Refolding of Brain-Derived Neurotrophic Factor from Guanidine Hydrochloride: Kinetic Trapping in a Collapsed Form Which Is Incompetent for Dimerization

John S. Philo,* Robert Rosenfeld, Tsutomu Arakawa, Jie Wen, and Linda Owers Narhi

Protein Chemistry Department, Amgen Inc., Amgen Center, Thousand Oaks, California 91320

Received April 2, 1993; Revised Manuscript Received July 26, 1993®

ABSTRACT: We have studied the pathway and kinetics of refolding of recombinant human brain-derived neurotrophic factor (BDNF), which is a very tightly-associated dimer in its native state. When BDNF unfolded in 6 M guanidine hydrochloride is diluted 20-fold into phosphate-buffered saline, a partially folded intermediate is rapidly formed (<1 min). Circular dichroism and fluorescence spectroscopy show that this intermediate has extensive secondary structure, but no well-defined tertiary structure. Size-exclusion chromatography with light scattering detection shows that it is compact and monomeric, and therefore corresponds to what is often called a "collapsed form" or "molten globule". This collapsed form disappears with a half-time of ~30 min, simultaneously with the appearance of native dimers, *without* accumulation of monomeric species with a native tertiary structure. Remarkably, the monomer–dimer association constant of the collapsed form is $\sim 10^{10}$ weaker than the native structure, and it has a low tendency to form large aggregates. Given the very large hydrophobic surface present at the dimer interface of nerve growth factor (and presumably in BDNF), these results indicate that these hydrophobic groups are *not* exposed in the collapsed form, and that it is therefore quite dissimilar from the native structure. A significant conformational change in the collapsed form is necessary to re-expose these hydrophobic groups to form the dimer interface, making this the rate-limiting step in reaching the native conformation.

Brain-derived neurotrophic factor (BDNF)[1] is a member of the neurotrophin family of growth factors which affect the development and survival of neuronal cells. The parent member of this family is nerve growth factor (NGF), whose crystal structure was recently reported (McDonald *et al.*, 1992). BDNF shares 55% sequence homology with NGF, and has similar spectroscopic markers for secondary and tertiary structure (Narhi *et al.*, 1993a; Radzeijewski *et al.*, 1992). Therefore, like NGF, BDNF is thought to be stabilized by three intrachain disulfides and to consist primarily of β-sheet and irregular secondary structure. In solution, the neurotrophins are strongly associated to dimers. For NGF, the dissociation constant of the native dimer was reported as 10^{-13} M, i.e., a binding energy of ~17 kcal/mol (Bothwell & Shooter, 1977). The dissociation constant for BDNF appears to be similar, since equilibrium ultracentrifugation studies show no evidence for BDNF monomers even at protein concentrations near 10^{-10} M (Narhi *et al.*, 1993a; Radziejewski *et al.*, 1992). This means that intersubunit contacts probably contribute more than half of the 26.4 kcal/mol conformational stability of BDNF measured by denaturation with guanidine hydrochloride (GdnHCl) (K. Neet, private communication). The corollary of this strong stabilization by intersubunit contacts is, of course, that a BDNF monomer is a fairly unstable species. Indeed, no accumulation of folded monomers was seen during the equilibrium denaturation studies, with an apparent two-state transition from native dimer to unfolded monomer (K. Neet, private communication).

If the monomer is relatively unstable, what is the *kinetic* pathway for folding and assembly of the native dimer? Does BDNF transiently form a "native monomer" (i.e., one with a tertiary structure like that in the native dimer) and then assemble into a dimer, or does it dimerize *prior* to the completion of folding, perhaps with the dimer interface serving as a nucleus around which the rest of the structure condenses?

To answer these questions, we have investigated the kinetics of refolding of BDNF denatured in 6 M GdnHCl, monitoring both the rates of appearance of structural markers and the state of oligomerization. We find that indeed the refolding of BDNF is strongly linked to its dimerization. Upon removal of GdnHCl, the secondary structure of BDNF returns rapidly. However, it then becomes kinetically trapped in a compact, monomeric folding intermediate with extensive secondary structure, but which lacks the necessary structural elements for dimer formation. Indeed, a large number of hydrophobic groups which occur at the dimer interface in the native state are internalized in this folding intermediate. Therefore, a significant conformational change which re-exposes these hydrophobic groups is needed before dimerization can occur, and the slow rate of this step dominates the assembly kinetics. In the following paper (Narhi *et al.*, 1993b), we have also investigated the acid-induced unfolding of BDNF, and show that it is similarly strongly linked to association and also produces a compact monomeric state with considerable secondary structure but no unique tertiary structure.

MATERIALS AND METHODS

BDNF Samples. Recombinant human BDNF was expressed in *Escherichia coli*, oxidized and refolded, and purified by sequential column chromatography. We have shown that its disulfide structure is identical to that of BDNF expressed in Chinese hamster ovary cells (Acklin et al., 1993). Further, we find that the circular dichroism (CD), Fourier transform infrared, and fluorescence spectra as well as the sedimentation properties of BDNF from *E. coli* are indistinguishable from

* To whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts*, September 15, 1993.

