RhNGF slow unfolding is not due to proline isomerization: Possibility of a cystine knot loop-threading mechanism

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Abstract

The unfolding of recombinant human β -NGF (NGF) in guanidine hydrochloride (GdnHCl) was found to be time dependent with the denaturation midpoint moving to lower GdnHCl concentration over time. Dissociation and extensive unfolding of the NGF dimer occurred rapidly in 5 M GdnHCl, but further unfolding of the molecule occurred over many days at 25 °C. Fluorescence spectroscopy, size-exclusion and reversed-phase HPLC, ultracentrifugation, and proton NMR spectroscopy were used to ascertain that the slow unfolding step was between two denatured monomeric states of NGF (M_1 and M_2). Proton NMR showed the monomer formed at early times in GdnHCl (M_1) had little β -sheet structure, but retained residual structure in the tryptophan indole and high-field methyl regions of the spectrum. This residual structure was lost after prolonged incubation in GdnHCl giving a more fully unfolded monomer, M_2 . From kinetic unfolding experiments in 5 M GdnHCl it was determined that the conversion of M_1 to M_2 had an activation energy of 26.5 kcal/mol, a half-life of 23 h at 25 °C, and the rate of formation of M_2 was dependent on the GdnHCl concentration between 5 and 7.1 M GdnHCl. These properties of the slow unfolding step are inconsistent with a proline isomerization mechanism. The rate of formation of the slow folding monomer M_2 increases with truncation of five and nine amino acids from the NGF N-terminus. A model for the slow unfolding reaction is proposed where the N-terminus threads through the cystine knot to form M_2 , a loop-threading reaction, increasing the conformational freedom of the denatured state.

Keywords: cystine knot; loop threading; neurotrophin; NGF; protein folding; proline isomerization; slow folding

Nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5) are growth factors required for the development and survival of specific neuronal populations (Barbacid, 1994). The crystal structure of the murine NGF homodimer (muNGF) reveals seven β -strands per monomer, six of which are arranged as pairs of antiparallel β -sheet, which combine to form a flat hydrophobic dimer interface. The three disulfide bonds in each monomer are clustered into a cystine knot (McDonald et al., 1991; McDonald & Hendrickson, 1993; Murray-Rust et al., 1993), an unusual structure where one disulfide bond (Cys¹⁵-Cys⁸⁰) threads through

a 14-amino acid loop formed by two other disulfide bonds (Cys⁵⁸-Cys¹⁰⁸ and Cys⁶⁸-Cys¹¹⁰). The neurotrophins share approximately 55% sequence homology, and all contain a cystine knot. The recent crystal structure of the NT-3 and BDNF heterodimer (Robinson et al., 1995) is very similar to that of muNGF, confirming that the structures of all the neurotrophins are very similar. The muNGF dimer/monomer equilibrium constant, $K_{D/M}$, has been studied by a variety of techniques and is reported to be $\leq 10^{-13}$ M at neutral pH (Bothwell & Shooter, 1977). Values of $K_{D/M}$ determined from equilibrium denaturation studies have also been reported (Timm & Neet, 1992; Timm et al., 1994; Woo et al., 1995). The unfolding of muNGF in GdnHCl was reported to show two-state unfolding behavior, with a dimer-to-unfolded monomer mechanism with no evidence of a native monomer (Timm & Neet, 1992; Timm et al., 1994). However, slow unfolding and refolding kinetics observed under certain solvent conditions for muNGF (Timm & Neet, 1992; Timm et al., 1994) complicate the interpretation of these denaturation studies.

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Abbreviations: DTT, dithiothreitol; GdnHCl, guanidine hydrochloride; muNGF, murine NGF; NGF, recombinant human NGF; RP-HPLC, reversed-phase HPLC; SEC, size-exclusion HPLC.

The unfolding and refolding of muNGF, NT-3, and BDNF from GdnHCl have been shown by a variety of techniques to be slow processes (Radziejewski et al., 1992; Timm & Neet, 1992; Philo et al., 1993; Kolbeck et al., 1994; Timm et al., 1994). MuNGF unfolding was reported to be complete within 1 min in 4 M GdnHCl at 25 °C, and this unfolding to be fully reversible after 24 h at 25 °C (Radziejewski et al., 1992; Timm & Neet, 1992; Timm et al., 1994). However, unfolding and refolding at intermediate GdnHCl concentrations were shown to take up to 24 and 72 h, respectively (Timm & Neet, 1992).

Here we characterize in detail the time dependence and GdnHCl concentration dependence of the recombinant human NGF slow folding process. Our results exclude proline isomerization, the most common explanation for a slow folding protein reaction (Brandts et al., 1975; Schmid, 1992), as a mechanism for this slow unfolding process. Results using NGF N-terminal truncation variants suggest another possible mechanism for slow unfolding.

Results and discussion

NGF in GdnHCl: Unfolding or just dissociation?

Murine NGF and BDNF dimers are known to dissociate in GdnHCl based on the protein concentration dependence of the fluorescence intensity signal and light scattering experiments (Timm & Neet, 1992; Philo et al., 1993). It is not clear, how-

ever, from these experiments whether NGF unfolds when it dissociates. NGF contains three Trp and two Tyr groups, all of which reside at the dimer interface. Upon dissociation, these aromatic groups may become solvent exposed, such that intrinsic fluorescence, near-UV CD, and UV studies are unable to distinguish between dissociation and denaturation. In addition, secondary structure determinations using far-UV CD are not very informative for NGF as spectra change little between 0 and 6 M GdnHCl.

Proton NMR experiments can distinguish between folded and unfolded proteins by the position and dispersion of the proton resonance frequencies. In a denatured protein, the side chain and backbone protons are in an environment defined by the solvent and the proton resonance frequencies are relatively unaffected by neighboring residues in the protein. In contrast, in the native state, the environment of many protons is defined by the surrounding atoms of the protein rather than by the solvent (McDonald et al., 1971).

