Acid-Induced Unfolding of Brain-Derived Neurotrophic Factor Results in the Formation of a Monomeric "A State"

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ABSTRACT: Recombinant human brain-derived neurotrophic factor in acid undergoes a slow loss of tertiary structure as monitored by both near-UV circular dichroism and fluorescence, and appears to retain some secondary structure, as monitored by far-UV circular dichroism and Fourier transform infrared spectroscopy. This loss of tertiary structure parallels a decrease in the weight average molecular weight, from dimer to monomer, when examined using light scattering. Increasing the temperature accelerates this slow reaction. This process may be described most simply as $N_2 \rightleftarrows 2D$ where N and D are the native and denatured forms of the protein, respectively. However, the acid denaturation strongly depends on the protein concentration, with higher concentration resulting in a lower rate and extent of denaturation. This suggests that the more complicated mechanism $N_2 \rightleftarrows 2N \rightleftarrows 2D$ more accurately describes the denaturation, where the dissociation into a native monomer is the rate-limiting step, and the conversion of N to D occurs relatively rapidly. Size-exclusion chromatography (at neutral pH) at several points during denaturation further demonstrated that the amount of tertiary structure remaining paralleled the dimer concentration and also that the monomer form was long-lived, remaining as monomer during the course of the chromatography. Size-exclusion chromatography and sedimentation velocity determination indicated that the acid-denatured form is a compact molecule. On the basis of the above data, the acid-denatured form may be considered to be a monomeric compact intermediate A state with no tertiary structure but considerable secondary structure. Guanidine hydrochloride-induced denaturation appears to be entirely different from the acid-induced denaturation in that the loss of tertiary structure occurs immediately upon addition of 6 M guanidine hydrochloride.

Brain-derived neurotrophic factor (BDNF)[1] is a member of a family of neurotrophic factors, of which NGF is the parent member. BDNF was isolated in 1982 (Barde *et al.*, 1982) and cloned in 1989 (Leitrock *et al.*, 1989) and is 55% homologous to NGF. All of the members of this family contain six cysteines which form three intrachain disulfide bonds. The sequence is strongly conserved in the region about the disulfides. The members of this family have been shown to form strong noncovalent dimers in solution such that they are dimers at physiologically active concentrations (Angletti *et al.*, 1971; Bothwell & Shooter, 1977; Radziejewski *et al.*, 1992; Narhi *et al.*, 1993).

The X-ray crystallography structure of murine β-NGF revealed a secondary structure rich in β-sheet, but with no α-helix (McDonald *et al.*, 1991). It also has a very hydrophobic face involved in dimer formation, which contains residues from noncontiguous segments of the amino acid sequence. On the basis of the high degree of sequence identity, BDNF is believed to adopt a similar three-dimensional structure. We have recently studied the refolding of GdnHCl-denatured BDNF, and found that the most abundant intermediate state is monomeric and compact, and contains secondary structure but no regular tertiary structure (a "molten globule") (Philo *et al.*, 1993), and it appears that the hydrophobic face present at the dimer interface is not exposed in this compact intermediate.

Recently, Benedek and Rosenfeld (1993) reported that recombinant human BDNF gives rise to two peaks during rp-HPLC analysis in acetonitrile/TFA. When the two rp-HPLC peaks are collected and reinjected onto the rp-HPLC column following prolonged incubation at room temperature, or following titration to neutral pH, both peaks are again observed. Further, the BDNF can be converted entirely to the second peak by prolonging the residence time on the column, or by performing the chromatography at elevated temperature. This result suggests that BDNF undergoes a slow conformational change induced by either acid, organic solvents, a nonpolar column matrix, or some combination of these. The observed slow denaturation may be related to the noncovalent dimerization of BDNF discussed above. Both peaks observed under the rp-HPLC conditions appear to be monomers from 90° light scattering done using an on-line fluorescence detector. Thus, both peaks appear to be at least partially unfolded monomers, with the second peak being more hydrophobic. In order to elucidate the role of solvent in this unfolding, we decided to examine the effect of acid, in particular TFA, on the BDNF in solution. In this paper, we report that acid alone is enough to induce the unfolding of BDNF to a nonnative monomer, and that this monomer is compact and possesses secondary structure similar to the intermediate state derived upon refolding from GdnHCl and in contrast to the end product of GdnHCl-induced denaturation.