[1] Abbreviations: BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; GdnHCl, guanidine hydrochloride; PBS, Dulbecco's phosphate-buffered saline; CD, circular dichroism; SEC, size-exclusion chromatography; LALLS, low-angle laser light scattering; ANS, anilinonaphthalenesulfonate.

0006-2960/93/0432-10812$04.00/0

the data reported previously (Narhi *et al.*, 1993a). A stock of unfolded BDNF with intact disulfides was prepared by cycles of dilution into 6 M GdnHCl followed by concentration using an Amicon Centricon 10, and then kept in the cold for >48 h to ensure complete unfolding.

Size-Exclusion Chromatography. BDNF unfolded in 6 M GdnHCl at protein concentrations of either 18 or 1.8 mg/mL was diluted 20-fold into Dulbecco's phosphate–buffered saline (PBS) (Gibco), pH 7.1, to initiate refolding. At various times, 15-μL aliquots of the refolding mixture were injected onto a TSK G2000SWXL (Toso Haas) size exclusion column (7.8 × 300 mm) with 1 mL/min flow and absorbance detection at 214 nm. A high ionic strength elution buffer consisting of 0.1 M phosphate and 0.5 M NaCl at pH 6.9 (buffer A) was used to minimize interactions with the column. The column was calibrated using γ-globulin, ovalbumin, myoglobin, and vitamin B-12 (Bio-Rad) as molecular weight markers.

Light Scattering. The on-line light scattering/chromatography system uses three detectors in series: an absorbance monitor at 280 nm (Knauer A293), a low-angle laser light scattering detector operating at 633 nm (Polymer Laboratories PL-LALLS, and a refractive index detector operating at 650 nm (Polymer Laboratories PL-RI). Base-line subtraction and interdetector delay corrections were made using the PL-LALLS software package (Polymer Laboratories). Size-exclusion chromatography on refolding mixtures using a TSK G2000SWXL column and buffer A was carried out as above, except that 200-μL aliquots were injected. Molecular weights were calculated from the ratio of the LALLS and RI signals, following procedures similar to those of Takagi (1990), after calibration using ribonuclease, ovalbumin, and bovine serum albumin monomer (Sigma) as molecular weight standards.

Circular Dichroism. BDNF at 18 mg/mL in 6 M GdnHCl was diluted 20-fold into PBS and the far- or near-UV CD spectrum immediately recorded. For the near-UV region, the sample was left in the 1-cm cuvette at ambient temperature, and spectra were recorded at the times indicated. For the far-UV region, in order to avoid degradation from UV radiation, the sample was diluted into a 1-mL Eppendorf tube which was incubated at ambient temperature. At the indicated times, 25-μL aliquots were placed into a 0.02-cm cuvette, the spectra were recorded, and the sample was discarded. The CD spectra were recorded using a Jasco J-500C spectropolarimeter. Near-UV CD spectra from 340 to 250 nm were recorded using a sensitivity of 50 mdeg/cm and a 50 nm/min scan speed and repeated 3×, while far-UV CD spectra from 150 to 190 nm were recorded using a sensitivity of 20 mdeg/cm and a 20 nm/min scan speed and repeated 3×. Molar ellipticities were determined assuming a mean residue weight of 113, and protein concentrations were determined from the absorbance spectrum assuming an $\epsilon_{280}^{0.1\%}$ of 1.6.

Fluorescence. The fluorescence spectra were determined using an SLM-Aminco SPF-500 spectrofluorometer, with slit widths set to obtain 5-nm resolution, using a cuvette with a path length of 0.5 cm. BDNF in 6 M GdnHCl was diluted into PBS as described for the CD determination. At the indicated times, including time zero, 45 μL of the refolding sample was removed and further diluted into 518 μL of PBS to minimize the inner filter effect. The fluorescence spectrum from 300 to 400 nm was recorded over 1 min with excitation at 280 nm. Sixty-six microliters of 1 mM anilinonaphthalenesulfonate (ANS) in PBS was then added to this same sample (final ANS concentration 105 μM), the time was noted, and the spectrum from 400 to 550 nm was recorded over 1.5 min with excitation at 380 nm. In separate experiments in which we tried to use ANS to perturb the refolding kinetics, the same dilution and sampling protocol was used, but 105 μM ANS was present either in the 6 M GdnHCl and throughout the refolding and spectroscopy or in the PBS used to initiate refolding and thereafter.

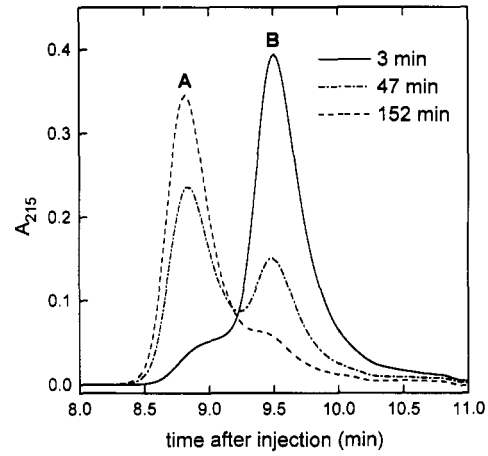


FIGURE 1: Size-exclusion chromatograms of BDNF refolding mixtures. Refolding was initiated by 20-fold dilution of BDNF in 6 M GdnHCl with PBS to give a BDNF concentration of 0.09 mg/mL. After 3, 47, or 152 min, aliquots of the refolding mixture were injected into the SEC column for analysis (see Materials and Methods).