A proton NMR spectrum for NGF in d3-acetate buffer at 37 °C is shown in Figure 1, spectrum a. The regions of the spectrum that indicated that the protein was in the native state were (1) amide and aromatic proton resonances at 7–9 ppm showed wide dispersion suggesting many different environments for these residues; (2) three resonances at ≥ 9.9 ppm were observed, likely due to each of the three tryptophan indole protons being in different environments; (3) proton resonances below 0.6 ppm generally result from methyl groups on hydrophobic residues be-

Fig. 1. Proton NMR spectra for NGF in (a) buffer and after incubation in 5 M GdnHCl for (b) 0.5 h and (c) 22.7 h at 37 °C. The characteristic dispersion of proton resonances in the native state disappears after brief incubation in 5 M GdnHCl. The intense peak near 7 ppm results from residual GdnHCl in the GdnHCl. Asterisk denotes the TSP chemical shift reference peak.

ing buried near the face of an aromatic residue, generally in the interior of a protein, where the ring-current fields cause distance and angle dependent changes in resonance positions (McDonald et al., 1971); (4) protons on α carbons in a β -sheet structure are shifted, on average, 0.4 ppm downfield from their random coil positions (Pardi et al., 1983; Szilágyi & Jardetzky, 1989; Wishart et al., 1991). The resonances between 5 and 6.1 ppm are suggestive of β -sheet structure based on chemical shifts reported by Wishart et al. (1991). The residual water peak, not completely eliminated by presaturation of the water resonance, is observed in the 4.5–5 ppm range.

The proton NMR spectrum for NGF in 5 M deuterated GdnHCl after 0.5 h at 37 °C is also shown in Figure 1. The tryptophan residues have collapsed to two peaks, suggesting one to be the random coil position and one to remain in a perturbed environment. The chemical shift dispersion near 7–9 ppm has collapsed to fewer resonance values and the high-field methyl resonances below 0.6 ppm have largely disappeared, though a small residual signal remains. In addition, the C α resonances between 5.5 and 6.1 ppm are much less intense, suggesting loss of β -sheet structure. Taken together, these changes in proton resonance frequencies show that after 0.5 h in 5 M GdnHCl the protein is denatured, with maintenance of some residual structure.

RhNGF denaturation in GdnHCl is time dependent

Proton NMR

Proton NMR spectra for NGF in 5 M deuterated GdnHCl were also collected over time at 37 °C. The spectra collected after 0.5 and 23 h of incubation in GdnHCl at 37° are compared with the native spectrum in Figure 1. All resonances between –0.03 and 0.6 ppm, indicative of residual structure, have disappeared from the spectrum after 24 h in GdnHCl. The rate of decay of the residual NGF structure in GdnHCl was determined by measuring the peak intensities of the methyl peaks at –0.28, 0.31, 0.43, 0.51, 0.62 ppm and the indole peaks at 10.02 and 10.10 ppm (peaks labeled A–G) over time. The decay in the peak intensities at 0.31 ppm (peak B) and 0.51 ppm (peak D) are shown in Figure 2. A first-order fit to these data yielded apparent first-order rate constants (Table 1). Peaks A–C decayed at a rate of 0.25 h^{–1}, peaks D, E, and G at 0.15 h^{–1}, and peak F grew at a rate of 0.094 h^{–1}. These rates are statistically different and may represent three local unfolding processes. Sequence specific NMR assignments have not been reported for NGF. The upfield-shifted resonances observed in GdnHCl cannot, therefore, be attributed to specific residues in the protein.

Intrinsic fluorescence

The GdnHCl denaturation of NGF was followed by intrinsic fluorescence after incubation at 25 °C for 1, 2, and 5 days. The concentration of GdnHCl at the midpoint of the denaturation curve (C_m) was observed to move to lower GdnHCl concentrations with an increase in incubation time, as shown in Figure 3 for the wavelength of maximum fluorescence intensity. A similar trend was observed for the fluorescence intensity data at 336 nm (data not shown). Evidence for the attainment of equilibrium was not obtained in this experiment. In addition, the 1.6-nm shift in fluorescence peak wavelength between 0 and 2 M GdnHCl, where the protein is dimeric, is much greater than

Fig. 2. Time-dependent loss in the NGF proton peak heights (intensities) in 5 M GdnHCl at 37 °C for (◆) peak B at 0.31 ppm and (▲) peak D at 0.51 ppm. The standard deviation of the intensity values due to thermal noise is approximately 0.076, which was determined at high field, outside the range of protein ¹H-NMR signals. Best fits to a first-order curve are drawn through the data.

expected due to solvent effects on Trp and Tyr fluorescence (Lakowicz, 1983). Time dependent changes in the fluorescence peak wavelength are also observed above 4 M GdnHCl. These data cannot be fit using the assumption of a two-state process at equilibrium.

Size-exclusion HPLC

Figure 4A shows a series of SEC chromatograms of NGF incubated in 0–5.0 M GdnHCl for 7 days at 25 °C and then assayed on a SEC column that was equilibrated in mobile phase with the same concentration of GdnHCl as each sample. Dis-

Table 1. Calculated parameters from the proton NMR decay curves

Peak	Chemical shift (ppm)	Type	Rate (h ^{–1})	Relative amplitude
A	–0.28	Methyl	0.28 ± 0.02	1.3
B	0.31	Methyl	0.25 ± 0.01	4.2
C	0.43	Methyl	0.23 ± 0.01	7.4
D	0.51	Methyl	0.15 ± 0.01	8.0
E	0.62	Methyl	0.14 ± 0.01	15.8
F	10.10	Indole	0.094 ± 0.02	0.9
G ^a	10.02	Indole	0.16 ± 0.01	n.a.

^a Rate calculated from the increase in intensity of the random coil signal.

Fig. 3. GdnHCl dependence of the wavelength of the maximum fluorescence emission intensity (peak wavelength) for NGF after incubation in GdnHCl for 24 (■), 48 (●), or 120 (◆) h at 25 °C. Lines are drawn to guide the eye.

sociation of the NGF dimer to monomer is observed with increasing GdnHCl concentration. The dimer has a shorter retention time than the monomer, consistent with a relatively compact, disulfide-crosslinked, monomer. When DTT is added to NGF in buffer, reducing the disulfide bonds, the dimer retention time is unchanged and the monomer elutes before (is larger than) the native dimer (data not shown), as would be expected for a random coil protein (Corbett & Roche, 1984; Herold & Leistler, 1991).