MATERIALS AND METHODS

Materials. Recombinant human BDNF derived from *Escherichia coli* was kindly supplied by Tom Boone and Jeff

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[1] Abbreviations: BDNF, brain-derived neurotrophic factor; TFA, trifluoroacetic acid; GdnHCl, guanidine hydrochloride; Tris, tris-(hydroxymethyl)aminomethane; CD, circular dichroism; rp, reverse phase; HPLC, high-performance liquid chromatography; SEC, size-exclusion chromatography; PBS, phosphate-buffered saline; FTIR, Fourier transform infrared.

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Hogan from Amgen. It was expressed in *E. coli*, refolded, oxidized, and purified by sequential column chromatography. The disulfide bond structure was identical to that of BDNF expressed in Chinese hamster ovary cells (Acklin *et al.*, 1993). The folded structures of the two molecules are identical as far as can be discerned using CD, FTIR, analytical ultracentrifugation, and GPC analysis (Narhi *et al.*, 1993). HPLC/spectrograde TFA was purchased from Pierce, ultrapure GdnHCl was purchased from Amresco, and Tris was obtained from BioRad. All other reagents were obtained commercially, and were of the highest grade generally available.

Acid-Induced Unfolding. The BDNF in PBS was dialyzed versus 1 mM Tris-HCl, pH 7.0, overnight at 4 °C. This BDNF stock solution was then diluted to the desired concentration with 1 mM Tris-HCl, pH 7.0, and the spectrum of the zero time point was recorded. The unfolding reaction was initiated by addition of concentrated acid to yield a final solution of 0.1% TFA (v/v), 10 mM phosphoric acid, or 10 mM HCl (final pH of 2.1), and the unfolding reaction was followed during incubation at appropriate temperatures as described below. The data are reported as the fractional change. This was obtained by plotting the percent of the final signal versus time, and fitting this plot with a single exponential. The end points of the reaction were determined from the resulting equation and used to determine the percent of the total change represented at each time point. The plot of this fractional change versus time is what is reported, fit by a single exponential.

Circular Dichroism. The CD spectra were recorded on a Jasco J-500C spectropolarimeter equipped with a water bath controlled thermal unit and using rectangular cuvettes with path lengths of 0.1 cm for the far-UV (250–190 nm) region and 1 cm for the near-UV region (340–240 nm). The far-UV spectrum for each time point was obtained as the average of three spectra collected over an elapsed time of 3 min, while the near-UV spectrum at each time point represented the average of two spectra collected over 10 min.

Infrared Spectroscopy. For infrared spectroscopy, a 30 mg/mL stock solution of BDNF in 1 mM Tris buffer, pD 7.0, in D_2O was prepared by buffer exchanging from a similar buffer in H_2O using Centricon-10 (Amicon) microconcentrators. Aliquots of the stock solution were diluted to 1 mg/mL either into 10 mM DCl/D_2O or into 1 mM Tris, pD 7.0. Spectra were collected immediately after dilution and again after incubating at 37 °C for 24 h. Samples of the 1 mg/mL solutions were placed in IR cells with CaF_2 windows and 100-μm spacers. Spectra were collected using a Mattson RS-1 FTIR spectrometer with an MCT detector. Four thousand scans were coadded at a resolution of 4 cm^{-1}.

Fluorescence. Fluorescence spectra were obtained using an SLM-Aminco SPF500 spectrofluorometer and a cuvette with a 0.5-cm path length in a water-cooled sample holder at 37 °C. The proteins were excited at 280 nm and the fluorescence spectra recorded from 280 to 420 nm over 2 min. The slit widths were set to provide a resolution of 5 nm.