Kinetic Analysis. Fitting of the SEC, CD, and fluorescence kinetics to single exponentials was done with the program Origin (Microcal Software). Simulations of kinetic reaction schemes were done by numerical integration of the differential equations using the Bulirsch–Stoer method and the program Diffeq (Micromath Scientific Software).

RESULTS AND DISCUSSION

Refolding Kinetics Monitored by Size-Exclusion Chromatography. BDNF was unfolded in 6 M GdnHCl, and the lack of secondary and tertiary structure was verified by CD spectroscopy (see below). Refolding was initiated by 20-fold dilution of either a high or low concentration stock of unfolded BDNF into pH 7.1 phosphate-buffered saline to give final protein concentrations of either 0.09 or 0.9 mg/mL. At various times, aliquots of the refolding mixture were analyzed by size-exclusion chromatography (SEC) in a high ionic strength mobile phase, as described under Materials and Methods. Several representative chromatograms are shown in Figure 1. At all times, two peaks are seen. The peak at ~8.8 min after injection (peak A) is barely visible when the sample is injected after 3 min of refolding. This peak increases as refolding time increases, while the peak at ~9.5 min after injection (peak B) decreases. Peak A elutes exactly at the position seen for native BDNF dimer (data not shown). Peak B elutes near the position expected for *folded* BDNF monomer, i.e., at an apparent molecular weight of 14 000 based on calibration with native globular proteins. Unfolded BDNF, if present, should not elute at all due to strong interactions with the column. Thus, these results also indicate that substantial refolding takes place in less than the ~12-min minimum time from initiation of refolding to observation by this technique.

As shown in Figure 2, the rate of the conversion from peak B to A is remarkably independent of protein concentration. Single-exponential fits of these data give decay times of 0.74 ± 0.04 and 1.0 ± 0.1 h at 0.9 and 0.09 mg/mL, respectively.[2]

[2] The reported uncertainty in fitted rates is twice the standard error determined from the fit, *i.e.*, an approximate 90% confidence interval.

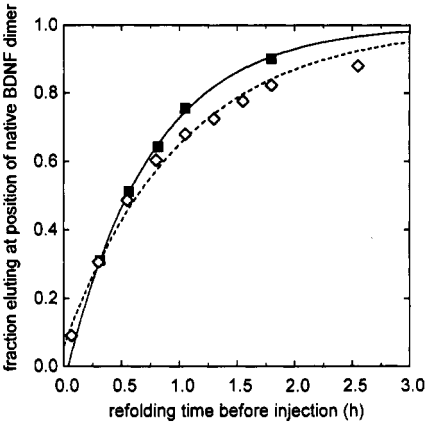


FIGURE 2: Fraction of BDNF eluting at native dimer position of SEC *vs* refolding time. The fraction was calculated from the integrated areas of peaks A and B (Figure 1) observed in each chromatogram, for refolding at the indicated BFNF concentrations. Note that the time axis does not include the ~10 min the sample is on the column prior to observation, during which further refolding may occur. Filled squares are for samples at 0.90 mg/mL after 20-fold dilution into PBS; open diamonds are for samples at 0.09 mg/mL. The curves are fits of single exponentials with decay times of 0.74 (solid) and 1.0 h (dashed), respectively.

Therefore, a 10-fold decrease in concentration has produced only a ~25% decrease in assembly rate. The data at the higher protein concentration are well represented by a single exponential, but at the lower concentration, the kinetics may be more complex. At the lower protein concentration, the total area of the two peaks remains constant within 5% over 2.5 h. At the higher protein concentration, the total area decreases with time to a final yield of ~60%, presumably due to aggregated material which is either lost to surfaces or remains bound to the column. Overall, the SEC results suggest that after a few minutes of refolding the predominant form of BDNF is a hydrodynamically compact, monomeric folding intermediate which gives rise to a dimeric species with a half-time of about 30 min at 0.9 mg/mL.

SEC with Light Scattering Detection. The SEC chromatograms *suggest* that the two peaks seen during refolding represent monomeric and dimeric species, but it is surprising that a monomer → dimer process would have a rate almost independent of concentration. Since SEC elution positions depend on conformation as well as molecular weight, it is critical to confirm this assignment. The SEC analysis was therefore repeated with on-line light scattering detection to unambiguously determine molecular weights. Injection at the higher protein concentration after 27 min of refolding gave the data shown in Figure 3. Clearly, peak A is approximately twice as intense in the scattering chromatogram as it is in the absorbance chromatogram (or the refractive index chromatogram, not shown), as expected if it has twice the molecular weight. This same pattern of relative signals was also observed for injection after 1 or 150 min of refolding. Quantitative analysis of the peak heights gives molecular weights of 27 300 ± 1000 and 13 700 ± 1000 for peaks A and B, respectively. Since the formula weight of BDNF monomer is 13 637, this confirms the peak assignments to dimers and monomers.