NGF eluted as a single peak at both low and high GdnHCl concentrations. Two peaks were observed at intermediate GdnHCl concentrations, the peak area shifting from the dimer to the monomer peak with increasing GdnHCl concentration. The retention times for these two peaks, as a function of GdnHCl concentration are shown in Figure 5A. The increase in retention time between 3 and 3.5 M GdnHCl was reproducible. Two runs are shown, the retention times for the second run were slightly greater than in the first run, at all GdnHCl concentrations. This was apparently due to irreversible changes in the SEC column after extended use in GdnHCl as this trend continued with longer use.

The retention times for both the dimer and monomer decreased with increasing GdnHCl concentration outside the denaturation transition range. This dependence of the retention time on GdnHCl concentration has been observed previously for polymers and for proteins that are unfolded or folded, through-

Fig. 4. A: Size-exclusion HPLC chromatograms for NGF in 0–5 M GdnHCl after 7 days of incubation at 37 °C. The concentration of GdnHCl in the mobile phase was the same as that in each incubated sample. GdnHCl concentration (M) is shown on each trace. Retention times for blue dextran and sodium azide were 5.56 and 11.8 min in 0 M GdnHCl and 5.60 and 12.0 min, respectively, in 5 M GdnHCl. B: NGF dimer dissociation curves in GdnHCl after 1 (■), 3 (●), and 7 (◆) days of incubation at 37 °C in GdnHCl. Lines are drawn to guide the eye.

Fig. 5. Peak retention times for the SEC experiment shown in Figure 4. **A:** Retention times for NGF incubated in GdnHCl for 18 h prior to SEC chromatography in a mobile phase with the GdnHCl concentration equivalent to that in the sample. The two sets of symbols represent two separate experiments, with $\blacktriangle$ assayed prior to $\blacksquare$. Closed symbols, early eluting peak; open symbols, late eluting peak (monomer). **B:** Retention times for NGF incubated in buffer and injected into the SEC column equilibrated at various GdnHCl concentrations. Data represented by triangles in A and B were determined in the same experiment.

out a broad range of GdnHCl concentration (Withka et al., 1987; Shalongo et al., 1989). This may arise from a change in the interaction of the protein with the column, a change in pore accessibility due to changes in solvent viscosity, a true swelling of the protein in denaturant or a combination of the above (Corbett & Roche, 1984; Withka et al., 1987; Shalongo et al., 1989; Shortle & Meeker, 1989; Herold & Leistler, 1991; Palleros et al., 1993). A decrease in retention time of approximately 0.2 min/M GdnHCl is observed for native and denatured proteins on a TSK2000SW XL column (Shalongo et al., 1989). For NGF, the decrease in retention time between 0.5 and 2.5 M GdnHCl was 0.20–0.24 min/M, suggesting that the change in dimer retention time is primarily a column/solvent effect and not due to large conformational changes. Protein incubated in 6 M GdnHCl and then refolded to GdnHCl concentrations between 1 and 5 M GdnHCl showed retention times equivalent to those determined during the unfolding experiment outside the unfolding transition region, whereas retention times in the transition region varied with incubation time (data not shown).

A second set of data was generated by analyzing NGF incubated in buffer on a SEC column equilibrated with mobile phase

containing various concentrations of GdnHCl. In this experiment, the protein was incubated in GdnHCl for only 8–9 min, the time on the column before elution. A single NGF peak was observed at all GdnHCl concentrations, peak retention times are shown in Figure 5B. At GdnHCl concentrations below the denaturation transition, the retention times in Figures 5A and B were superimposable. As in Figure 5A, an increase in the “dimer” retention time above 3 M GdnHCl was observed. These data suggest that NGF dimer (D) is in equilibrium with at least two monomeric species of NGF (M_1 and M_2), one of which (M_1) is in fast exchange with dimer. In fast exchange, only a single peak will be observed with a retention time that is the weighted average of the retention times of the dimer and monomer peaks. After short equilibration times in GdnHCl, only dimer and monomer in fast exchange are present resulting in a single peak at all GdnHCl concentrations (Fig. 5B). With increasing incubation time a second monomer (M_2), which is not in fast exchange with dimer, is formed and appears as a second peak in the chromatogram (Figs. 4A, 5A). This SEC experiment was repeated after 1, 3, and 7 days of incubation at 25 °C and the resulting denaturation curves are shown in Figure 4B. A decrease in the denaturation midpoint is observed with increasing incubation time.

RP-HPLC

Similar conclusions about the presence of two monomeric forms of NGF can be reached from RP-HPLC experiments. Representative chromatograms for NGF incubated in various concentrations of GdnHCl for 7 days at 25 °C are shown in Figure 6A. For samples equilibrated in buffer, 98% of the protein eluted with a main peak retention time of 11.1 min, whereas 2% eluted at 16.1 min. Both these peaks previously have been shown to be NGF monomers (Schmelzer et al., 1992), as also shown for BDNF (Philo et al., 1993). This second, late eluting, peak is formed in the acidic acetonitrile mobile phase (Schmelzer et al., 1992; Philo et al., 1993; Rosenfeld & Benedek, 1993) and is not created by chemical modification or cleavage of the parent molecule, as it can be converted back to the first peak by incubation in buffer (Schmelzer et al., 1992; for BDNF, Rosenfeld & Benedek, 1993).

Increased concentrations of GdnHCl resulted in a shift in peak area from the early to late eluting peak. This effect of GdnHCl was observed previously for NGF (Schmelzer et al., 1992) and BDNF (Philo et al., 1993). Unfolding curves determined by RP-HPLC after 1, 3, and 7 days of incubation in GdnHCl are shown in Figure 6B. Again, as in the fluorescence and SEC experiments, the midpoint of the denaturation curve decreases with an increase in incubation time. In contrast to the SEC experiments where only a single peak is observed at high GdnHCl concentrations (M_1 and M_2 indistinguishable), two peaks are observed in 5 M GdnHCl by RP-HPLC (M_1 = early eluting peak, M_2 = late eluting peak). After 1 day in 5 M GdnHCl at 25 °C, approximately 50% of the NGF is converted to the late eluting, more hydrophobic, monomer M_2 ; after 3 days, 90% is converted to M_2 .