Size-Exclusion Chromatography. The relative amounts of BDNF dimer and monomer were determined by HPLC size-exclusion chromatography. The time course of unfolding was followed by using a WISP autosampler to inject 160 μL of sample onto a Phenomenex G2000 SWXL column equilibrated in 0.1 M sodium phosphate/0.5 M NaCl, pH 7.0, connected to a Beckman System Gold and developed using a flow rate of 1 mL/min. The absorbance at 214 nm was monitored, and data analysis was performed using the Maxima Workstation from Waters. The chromatography was performed at room temperature.

Light Scattering. The light scattering system employed consists of three detectors in series: an absorbance monitor at 280 nm (Knauer A293), a 633-nm low-angle laser light scattering detector (Polymer Laboratories PL-LALLS), and a 650-nm diode laser refractive index detector (Polymer Laboratories PL-RI). This system is normally used with a size-exclusion column, but to avoid disturbance of the species distributions by the column, these experiments were performed in batch mode. The flow system and the reference cell of the refractive index detector were preloaded with the solvent for the BDNF sample described below and the base-line readings of the detectors recorded. A stock solution of BDNF in 1 mM Tris-HCl, pH 7.0, was mixed with concentrated TFA to make a final solution containing 0.1% TFA at 1.8 mg/mL protein and incubated at 37 °C. At various times during the incubation, aliquots of 1 mL were slowly manually injected into the detectors at room temperature. To avoid distortion of the scattering data from protein aggregates, dust, etc., the samples and buffer were injected through 0.02-μm Anotek filters (Whatman). Typically, it took about 10 min from the start of injection before a stable reading was obtained in the light scattering detector. In some cases, the sample remained in the system for long times to monitor the changes in the scattering signal with time. At the conclusion of each experiment, the sample was washed out with solvent to obtain post-run base-line readings. Molecular weights were computed using the ratio of the light scattering to refractive index signals following procedures similar to those of Takagi (1990). The system was calibrated using ribonuclease, ovalbumin monomer, and BSA monomer as molecular weight standards.

Sedimentation Velocity. Sedimentation velocities were measured in a Beckman XL-A analytical ultracentrifuge at protein concentrations of 0.125 mg/mL. At this low protein concentration, the sedimentation rate is independent of concentration. Therefore, for each sample the absorbance vs radius data at 9 different time points (about 5000 data points total) were fitted to the Faxén solution of the Lamm equation (Schachman, 1959) with the sedimentation and diffusion coefficients and initial concentration as fitting variables. The raw sedimentation coefficients were then corrected to standard conditions, 20 °C in pure water, using solvent densities determined with an Anton Paar DMA 602 densimeter, a partial specific volume of 0.728 mL/g calculated from the amino acid composition, and calculated viscosities (Laue *et al.*, 1992).

RESULTS

Acid-Induced Unfolding. The acid-induced unfolding of BDNF was examined using 0.1% TFA (v/v) as the acid, a protein concentration of 0.125 mg/mL, and a reaction temperature of 33 °C. The fluorescence spectra collected at several different times during the unfolding reaction are shown in Figure 1. The spectra indicate that the BDNF slowly loses its three-dimensional structure, as indicated both by the shift of the emission maximum from 343 to 350 nm and also by the quenching of the fluorescence. Both of these changes are consistent with the fluorescent Trp(s) being partially buried in the native molecule, and becoming increasingly exposed to solvent as the molecule unfolds.