The fact that peak B is both monomeric and compact might seem to imply that it represents monomers with native secondary and tertiary structure. However, native monomers would be expected to associate to dimers very rapidly. Protein subunit association rates are generally relatively insensitive to structural details, and assembly studies of a wide variety of multimeric proteins yielded association rate constants of

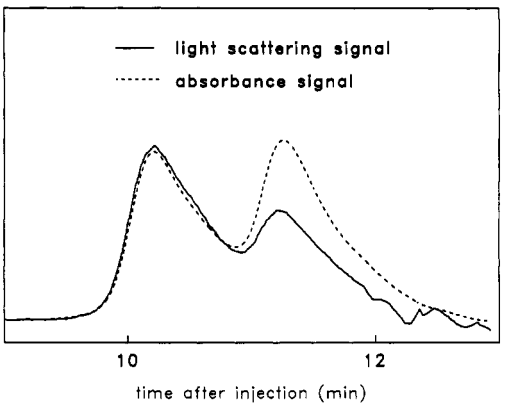


FIGURE 3: SEC chromatography with light scattering and absorbance detection. (Solid line) Low-angle laser light scattering intensity; (dashed line) absorbance at 280 nm. The vertical scales have been adjusted to match the height of the first peak. The elution times and peak shapes differ from Figure 1 because this is a different chromatography system and an older TSK SW2000XL column with poorer resolution.

at least 10^3 M^{-1} s^{-1}, and often 1–3 orders of magnitude faster (Jaenicke, 1987; Turner *et al.*, 1981). Therefore, the half-time for assembly of a BDNF dimer from native monomers at the higher protein concentration should be at most 10 s, and probably is ~0.1 s. Further, even if the association rate of native BDNF monomers is orders of magnitude slower than that of other proteins, the dimer formation rate would still be strongly concentration dependent, contrary to the data in Figure 2.

Therefore, peak B cannot be native monomer, and the unexpected result is that peak B must represent some long-lived folding intermediate which is hydrodynamically compact yet lacks sufficient structure to assembly into a dimer. To further characterize the structure of these folding intermediates, the refolding kinetics were studied by CD and fluorescence spectroscopy.

Circular Dichroism. The refolding of BDNF was followed by both far-UV CD (secondary structure) and near-UV CD (tertiary structure) and is shown in Figure 4. Figure 4A shows the near-UV CD spectra resulting during the gradual refolding of BDNF. The large negative trough near 283 nm, attributable to disulfide bonds, is totally absent in the presence of GdnHCl, and barely evident immediately upon dilution into PBS (6-min spectral acquisition time). This peak has substantially re-formed by 20 min, and attains the intensity of the signal from the native dimer within 2 h. This indicates that the tertiary structure of the BDNF is totally unfolded in the GdnHCl, remains unfolded initially in the PBS, and refolds within 2 h. These results are shown graphically in Figure 5, where the data at 283 nm have been normalized to show the fractional change in ellipticity. Fitting these data to a single exponential gives a decay time of 0.57 ± 0.07 h. While this is ~20% faster than the fit to the corresponding SEC data, we do not believe this is truly a significant difference, given the scatter in the data sets and the 6-min spectral acquisition time for the CD data.

The refolding of the secondary structure of BDNF is shown in Figure 4B. As previously reported (Radziejewski *et al.*, 1992; Narhi *et al.*, 1993a), the far-UV CD spectrum of BDNF indicates that it is a β-sheet protein, with characteristic ellipticity in the 210–225-nm region of the spectrum. However, this protein also has a characteristic positive ellipticity in the 230–240-nm region which has been attributed to either secondary (Radziejewski *et al.*, 1992) or tertiary (Narhi *et al.*, 1993a) structure. As indicated in Figure 4B, the spectrum