The SEC, RP-HPLC, and fluorescence methods all show a shift in the midpoint of denaturation to lower concentrations of GdnHCl with an increase in incubation time in GdnHCl. Taken together with the NMR data, these data support the hypothesis that there are at least two monomeric species formed in GdnHCl. The simple model below, which is consistent with

Fig. 6. **A:** RP-HPLC chromatograms for NGF incubated in GdnHCl at 25 °C for 7 days. The GdnHCl concentration (M) is shown on each trace. Minor peaks are chemical degradation products of NGF (e.g., Met oxidation). **B:** RP-HPLC peak areas for the early eluting NGF monomer after 1 (■), 3 (●), and 7 (◆) days incubation in various concentrations of GdnHCl at 25 °C. Lines are drawn to guide the eye.

this data, is used as a working model to explain the experimental data presented.

where D is the native dimer, M_1 is a monomer which is in fast exchange with dimer on SEC, and M_2 is the more hydrophobic monomer on RP-HPLC which chromatographs as a separate peak on SEC. In this model, M_1 is formed rapidly in 5 M GdnHCl, whereas M_2 is formed slowly. In buffer, M_1 folds and associates rapidly to form dimer and M_2 folds slowly to M_1 . By proton NMR M_2 would be the more fully unfolded monomer, with no residual structure.

Sedimentation equilibrium was used to confirm that both M_1 and M_2 are monomers in 5 M GdnHCl. After 7 days of incubation at ambient temperature, NGF was found to be dimeric at 0 M (MW = 26.8 kDa), 1 M, and 2 M (MW = 24.1 kDa) GdnHCl and monomeric at 4.7, 5.3 (MW = 12.1 kDa) and 5.7 M GdnHCl. The temperature dependence studies reported below show NGF to be in the slow folding state M_2 after 7 days at 25 °C in GdnHCl, therefore M_2 is confirmed by ultracentrifugation to be monomeric. NGF in 5 M GdnHCl held at 5 °C for 24 h rapidly refolds to dimer on buffer dilution, being in state M_1 . The sedimentation equilibrium studies for NGF held at 5 °C for 24 h gave molecular weights consistent with dimer at 0 M GdnHCl (MW = 26.7 kDa) and monomer at 5.3 (MW = 13.3 kDa) and 5.9 M GdnHCl. Both M_1 and M_2 can therefore

be studied independently and both are monomeric states of NGF.

Does the slow folding monomer result from proline isomerization?

The most common explanation for protein slow folding is proline *cis-trans* isomerization (Brandts et al., 1975; Schmid, 1992). Examination of the NGF crystal structure (McDonald et al., 1991) shows that all the Pro residues are in the *trans* configuration in the native state. Several experiments were undertaken to ascertain whether proline isomerization in the denatured state plays a role in NGF slow unfolding. Two methods were used to determine rate constants for the formation of the NGF slow folding monomer at several temperatures for determination of an activation energy for this process. RP-HPLC was used directly to quantitate the rate of formation of the slowly formed monomer (M_2 , the late eluting peak) in GdnHCl. The M_1 and M_2 monomers are distinguishable by SEC under refolding conditions as their rates of refolding differ. Double-jump SEC experiments, where protein unfolded in 5 M GdnHCl is refolded in 0.5 M GdnHCl on the SEC column, were used as a second method to determine these rate constants.

The rate of formation of the slow folding monomer, M_2 , in the double-jump experiment at 0.35 mg/mL NGF, was determined at 5, 15, 25, and 37 °C. SEC chromatograms for the double-jump experiment at 37 °C are shown in Figure 7A. In

Fig. 7. **A:** SEC chromatograms for the double-jump experiment after (—) 0.01, (---) 2.2, (----) 5.2, and (— · —) 24.0 h in 5 M GdnHCl at 37 °C. The mobile phase contained 0.5 M GdnHCl. **B:** Loss of NGF dimer in the double-jump experiment with increasing incubation time in GdnHCl at (◆) 5 °C, (■) 15 °C, (●) 25 °C, and (▲, △) two independent experiments at 37 °C. Best fits to a first-order curve are drawn through the data.

these experiments, protein converted to M_2 in 5 M GdnHCl eluted last, whereas protein in the M_1 state in GdnHCl eluted first (as dimer) due to its rapid refolding to dimer on the column. With an increase in incubation time in GdnHCl, less dimer (less M_1) and more monomer (more M_2) appeared in the SEC chromatograms. At long incubation times only 2% of the NGF assayed as dimer (Fig. 7A). A small amount of aggregate, representing 0–2% of the total UV 280 nm signal, was observed in some chromatograms. There was no loss in NGF total peak area in any of the chromatograms. The time dependent loss of M_1 in GdnHCl (quantitated as dimer in the double-jump experiment and which is equivalent to the formation of M_2) with incubation time in GdnHCl is shown in Figure 7B at 5, 15, 25, and 37 °C. The data were well fit with the first-order equation, $A = A_0 e^{-kt} + 1.2$, using a terminal value for % M_1 of 1.2%, the average of the values determined at 25 and 37 °C (values ranged from 0.6 to 2.4).

RP-HPLC was also used to determine the rate of conversion of M_1 to M_2 . Representative chromatograms are shown in Figure 8A. The early (M_1) and late (M_2) eluting monomers were quantitated and the loss of M_1 with time and temperature is shown in Figure 8B. First-order curves were fit to the data ($A = A_0 e^{-kt}$), which were again consistent with the conversion of M_1 to M_2 being a first-order process. No loss in NGF RP-HPLC total peak area was observed at any time or temperature.

Apparent first-order rate constants for the SEC double-jump and RP-HPLC experiments are listed in Table 2. The apparent

rate constants for formation of the slow folding monomer increased with an increase in temperature. For RP-HPLC, 100% of M_1 was converted to M_2 ; in SEC, 98.8% was converted. Proline isomerization is an equilibrium reaction and complete conversion of prolines from the *trans* to the *cis* conformation would be unusual.