The effects of exposure to TFA on the secondary and tertiary structure of BDNF were also examined by CD, and the results are shown in Figure 2. The near-UV CD spectrum, which is sensitive to tertiary structure (Figure 2A), loses both the trough at 300–270 nm and the positive signal at 250–240 nm during

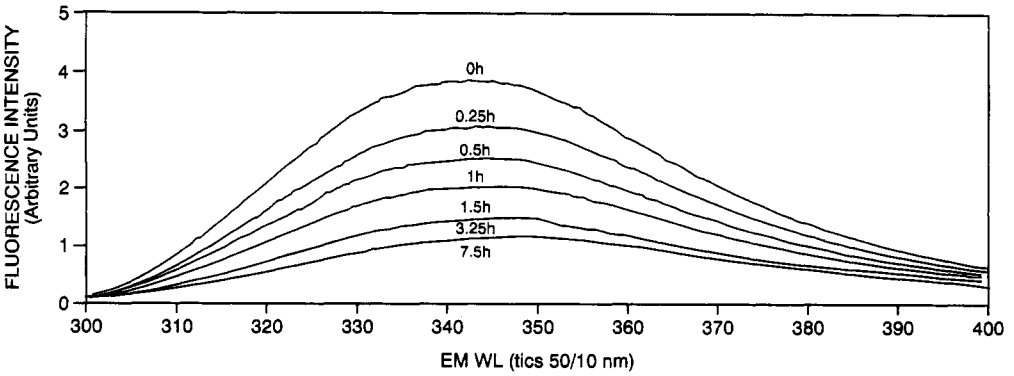

FIGURE 1: Fluorescence spectra of BDNF during acid-catalyzed unfolding. BDNF at 0.125 mg/mL was incubated in 0.1% TFA (v/v) at 33 °C. The fluorescence emission spectrum obtained upon excitation at 280 nm was recorded as described under Materials and Methods at the times indicated above the spectra. The spectra obtained at 3.25 and 7.5 h were identical.

incubation in TFA. This demonstrates that acid induces the loss of the tertiary structure of BDNF, confirming the fluorescence results. In the far-UV CD region (240–200 nm), the peak at 233 nm, believed to result from tertiary structure elements (Narhi *et al.*, 1993; Philo *et al.*, 1993), also disappears with time in TFA. However, the ellipticity from 220 to 200 nm, attributable to the β-sheet structure of BDNF, does not change significantly upon incubation with TFA. The spectrum of the end point structure in TFA is very different from that of the mostly disordered structure obtained in GdnHCl; the latter consists of a flat line from 240 to 215 nm, which then becomes increasingly negative to 200 nm [see Philo *et al.* (1993)]. This suggests that much of the secondary structure of BDNF remains following TFA-induced denaturation. The rate of these changes is shown in Figure 3; the three data sets all seem to fit well to a single exponential. The change in ellipticity at 284 nm corresponds to a decay time of 1.1 ± 0.30 h, the decrease in ellipticity at 233 nm is well represented by a single exponential with a decay time of 0.79 ± 0.26 h, and the decrease in fluorescence intensity can be fit with a single exponential with a decay time of 0.80 ± 0.28 h. Thus, the changes in structure monitored by these techniques appear to occur with an identical rate. This suggests that these techniques are following the same reaction, the unfolding of the tertiary structure of BDNF. The ellipticity at 222 nm is a combination of the contribution from the tertiary structure feature which maximizes at 233 nm and the secondary structure contributions. As the molecule unfolds and the 233-nm peak disappears, the ellipticity at 222 nm no longer contains any contributions from tertiary structure, but reflects the secondary structure alone. It is therefore impossible to determine if the small changes from 200 to 225 nm seen upon incubation with TFA can be accounted for by the decrease in tertiary structure contributions to this spectral region alone, or if there are differences due to some slight alterations in secondary structure as well. Thus, the kinetics at 222 nm are impossible to interpret, and are not shown.

In order to ascertain whether the TFA-induced unfolding of BDNF is the result of the pH change, or is specific to TFA, BDNF was incubated under the same conditions in 10 mM phosphoric acid and in 10 mM HCl. The rate of the denaturation was similar with all three acids, and the end point structure appeared to be identical (data not shown). This implies that the unfolding of BDNF described here is induced by acid and is not due to any specific ionic effects.