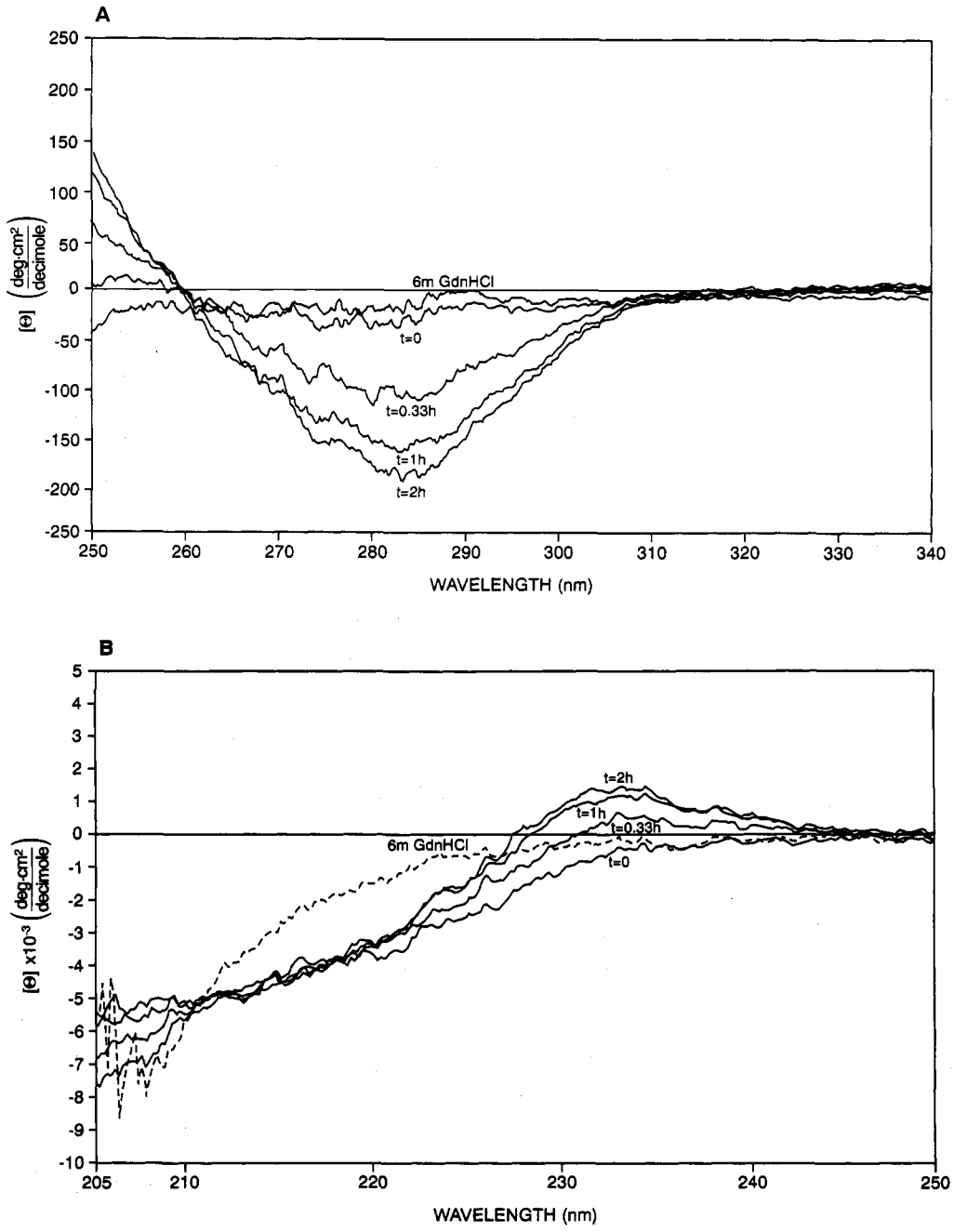


FIGURE 4: Changes in CD spectra during refolding. (A) Near-UV region; (B) far-uv region. Spectra were taken at the indicated times after 20-fold dilution of 18 mg/mL BDNF in 6 M GdnHCl into PBS, and required 6 min for data acquisition. Reference spectra of BDNF unfolded in 6 M GdnHCl are also shown.

of the protein in 6 M GdnHCl lacks either of these features, indicating that the molecule is fully unfolded. Immediately upon dilution into PBS (6-min spectral acquisition time), the 233-nm peak is still absent, but the characteristic spectrum in the 220-nm region has already been formed. The secondary structure features in the 220-nm region remains essentially unchanged as refolding proceeds, except possibly for some small changes due to overlap with the broad 233-nm feature. The refolding kinetics at 233 nm are also shown in Figure 5. Single-exponential fits to these data give a decay time of 0.96 ± 0.33 h, which is not significantly different from that for the 283-nm or SEC data.

From this experiment, three conclusions were reached. First, immediately upon dilution into PBS, the BDNF appears to have considerable secondary structure but no well-defined tertiary structure. Since the SEC and light scattering data show this form is also monomeric and hydrodynamically compact, it has all the characteristics generally considered to define the state known as a "collapsed form" (Kim & Baldwin, 1990). This state is also sometimes referred to as a "compact intermediate" or "molten globule" (Goto *et al.*, 1990; Kuwajima, 1989). Second, the time course of appearance of the 233-nm peak corresponds to that of the 283-nm peak, not the 222-nm peak, confirming that this feature must be due to tertiary rather than secondary structure. The 283-nm feature could be due to stacking of aromatic rings, as has been described for *fd* bacteriophage (Arnold *et al.*, 1992). Lastly, the similarity of the refolding time courses seen by CD and SEC, even though the SEC was done in a high-salt buffer, indicates that the higher ionic strength during the SEC analysis did not significantly alter the species distribution that was present prior to the chromatography.