The interconversion of dimer, M_1 , and M_2 were reversible as determined by SEC and RP-HPLC. For example, after 24.8 h of incubation in 5 M GdnHCl at 25 °C, where 50% of the protein had converted to M_2 , an aliquot of the NGF solution was diluted fivefold with buffer to a final GdnHCl concentration of 1 M. The folding of M_2 to M_1 at 25 °C, monitored by RP-HPLC, is shown in Figure 9. A first-order fit of the data using the equation $A = A_0 e^{-kt} + 4.8$, where 4.8 equals the terminal value of M_2 , gave an apparent first-order rate constant for the refolding of M_2 to M_1 of $0.064 \pm 0.007 \text{ h}^{-1}$.

Arrhenius plots of the SEC and RP-HPLC data in Table 2 are shown in Figure 10. The Arrhenius plots are linear. Activation energies of 27.0 ± 0.61 and 26.2 ± 0.15 kcal/mol were determined from the slopes of SEC and RP-HPLC curves, respectively. These activation energies are greater than typical values of 18–22 kcal/mol reported for proline isomerization.

Rate dependence on pH, reducing agent, GdnHCl and protein concentration

The protein concentration dependencies of the rate constants determined in the SEC double-jump and RP-HPLC experiments

Fig. 8. A: RP-HPLC chromatograms for NGF after various incubation times (shown in h on HPLC tracings) in 5 M GdnHCl at 37 °C. B: Loss of early eluting monomer with increasing incubation time in GdnHCl at (◆) 5 °C, (■) 15 °C, (●) 25 °C, and (▲) 37 °C. Best fits to a first-order curve are drawn through the data.

are shown in Table 3. The rate constants for formation of slow folding monomer were independent of protein concentration between 0.02 and 0.1 mg/mL. A small decrease in rate was observed with a further increase in protein concentration, up to the maximum concentration tested of 2 mg/mL. No significant aggregation was observed at any of these protein concentrations. The origin of the small protein concentration dependence is unknown. The similarity of the protein concentration dependence for SEC and RP-HPLC suggests that this is not an artifact of the chromatography. Philo et al. (1993) found a similar small protein concentration dependence in the refolding of BDNF from GdnHCl. The rate limiting step appears to be dominated by a monomer-to-monomer reaction with a small contribution by dimer or larger aggregate at protein concentrations above 0.1 mg/mL.

Table 2. Temperature dependence of the apparent first-order rate constants for conversion of M_1 to M_2

Temp. (°C)	SEC rate (h^{-1})	RP-HPLC rate (h^{-1})
5	$1.0 \times 10^{-3} \pm 4 \times 10^{-5}$	$1.1 \times 10^{-3} \pm 1 \times 10^{-5}$
15	$6.9 \times 10^{-3} \pm 3 \times 10^{-4}$	$6.1 \times 10^{-3} \pm 9 \times 10^{-5}$
25	$3.2 \times 10^{-2} \pm 5 \times 10^{-4}$	$3.0 \times 10^{-2} \pm 5 \times 10^{-4}$
37	$1.8 \times 10^{-1} \pm 4 \times 10^{-3}$	$1.6 \times 10^{-1} \pm 2 \times 10^{-3}$

Fig. 9. RP-HPLC peak areas for the time-dependent refolding of the NGF late eluting monomer in 1 M GdnHCl at 37 °C. NGF was unfolded for 24.8 h in 5 M GdnHCl at 37 °C prior to initiation of the refolding experiment. A best fit to a first-order curve is drawn through the data.

Fig. 10. Arrhenius plot for the conversion of M_1 to M_2 in 5 M GdnHCl as determined by (□) SEC double-jump and (▲) RP-HPLC. Standard errors in $\ln k$ are smaller than the size of the symbols.

The three prolines in NGF are at positions 5, 61, and 63. Pro⁶¹ and Pro⁶³ are located within the cystine knot, in the 14-amino acid disulfide-bonded loop. It is possible that this region of the protein is constrained, leading to a greater activation energy and/or time constant for isomerization of these Pro residues. To further test the proline isomerization mechanism, the GdnHCl concentration dependence of the rate of formation of the slow folding monomer was determined. The rate of formation of M_2 was measured using 5.0, 5.7, 6.5, and 7.1 M GdnHCl in the double-jump experiment at 37 °C. The terminal amount of dimer, at long incubation times, was indepen-

Table 3. Protein concentration and guanidine hydrochloride concentration dependence of the rate of conversion of M_1 to M_2 at 37 °C

[GdnHCl] (M)	[NGF] (mg/mL)	SEC rate (h ⁻¹)	RP-HPLC rate (h ⁻¹)
5	0.02	0.22 ± 0.02	
5	0.05	0.23 ± 0.01	0.19 ± 0.01
5	0.1	0.23 ± 0.02	
5	0.35	0.20 ± 0.004	0.16 ± 0.002
5	0.85		0.15 ± 0.003
5	1.0	0.16 ± 0.003	
5	2.0	0.16 ± 0.01	
5.7	0.35	0.15 ± 0.003	
6.5	0.35	0.12 ± 0.001	0.12 ± 0.006
7.1	0.35	0.095 ± 0.003	

dent of GdnHCl concentration. The rate constant for formation of slow folding monomer decreases with increasing GdnHCl concentration (Table 3); the same trend was observed by RP-HPLC. These data are inconsistent with a proline isomerization mechanism, where no GdnHCl concentration dependence would be observed.

Proline isomerization does not appear to be the mechanism for the NGF slow unfolding process. Typical activation energies for proline isomerization range between 18 and 22 kcal/mol (Schmid & Baldwin, 1979), smaller than the 26–27 kcal/mol observed here. The rate of formation of the slow folding monomer, with a time constant (1/rate) of 112,000 s at 25 °C, is much slower than typically observed isomerization rates in the 10–100-s time range. Finally, proline isomerization is independent of GdnHCl concentration (Schmid & Baldwin, 1979) and here the conversion of M_1 to M_2 is not. The data are, however, consistent with a sequential reaction where all protein converts to M_1 before slowly forming M_2 , because at very early incubation times, no M_2 is observed by SEC. Human lysozyme has also been reported to undergo a slow refolding step which cannot be ascribed to proline isomerization (Herning et al., 1991).