Effect of Protein Concentration and Temperature. The acid-induced unfolding of BDNF at 37 °C was examined at several different protein concentrations, and the rate of denaturation was observed to be concentration dependent, as shown in Figure 4. At protein concentrations of 1 mg/mL and higher, the reaction proceeds quite slowly, taking well over 20 h to reach equilibrium at 37 °C, and at the highest protein concentration, a significant amount of native dimer is present even after a 20-h incubation. At lower protein concentrations, the denaturation proceeds very rapidly. The exponential decay times are 0.28 ± 0.05 h at a protein concentration of 0.05 mg/mL, 0.48 ± 0.12 h at 0.25 mg/mL, and 2.8 ± 1 h at 1.8 mg/mL. Native BDNF exists as a dimer; thus, the concentration dependence of acid-induced unfolding suggests that the dimer to monomer equilibrium is rate-limiting for this process.

The temperature dependence of the acid denaturation, at constant concentration (0.125 mg/mL), was also examined. At temperatures of 15 °C or less, little change in structure is seen over 48 h, while at 50 °C and higher the loss of tertiary structure occurs very rapidly. The higher temperatures were also observed to shift the equilibrium entirely to the denatured form at the high protein concentration.

Association State of BDNF during Unfolding. As described above, the rate-limiting step in the acid-induced unfolding of BDNF appears to be the conversion of the dimer to monomer. To examine this further, the time course of acid denaturation was followed by laser light scattering in a batch mode as described under Materials and Methods. The protein concentration for these experiments was 1.8 mg/mL, due to the limits of sensitivity of this light scattering detector. The results are summarized in Table I. It is evident that the weight average molecular weight slowly decreases with time from that of dimer at time zero to that of monomer after about 22.5 h in TFA at 37 °C. Intermediate values are observed at 1 and 2.75 h, indicating that at these times a mixture of both monomer and dimer exists.

The observed change in association state can also be characterized by SEC if the denatured monomer is resolved from the dimer peak. This experiment was carried out with 0.125 mg/mL BDNF incubated in 0.1% (v/v) TFA at 37 °C (the column itself was equilibrated in 0.1 M sodium phosphate/0.5 M NaCl, pH 7.0). The results are shown in Figure 5. BDNF which has been briefly exposed to 0.1% TFA elutes as a single peak with an elution time of 9.2 min. After 2 h in 0.1% TFA, all of the protein elutes at 10.1 min. At intermediate times in 0.1% TFA, both peaks are present at various ratios. We have extensively characterized these two peaks as reported elsewhere (Philo *et al.*, 1993) and showed that the first and second peaks correspond to dimer and monomer, respectively. Therefore, it appears that after brief exposure to 0.1% TFA much of the protein remains as dimer, and also that the denatured monomer in 0.1% TFA remains