Fluorescence. The refolding of BDNF was also monitored both by intrinsic fluorescence and by the hydrophobic

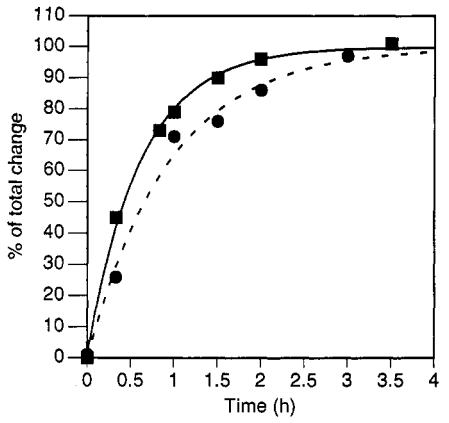


FIGURE 5: Time dependence of the fractional change in CD intensity. (Solid squares, solid line) 283 nm; (solid circles, dashed line) 233 nm. CD intensities were fitted to a single exponential to give the curves shown, with decay times of 0.57 and 0.96 h for 283 and 233 nm, respectively. The fitted intensities at $t = 0$ and $t = \infty$ were used to convert the data to fractional change for easier comparison of the data. The actual ellipticities ranged from −39 to −201 deg·cm²/dmol at 283 nm, and from −500 to 1590 deg·cm²/dmol at 222 nm.

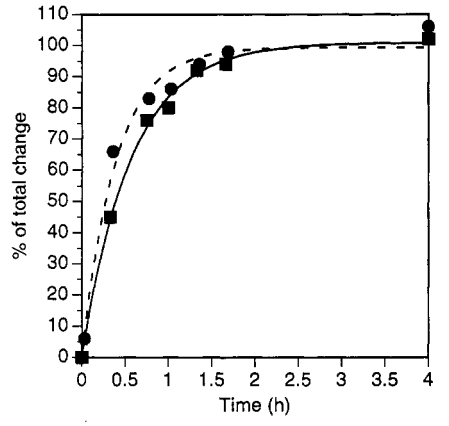
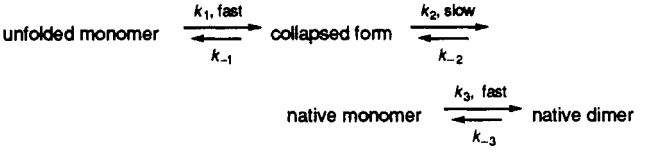


FIGURE 6: Time dependence of the fractional change in intrinsic and ANS fluorescence. (Solid squares, solid line) Intrinsic fluorescence intensity with excitation at 280 nm, observed at the emission peak, which changes from 350 to 344 nm during this time period. (Solid circles, dashed line) ANS fluorescence intensity with excitation at 380 nm, observed at the emission peak, which changes from 500 to 523 nm during this time period. Spectra were taken at the indicated times after 20-fold dilution of 18 mg/mL BDNF in 6 M GdnHCl into PBS, and required 1–1.5 min for data acquisition. The fluorescence intensity data were fitted to single exponentials and the data converted to fractional change as in Figure 5. The fitted exponential decay times were 0.61 and 0.40 h for the intrinsic and ANS fluorescence, respectively. The actual relative fluorescence intensities ranged from 4.0 to 8.2 for the intrinsic fluorescence and from 8.3 to 3.8 for the ANS fluorescence.

fluorescent probe ANS, as described under Materials and Methods. The intrinsic fluorescence spectrum of the BDNF at time zero (1-min spectral acquisition time) has a peak at 350 nm. After 80 min, the spectrum has shifted to a peak at 344 nm, and the relative intensity has doubled (data not shown). The time course of refolding as monitored by the fractional change in intrinsic fluorescence intensity is shown in Figure 6. These data are well represented by a single exponential with a decay time of 0.61 ± 0.06 h, a rate which is indistinguishable from that for the CD at 283 or 233 nm. This demonstrates that the environment around the fluorescent Trp(s) is changing in concert with the overall tertiary structure of the molecule.

ANS is a hydrophobic fluorescent probe which binds to hydrophobic patches on proteins and, when bound, fluoresces upon excitation at 380 nm. The intensity and wavelength of the resulting fluorescence indicate the extent of hydrophobicity of the protein region involved. When BDNF is initially diluted into PBS (1.5-min spectral acquisition time), the ANS spectrum has a peak at 500 nm and a relative fluorescence intensity of 8. This indicates substantial interaction between the probe and protein, consistent with a collapsed form, but the wavelength indicates a relatively hydrophilic surface. As the protein refolds, the fluorescence peak red-shifts to 523 nm, and the relative intensity decreases to 3.80. BDNF which was never unfolded has an ANS spectrum similar to that after 4 h of refolding (data not shown). Thus, the folded, intact dimer does not interact substantially with ANS, and the protein surface is very hydrophilic. The kinetics of the fractional change in ANS fluorescence intensity at the emission maximum are also shown in Figure 6. Fitting these data to a single exponential gives a decay rate of 0.40 ± 0.16 h. While this is faster than the intrinsic fluorescence data, we do not believe this is truly a significant difference, but rather reflects the poorer signal/noise ratio for ANS fluorescence and the difficulty of accurately measuring the intensity of this rapidly shifting peak.