Another possible mechanism that could lead to time dependent changes in the unfolded state is disulfide exchange. The three disulfide bonds in NGF are clustered and it is possible that these disulfide bonds break and reform to form alternative disulfide bond configurations. The rate of disulfide exchange is expected to increase with increasing pH due to the participation of the thiolate anion ($pK_a \approx 8.6$) in the exchange mechanism (Gilbert, 1990). The pH dependence of the rate of formation of M_2 in the double-jump experiment at 37 °C (with 5 M GdnHCl in the first step) was determined at pH 4.5, 5.5, 6.5, and 7.5 (20 mM potassium phosphate was used in addition to the 200 mM Na acetate for this experiment). There was no dependence of the rate on the pH in this range, eliminating disulfide exchange as a possible mechanism.

The effect of DTT on the unfolding of NGF was also investigated. NGF was incubated at 25 °C in 5 M GdnHCl, 20 mM DTT, pH 5.5 or 7.5, and then injected onto the SEC column running a 5 M GdnHCl, 20 mM DTT mobile phase, pH 5.5, and a flow rate of 0.5 mL/min. The NGF monomer at time zero eluted at the same position as in the absence of DTT with a retention time of 16.5 min. With an increase in incubation time a peak at 14.8 min increased in area and the 16.5-min peak decreased in area. The pseudo-first-order rate constant for the conversion of the compact monomer to an expanded monomer was 0.83 h⁻¹ at pH 7.5 and 0.043 h⁻¹ at pH 5.5, the rate increasing with pH as expected from the mechanism of action of DTT. Again, the rate of M_1 to M_2 conversion is independent of pH as discussed above. The double-jump experiment cannot be done in the presence of DTT as it is difficult to refold NGF from the reduced state.

Why do the midpoints of the denaturation curves move to lower GdnHCl concentration with time?

SEC, RP-HPLC, and fluorescence studies all showed that the denaturation midpoint moves to lower GdnHCl concentration over time. The SEC, RP-HPLC, and NMR kinetic experiments show that this is due to a slow unfolding step that occurs for protein in the denatured state. Essentially all protein molecules are able to undergo the slow unfolding reaction at high GdnHCl

concentrations; at the denaturation midpoint, half the molecules are. With an increase in incubation time a fraction of the denatured protein in M_1 converts to M_2 . By the law of mass action, more of the native protein then denatures to repopulate M_1 . The total amount of protein in the denatured states ($M_1 + M_2$) therefore increases with time, shifting the denaturation curve to lower denaturant concentrations.

M_2 formation in NGF N-terminal variants

Two variants of NGF with five ($\Delta 5$ -NGF) and nine ($\Delta 9$ -NGF) residues truncated from the N-terminus were also analyzed in the double-jump experiment using 5 M GdnHCl in the denaturing step. The rate constant for conversion of M_1 to M_2 increased with a decrease in N-terminal length as shown in Figure 11. The $\Delta 5$ -NGF also showed an increase in rate of conversion of M_1 to M_2 with an increase in temperature, similar to full-length NGF (data not shown). However, a single first-order curve did not accurately fit the $\Delta 5$ -NGF data, suggesting a more complex mechanism (the $\Delta 5$ -NGF data in Figure 11 are fit with a biexponential curve). Kolbeck et al. (1994) have shown previously by RP-HPLC that the GdnHCl induced conversion of NT-3 from what they described as dimer to monomer (which we believe to be fast folding monomer to slow folding monomer) is accelerated by truncation of five residues from the N-terminus. In addition, a variant of BDNF with a mutation in the pro sequence which prevented the normal cleavage to the parent molecule (the residue preceding the N-terminus was mutated from Arg to Lys) resulted in a monomer with 19 additional residues attached to the N-terminus. Upon incubation in GdnHCl, Kolbeck et al. (1994) found a small decrease in the rate of BDNF conversion from the "dimer" to the "monomer" species. Woo et al. (1995) reported that the conformational stability of murine NGF was

not affected by $\Delta 9$ or $\Delta 9$ -13 deletions, as measured in GdnHCl denaturation studies. However, whereas their data showed little change in the denaturation midpoint, the slope in the transition range and the behavior in the pre and post denaturation regions for these mutants varied greatly from that of the parent NGF.

Could N-terminal loop threading be the slow unfolding mechanism?

NGF has a cystine knot, an unusual configuration of disulfide bonds common to the neurotrophins, transforming growth factor- $\beta 2$, and platelet-derived growth factor (Murray-Rust et al., 1993). A 14-amino acid loop is formed in the NGF chain by disulfide bonds between Cys⁵⁸-Cys¹⁰⁸ and Cys⁶⁸-Cys¹¹⁰. The loop is comprised of residues 58–68, the two disulfide bonds, and residues 108–110. Cys¹⁵, close to the N-terminus, is bonded to Cys⁸¹ through this disulfide loop. The proximity of the N-terminus to the disulfide bonded loop suggests that an N-terminal "loop-threading" reaction may explain the slow unfolding step observed for NGF. If the disulfide bonds are held in a native-like configuration upon denaturation, due to the constraints of the cystine knot, a relatively high free energy for the denatured state would be expected due to restrictions on the chain conformational freedom. In particular, residues 69–80 are held in a constrained loop. If the disulfide bond Cys¹⁵-Cys⁸¹, the N-terminal 14 residues, and a portion of the chain beyond residue 16, migrate through the disulfide loop in the unfolded state, the conformational freedom of the protein would increase significantly, lowering the free energy of the denatured state. This mechanism can be visualized easily by constructing a pipe cleaner model of the protein, where only loops and disulfide bonds are represented. A drawing of " M_1 " is shown in Figure 12. Here, the murine NGF disulfide knot, taken from the Brookhaven Protein Data Bank (Coordinate filecode 1BET, McDonald et al., 1991) is shown with all other residues represented by unstructured loops. M_1 is modeled here keeping the disulfide knot in the same configuration as in the native state. In M_2 , the disulfide bond Cys¹⁵-Cys⁸¹ and the N-terminus would be pulled through the loop, with residues beyond Cys¹⁵ also being drawn through the loop. This N-terminal loop threading would result in a significant increase in the size of the loop at the top of the figure which contains residues 69–80. Truncation of the N-terminus of NGF, as with the variants $\Delta 5$ -NGF and $\Delta 9$ -NGF, would be expected to increase the rate of conversion to the slow folding monomer due to greater ease of migration of the N-terminus through the disulfide-bonded loop.

