetic intermediate(s) accumulates at lower concentration of GdnHCl (<1.2 M). These results suggest that the equilibrium intermediate accumulated in 0.96 M GdnHCl probably also exists in the kinetic refolding pathway of hFGF-1.