Scheme I



Scheme II

Overall Folding and Assembly Pathway. The combined spectroscopic, SEC, and light scattering data specify the appearance of secondary, tertiary, and quaternary structure. Upon dilution into refolding conditions, secondary structure rapidly (<1 min) re-forms, giving a monomeric collapsed form. Thereafter, all CD and fluroescence markers for native tertiary structure appear to increase at the same rate, and to closely track the appearance of the dimers seen by SEC. Thus, there is no evidence for accumulation of native monomers. The marked lack of concentration dependence shown in Figure 2 implies that the rate-limiting step must occur prior to reaching the native tertiary structure from the collapsed form, with dimer assembly proceeding relatively rapidly once a native tertiary structure is reached. It appears that two reaction schemes for assembly may be able to explain these results (Schemes I and II). The key difference between these schemes is that in Scheme II the collapsed form is a side path, *not* on the pathway to a native structure, while in Scheme I the collapsed form is a true intermediate between the unfolded and native states.

We have simulated both kinetic schemes by numerical methods, trying to reproduce the three key features of the kinetics: (1) the initial rapid disappearance of unfolded monomer and the rapid appearance of the collapsed form; (2) the slow disappearance of the collapsed form and conversion to native dimer without accumulation of native monomer; and (3) the only ~25% decrease in the rate of step 2 when the protein concentration is decreased from 0.9 to 0.09 mg/mL. The simulations show that both schemes can reproduce these features with plausible rates for the individual reactions,

although the mechanisms leading to the overall slow formation of native dimer are different. For Scheme I, the slow conversion from the collapsed form to native monomer is the primary rate-limiting step. The data from Figure 2 are reproduced well with $k_1 = 1 \times 10^{-1}$ s^{-1}, $k_{-1} = 1 \times 10^{-4}$ s^{-1}, $k_2 = 3.5 \times 10^{-4}$ s^{-1}, $k_{-2} = 1 \times 10^{-3}$ s^{-1}, $k_3 = 5 \times 10^4$ M^{-1} s^{-1} and $k_{-3} = 5 \times 10^{-9}$ s^{-1}, but these values are *not* unique. For Scheme II, where unfolded monomer can convert directly to native monomer, the fact that we do not see an initial burst of native monomer or dimer implies that k_1 is significantly faster than k_2. This, in turn, implies that when the collapsed form reverts to unfolded monomer, it is more likely to return to the collapsed form than to fold to a native monomer. Therefore, for Scheme II, the overall slow conversion to the native state is a result of kinetic competition between the two folding pathways for an unfolded monomer, as well as a required slow rate of folding to the native monomer (k_2). Simulations for Scheme II show that the data from Figure 2 are reproduced well with $k_1 = 1 \times 10^{-1}$ s^{-1}, $k_{-1} = 1.3 \times 10^{-2}$ s^{-1}, $k_2 = 2 \times 10^{-3}$ s^{-1}, $k_{-2} = 1 \times 10^{-3}$ s^{-1}, $k_3 = 5 \times 10^4$ M^{-1} s^{-1}, and $k_{-3} = 5 \times 10^{-9}$ s^{-1}, but again these values are not unique.

Since both schemes seem to be able to reproduce the main features of the data, how could we distinguish between them? For Scheme II, there can be an initial small burst of native dimer during the time that the equilibrium is being established between unfolded monomer and the collapsed form, while for Scheme I at early times there is always a small lag in formation of native dimer. Unfortunately, the simulations suggest that the size of this initial burst, if present, is likely to be only a few percent of the total, and therefore to be too small to be seen by spectroscopic techniques. The putative initial burst of native dimer would occur on a time scale of ~1 min or less, which is too fast to be seen by separation techniques such as SEC.

We also attempted to resolve these two schemes by using ANS to perturb the kinetics. Since ANS is expected to bind strongly and extensively to the unfolded state, and we have shown that only a small amount of ANS binds to the collapsed form, we thought that ANS might substantially slow the folding of the unfolded state. (That is why it was added only immediately before the measurements for the data in Figure 6.) If so, the addition of ANS after 1–2 min of refolding would have little effect on Scheme I, but would significantly disturb Scheme II, where the collapsed form must pass back through the unfolded state to reach the native conformation. However, we find that ANS has no significant effect on the refolding kinetics if added either directly to the unfolded material in GdnHCl or after 1 min of refolding. We believe this indicates that the ANS probably cannot bind in the presence of GdnHCl, and that upon dilution into refolding conditions the rate of folding of BDNF is much more rapid than the binding of ANS to the unfolded state. Alternatively, ANS may bind to the unfolded state but be unable to significantly perturb its folding kinetics. In conclusion, we are unable at present to experimentally distinguish between these two reaction schemes.

A Significant Structural Rearrangement of the Collapsed Form Is Required To Form a Dimer Interface. It is remarkable that dimers are not formed until so late in the folding. The monomer–dimer association in native BDNF appears to be extremely strong. Two studies by analytical ultracentrifugration have failed to show any evidence for BDNF monomer, even at concentrations near 10^{-10} M (Narhi *et al.*, 1993a; Radziejewski *et al.*, 1992). Assuming the dissociation constant for BDNF dimer is similar to the 10^{-13} M reported for nerve growth factor (NGF) (Bothwell & Shooter, 1977), and given that the collapsed form forms less than 10% dimer even at the ~0.1 mM concentration used in these experiments, the monomer → dimer association of the collapsed form must be ~10^{10} times weaker than that of the native structure (a free energy difference of ~13 kcal/mol).