This N-terminal loop-threading reaction can explain the data presented herein. The slow rate for M_1 to M_2 conversion would not be unexpected for such a loop-threading reaction. The 100% conversion of M_1 to M_2 at high GdnHCl concentrations would be driven by the higher conformational freedom and lower free energy of the M_2 state. M_2 would be more fully unfolded than M_1 and would therefore have more hydrophobic surface exposed (and elute later on RP-HPLC). The decreased rate for formation of M_2 from M_1 with an increase in GdnHCl concentration may be explained by a GdnHCl solvent effect. As the GdnHCl concentration increases, both M_1 and M_2 become more favorable (the solubility of most polar and nonpolar amino acid side chains, and the peptide backbone, increase with GdnHCl concentration [Robinson & Jencks, 1965; Nandi &

Fig. 11. Loss of NGF dimer with increasing incubation time in 5 M GdnHCl in the SEC double-jump experiment at 5 °C for (●) NGF, (▲) $\Delta 5$ -NGF, and (■) $\Delta 9$ -NGF. The NGF and $\Delta 9$ data are fit with a single exponential curve, $\Delta 5$ with a biexponential curve.

Fig. 12. Backbone representation of the unfolded state of NGF. The disulfide knot structure was taken from the Brookhaven Data Base (Coordinate filecode 1BET, McDonald et al., 1991). Loops replace all portions of the molecule outside the knot. Loops are not to scale. Red and orange residues depict the loop in the cystine knot; disulfide-bonded residues in orange. The loop-threading disulfide-bonded residues Cys¹⁵ and Cys⁸¹ are shown in yellow, N and C termini in dark blue. The N-terminus can be imagined to thread through the disulfide loop if the yellow disulfide is grabbed at the * and pulled in the direction of the arrow. If the N-terminus and residues 16 and up are pulled through the loop, the small blue loop at the top of the figure, containing residues 69–80, will be enlarged, increasing the conformational freedom of the chain. The loop containing residues 16–57 will also decrease in size though conformational constraints in this large loop will be small.

Robinson, 1984]). The unfavorable entropic contribution to the M_1 conformation would become a smaller contribution to the free energy with increasing GdnHCl concentration and therefore the rate of conversion to M_2 would decrease. If an N-terminal loop-threading mechanism is correct, the rate of M_1 to M_2 conversion could be affected by the compactness of the denatured state, which would be expected to increase with a decrease in denaturant concentration (Dill & Shortle, 1991).

A potential challenge to this theory is the significantly different rate of formation of M_2 for muNGF, as compared to rhNGF, despite the 95% sequence homology for these two molecules. The rate of formation of M_2 for muNGF and NGF were compared in a SEC double-jump experiment at pH 5.5, 25 °C, using 5 M GdnHCl. First-order fits to the data gave rates for formation of M_2 of $0.30 \pm 0.004 \text{ h}^{-1}$ for muNGF and $0.033 \pm 0.0006 \text{ h}^{-1}$ for rhNGF. RhNGF and muNGF differ at only 12 of 118 residues (Ullrich et al., 1983). Three of these amino acid differences are in the 15 N-terminal amino acids and three are in the knot residues 59–67. Differences in the number of charged amino acids in the N-terminal and cystine knot regions, as well

as the presence of two proline residues in the rhNGF knot (Pro⁶¹ and Pro⁶²), compared to only one (Pro⁶³) in muNGF, may be responsible for the different rates of formation of the slow folding species. Future comparisons with the slow unfolding reactions observed for BDNF and NT-3 are also of interest.

Conclusions

The unfolding of NGF in GdnHCl is not a two-state process. A slow unfolding step is observed that results in time dependent changes in the proton NMR, SEC, RP-HPLC, and fluorescence spectroscopy data. These changes are consistent with the formation of two monomeric states on denaturation, one that forms rapidly (M_1) and one that forms slowly (M_2) under denaturing conditions. M_1 folds and dimerizes rapidly on transfer to refolding conditions, whereas M_2 is unable to dimerize. The activation energy, time constant, extent of reaction, and rate dependence on GdnHCl concentration, all disagree with values expected for proline isomerization. An N-terminal cystine knot loop-threading mechanism is proposed as a slow unfolding step for the denatured protein. Conformational entropy is suggested as the driving force for threading of the N-terminus through the cystine knot in the denatured protein resulting in the formation of a more slowly folding denatured state. Truncation of the N-terminus results in an increased rate of formation of the slow folding species, consistent with this mechanism.

Materials and methods

Recombinant human NGF was produced in transfected Chinese hamster ovary (CHO) cells as described previously (Burton et al., 1992; Schmelzer et al., 1992). A NGF variant with five residues truncated from the N-terminus ($\Delta 5$ -NGF) was generated by in situ proteolysis during conditioning of the CHO cell media by an unidentified pathway (Shih et al., 1994). A variant with nine residues truncated from the N-terminus was prepared by limited proteolysis ($\Delta 9$ -NGF) (Burton et al., 1992). NGF and both variants were purified on reversed-phase and cation-exchange chromatography (Burton et al., 1992; Shih et al., 1994). Murine (muNGF) was prepared from mouse submaxillary glands as described previously (Burton et al., 1992). Ultrapure GdnHCl was purchased from Sigma or Boehringer Mannheim; results were independent of vendor. Deuterated d5-GdnDCl was purchased from Isotec, Inc. For all experiments except NMR, NGF was prepared in GdnHCl and buffer solutions containing 0.02 M Na acetate, 0.45 M KCl, and 0.14 M NaCl adjusted to pH 5.5 with HCl. The KCl was included in all samples and the SEC mobile phase to prevent NGF adsorption to the size-exclusion column. GdnHCl concentrations were determined using refractive index measurements.