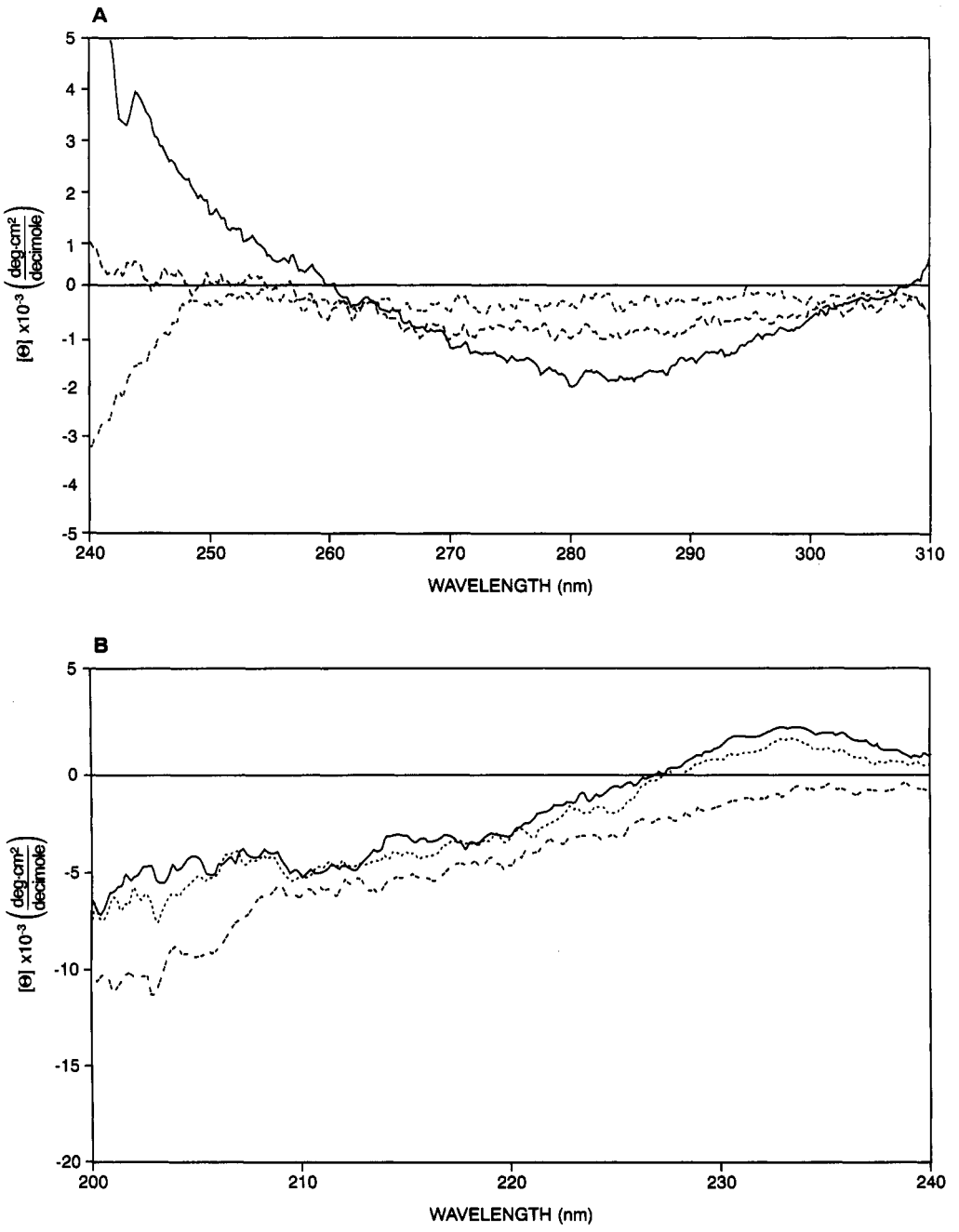

FIGURE 2: Effects of exposure to TFA on the near-UV CD spectra (A, tertiary structure) and far-UV CD spectra (B, secondary structure) of BDNF. Incubation conditions were as described in Figure 1. CD spectra shown were recorded at t = 0 (—), t = 1 h (- - -), and t = 3 h (– –) for the near-UV CD region and at t = 0 (—), t = 0.5 h (···), and t = 3.5 h (- - -) in the far -UV as described under Materials and Methods.

in the monomeric form during the time course of the chromatography. This monomeric form is relatively stable, since it does not reassociate to dimer during the course of the chromatography. Thus, the relative amount of the dimer remaining in 0.1% TFA can be estimated using SEC. When the relative area of the peak corresponding to dimer is plotted versus time, it parallels the near-UV CD ellipticity and fluorescence spectral shifts (data not shown). This confirms that the dissociation is a slow process and occurs concurrently with the loss of tertiary structure of the protein. It appears that the BDNF in acid slowly converts to a stable monomer with no detectable tertiary structure, but considerable secondary structure.