The inability of the collapsed form to dimerize is surprising in view of what is known about the structure of NGF. A recent crystal structure of NGF has shown the NGF monomer to have an extensive flat hydrophobic surface constituting the dimer interface. The formation of dimers was estimated to bury over 2300 Å^2 of surface area, and the hydrophobic free energy to contribute ~58 kcal/mol to the stabilization of the dimer (McDonald *et al.*, 1992). With such a strong hydrophobic driving force, it would seem likely that dimerization would occur prior to completion of folding.[3]

Given the large hydrophobic area expected on the surface of a native monomer, the poor dimerization of the collapsed form has important implications about its structure. The relatively hydrophilic surface of the collapsed form seen by ANS fluorescence, as well as the weak association to dimers, implies that most of these hydrophobic groups are internalized in the collapsed form. This view is further supported by the relatively low tendency of the collapsed form to form insoluble aggregates even at 1 mg/mL concentrations. Therefore, an important conclusion from these studies is that a significant structural arrangement of the collapsed form is needed in order to form a dimer interface.

Furthermore, the probable high activation energy for such a structural change can easily explain why this is the rate-limiting step in refolding in Scheme I. On the other hand, this significant structural difference between the collapsed form and the native state may also be viewed as evidence that this intermediate is not on the pathway to the native state, and therefore as supportive of reaction Scheme II.

Another question raised by these data is whether proline isomerization plays a role in these kinetic and structural phenomena. Certainly it is possible that one of the structural differences between the collapsed form and a native monomer is a difference in the *cis*↔*trans* isomerization of one or more of the three prolines in BDNF. However, a localized change in proline conformation is clearly insufficient to explain the large change in exposure of hydrophobic groups, so putative proline isomerization(s) would have to be strongly coupled to conformational changes in many residues. Also, if proline isomerization was the primary factor responsible for the slow refolding kinetics, then the homogeneity of the kinetics, and the lack of an initial burst of native dimer, would require that essentially all unfolded BDNF molecules exist as the nonnative isomer, which seems unlikely. On the other hand, we have observed that the refolding kinetics depend on the length of exposure to GdnHCl [see Narhi *et al.* (1993b)], which is highly suggestive that proline isomerization may be important.

Significance. We have shown that BDNF is kinetically trapped in a monomeric collapsed form which cannot dimerize. BDNF is certainly not the only oligomeric protein in which nonnative monomers accumulate during refolding. However, for BDNF, the nonnative monomer is both usually long-lived and unusually soluble (Jaenicke, 1987). For the first time,

[3] In fact, these data do not totally exclude the possibility that dimerization occurs before the final, native structure is reached. They do, however, require that the rate of folding from a putative nonnative dimer to the native dimer is rapid compared to the rate at which the collapsed form enters a state capable of dimerization.

this has permitted us to demonstrate that a collapsed form has an extremely large difference in association constant from the native structure and that reaching the native tertiary structure requires not just a "reshuffling" or "annealing" step, but rather a significant re-exposure of surface hydrophobic groups. Indeed, it appears that on entering strongly refolding conditions, unfolded BDNF not only rapidly collapses to a compact state but also actually overshoots the internalization of hydrophobic groups needed to form the native structure.

ACKNOWLEDGMENT

We thank Thomas Boone and Jeff Hogan for providing the BDNF used in these studies.

REFERENCES

Acklin, C., Stoney, K., Rosenfeld, R. A., Miller, J. A., Rohde, M. F., & Haniu, M. (1993) *Int. J. Pept. Protein Res. 41*, 548–552.

Arnold, G. E., Day, L. A., & Dunker, A. K. (1992) *Biochemistry 31*, 7948–7956.

Bothwell, M., & Shooter, E. (1977) *J. Biol. Chem. 252*, 8532–8536.

Goto, Y., Takashi, N., & Fink, A. L. (1990) *Biochemistry 29*, 3480–3488.

Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol. 49*, 117–237.

Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631–660.

Kuwajima, K. (1989) *Proteins: Struct., Funct., Genet. 6*, 87–103.

McDonald, N. Q., Lopatto, R., Murray-Rust, J., Gunning, J., Wlodawei, A., & Blundell, T. L. (1992) *Nature 341*, 149–152.

Narhi, L. O., Rosenfeld, R., Talvenheimo, J., Prestrelski, S. J., Arakawa, T., Lary, J. W., Kolvenbach, C. G., Hecht, R., Boone, T., Miller, J. A., & Yphantis, D. A. (1993a) *J. Biol. Chem. 268*, 13309–13317.

Narhi, L. O., Rosenfeld, R., Wen, J., Arakawa, T., Prestrelski, S. J., & Philo, J. S. (1993b) *Biochemistry* (following paper in this issue).

Radziejewski, C., Robinson, R. C., DisStefano, P. S., & Taylor, J. W. (1992) *Biochemistry 31*, 4431–4436.

Takagi, T. (1990) *J. Chromatogr. 280*, 409–416.

Turner, B., Pettigrew, D., & Ackers, G. K. (1981) *Methods Enzymol. 76*, 596–628.