NMR experiments

¹H NMR spectra were acquired on a Bruker AMX 500 spectrometer at 37 °C. Samples were prepared at pH 5.5, with and without 5 M d5-GdnDCl, with a protein concentration of 10.4 mg/mL (0.4 mM dimer) in 20 mM d3-sodium acetate buffer containing 50 mM NaCl. D₂O at 10% (v/v) was added for the spectrometer field frequency-lock and 0.1 mM d₄-sodium 3-(trimethylsilyl)-propionate (TSP) was added for chemical shift calibration. One-dimensional ¹H NMR spectra were acquired

consecutively over a period of 24 h for the NGF sample containing 5 M d5-GdnDCI. The 90° observation pulse was preceded by 1.5 s of weak phase-coherent irradiation to suppress the water signal and followed by a Hahn-echo to improve the quality of the baseline (Rance & Byrd, 1983). Five-hundred twelve transients were collected, requiring an acquisition time of 15.2 min for each spectrum. The spectra were processed using the program FELIX (Biosym Technologies, San Diego, California). Free induction decays were multiplied by weak Lorentz to Gaussian transformations prior to Fourier transformation; baseline distortions were corrected with a second-order polynomial routine. Each 1D spectrum was zero-filled to 4,096 complex points.

Several upfield-shifted methyl resonances and a downfield-shifted indole resonance were evident immediately after addition of guanidine hydrochloride, presumably arising from incompletely unfolded protein. Rate constants for the decay of these nonrandom coil signals were obtained by performing a nonlinear first-order fit of the form $y = A \exp^{-kt} + B$ to the peak intensities as a function of time (the peak intensities were the sum of eight data points spanning the peak maximum). The amplitude (A), offset (B), and rate constant (k) were obtained from the fit.

Ultracentrifugation studies

Sedimentation equilibrium was used to determine the average molecular weight of NGF in buffer and GdnHCl. The sedimentation equilibrium experiments for NGF samples equilibrated at ambient temperature for 7 days were performed at 28,000 rpm and 25 °C in a Beckman XLA ultracentrifuge; NGF samples equilibrated at 5 °C were assayed immediately after preparation at 30,000 rpm. The partial specific volumes of NGF in GdnHCl and buffer were calculated from their amino acid compositions and their preferential interaction with GdnHCl (Lee & Timasheff, 1979). The NGF weight average molecular weight was determined using a nonlinear least squares fitting program, NONLIN (Johnson et al., 1981) by fitting the data as a single ideal species.

Fluorescence studies of GdnHCl denaturation of NGF

The time dependent unfolding of NGF in GdnHCl was monitored by intrinsic fluorescence spectroscopy. Denaturation curves were generated for 50 µg/mL NGF in 0–5.8 M GdnHCl after 1, 2, and 5 days of incubation at 25 °C. Fluorescence was measured on a SLM 8000 fluorimeter using an excitation wavelength of 280 nm. The excitation and emission bandpasses were 2 nm. The wavelength of maximum fluorescence emission intensity (peak wavelength) was determined at each GdnHCl concentration. Excitation at 295 nm gave similar results. The fluorescence spectra were ratioed against rhodamine to correct for variations in the lamp intensity. In addition, the emission spectra were corrected for the wavelength dependent efficiency of the detection system.

SEC and RP-HPLC denaturation studies

SEC experiments were done using a Tosoh TSK 2000 SWXL, 5 µm, 7.8 × 300 mm column. The mobile phase contained 0.45 M KCl and 0.2 M Na acetate, pH 5.5, with GdnHCl concentrations from 0 to 5.8 M. The column excluded volume and

inclusion volume were determined using blue dextran and sodium azide as high and low molecular weight markers. A YMC, C₄, 5 µm, 4.6 × 250 mm column was used for RP-HPLC. A linear gradient of 25–30% B (A = 0.05% TFA in water; B = 0.05% TFA in acetonitrile) was run between 0 and 10 min followed by 30–40% B in 6 min and then a 9-min reequilibration at 25% B. Both HPLC methods were run on a HP1090 system at ambient temperature using a flow rate of 1.0 mL/min and UV detection at 280 nm for SEC and 214 nm for RP-HPLC. Data were collected and analyzed using Turbochrome software.

SEC and RP-HPLC were used to monitor NGF dissociation and conformational changes. Sterile solutions of 0.35 mg/mL NGF in 0.2 M Na acetate, 0.45 M KCl, with 0–5 M GdnHCl were incubated at 25 °C for 1, 3, and 7 days. These samples were analyzed by SEC in two ways. In the first run, the mobile phase contained the same concentration of GdnHCl (0–5 M) as the sample to be injected (an “equilibrium unfolding” experiment). In a second run, NGF equilibrated in buffer was injected into the column equilibrated in 0–5 M GdnHCl (a “kinetic unfolding” experiment). The desired mobile phase GdnHCl concentration was obtained by having the HPLC pump mix buffer and 6 M GdnHCl. At each mobile phase GdnHCl concentration, two samples were injected into the column and then the solvent mix was changed to increase the GdnHCl concentration by ≤0.5 M. Retention times for the dimer and monomer peaks were constant after the first injection, indicating that the column had equilibrated during the first 13-min run. GdnHCl concentration was confirmed by refractive index measurements. The NGF samples incubated in GdnHCl (0–5.5 M) were also analyzed by RP-HPLC. HPLC analysis for the full sample set required 6–7 h for each chromatographic method. NGF (0.78 mg/mL) was also incubated for various times at 25 °C in 5 M GdnHCl containing 20 mM DTT, pH 7.5, and then assayed on the SEC column running a 5 M GdnHCl, 20 mM DTT mobile phase, pH 5.5.

Kinetic unfolding experiments

Solutions of 0.35 mg/mL NGF were incubated at 5, 15, 25, and 37 °C in 5.0 M GdnHCl and at 37 °C in 5.7, 6.5, and 7.1 M GdnHCl. At time intervals, an aliquot of these samples were analyzed on a SEC column equilibrated in 0.5 M GdnHCl (a “double-jump” experiment). The amount of dimer and monomer were quantitated using peak areas. The protein concentration dependence of the double-jump experiment at 37 °C was determined for 0.02, 0.05, 0.1, 0.35, 1, and 2 mg/mL NGF in 5 M GdnHCl. A subset of these samples were also analyzed on RP-HPLC and the peak areas of the two monomers quantitated.

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