Structure of Unfolded BDNF. From the above results, it appears that the acid-denatured monomer of BDNF has structural characteristics of what is referred to as the "A state" (Kuwajima, 1977, 1989) and is compact, and thus could be called a "collapsed form" (Kim & Baldwin, 1990), "compact intermediate", or "molten globule" (Goto & Fink, 1989; Christenson & Pain, 1991; Kuwajima, 1989). As discussed above, far-UV CD suggested the presence of β-sheet structures in the acid-denatured BDNF. To further clarify the secondary structure, we employed FTIR, which measures β-sheet structure more accurately. Figure 6 shows the second-derivative spectra in the amide I region of BDNF in 10 mM DCl and as a control in 1 mM Tris, pD 7.0, after incubating at 37 °C for 24 h. The control spectrum is essentially the same as that taken prior to incubation, indicating that no conformational changes take place at neutral pH. In contrast, the spectrum of BDNF in 10 mM DCl is markedly different indicating that a conformational transition has occurred at the acid pH. The spectrum of BDNF at low pH still shows

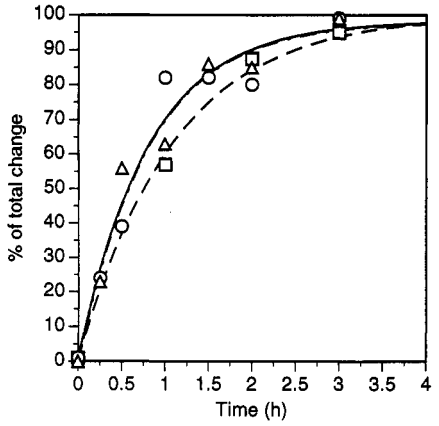

FIGURE 3: Rate of acid-induced unfolding of BDNF. The rate of the fractional change of unfolding of BDNF following the addition of TFA is plotted as determined from the far-UV CD spectra by following changes in the molar ellipticity at 233 nm (tertiary structure, ○), from the near-UV CD spectra by following changes in the molar ellipticity at 284 nm (tertiary structure, □), and from changes in the fluorescence intensity at 350 nm (△). CD and fluorescence spectra were obtained as described under Materials and Methods, and in Figures 1 and 2. The time shown is the time after TFA addition when the accumulation of the spectra began. Each far-UV CD spectrum took 3 min to accumulate, the near-UV CD spectra took 10 min, and the fluorescence spectra were accumulated over 1.5 min. The absolute ellipticities ranged from −181 to −27 at 284 nm and from 2300 to −870 at 233 nm, and the fluorescence at 350 nm ranged from 7.0 to 2.22.

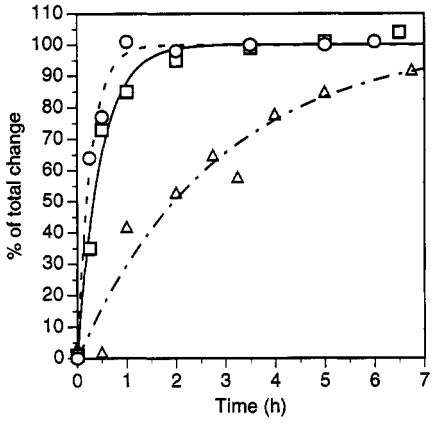

FIGURE 4: Effect of protein concentration on the rate of acid-catalyzed unfolding of BDNF. BDNF solutions at protein concentrations of 1.8 (△), 0.25 (□), and 0.05 mg/mL (○) were made 0.1% in TFA (v/v) and incubated at 37 °C, and the fluorescence spectra were determined at various times as described in Figure 2. The fractional change in fluorescence intensity is plotted versus time. Fluorescence intensities ranged from 8.28 to 4.26 at 1.8 mg/mL, from 8.1 to 2.5 at 0.25 mg/mL, and from 8.4 to 2.6 at 0.05 mg/mL.

Table I: Weight Average Molecular Weight of Acidified BDNF Determined by Light Scattering Values[a]

time (h) in TFA at 37 °C	molecular weight × 10^{-3}
0	23.1[b]
1	20.0
2.75	18.0
22.5	13.6

[a] The times indicated do not include the ~15 min at room temperature for injection and observation; thus, some further denaturation may occur prior to the reading. [b] All values ±1.

the major peaks present in the control spectrum near 1636 and 1685 cm^{-1}. However, these peaks are much broader and the minor peaks are poorly resolved. This indicates that at low pH, BDNF contains the major elements of secondary structure, i.e., β-sheet structure, but that the molecule has a

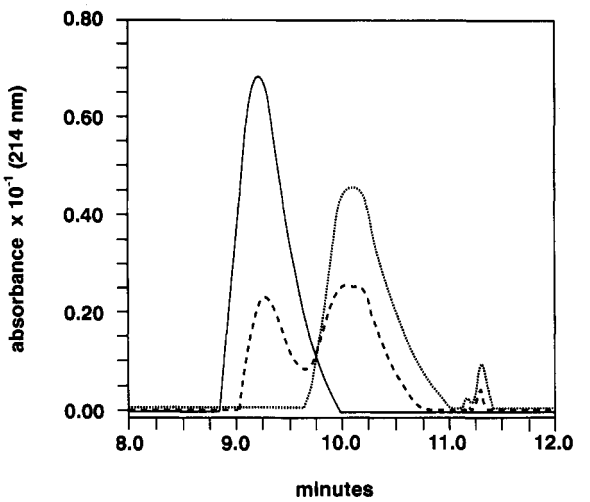

FIGURE 5: Effect of TFA on the size of BDNF. BDNF at 0.125 mg/mL was incubated at 37 °C in 0.1% TFA (v/v), aliquots were injected onto a TSK 2000 SW SEC column developed in 0.5 M NaCl/0.1 M sodium phosphate, pH 7, and the absorbance at 215 nm was recorded at t = 0 (—), t = 1 h (---), and t = 3 h (···).

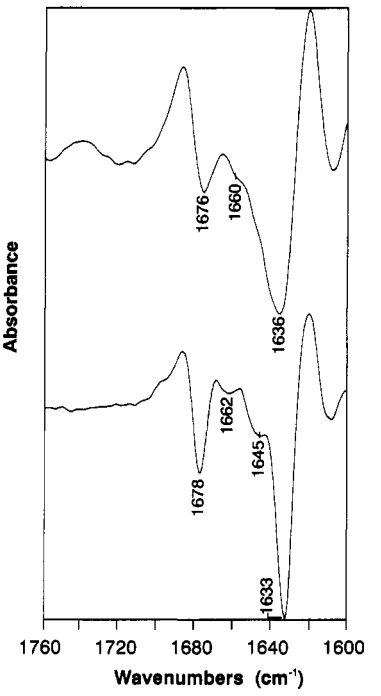

FIGURE 6: Second-derivative infrared spectra in the amide I′ region for BDNF in (top) 10 mM DCl and (bottom) 1 mM Tris buffer, pD 7.0. The protein concentration is 1 mg/mL. Spectra were smoothed with a nine-point smoothing function. The broad peak near 1715–1720 cm^{-1} in the top spectrum arises from the C=O stretch vibrations of side-chain carboxyl groups which do not ionize at the low pH.

high degree of conformational microheterogeneity indicating flexibility or enhanced mobility, consistent with the formation of a molten globule.

We have also examined the compactness of the protein by sedimentation velocity. The sedimentation velocity of a BDNF sample equilibrated in 1 mM Tris-HCl, pH 7.0, then adjusted to 0.1% in TFA (v/v), and held at 37 °C for 24 h was measured, also at 37 °C. The same run also included a sample of native BDNF in 1 mM Tris-HCl, pH 7. These data gave $s_{20,w}$ values of 1.67 S for monomeric BDNF in acid and 2.57 S for the native BDNF dimer. A typical globular protein of M_r 13 500 and $\bar{v}$ = 0.728 mL/g would have an $s_{20,w}$ of 1.59 S (van Holde, 1975). Therefore, the 1.67S value for acid-denatured BDNF indicates a compact structure. A better comparison can be

made with the value for the native BDNF dimer. If it is assumed that the hydration of monomeric BDNF in acid is the same as that for dimeric native BDNF, and that both are approximately spherical, the 2.57S value for the dimer predicts a value of 1.62 S for a native BDNF monomer, while treating the dimer as a pair of spheres implies 1.71 S for a native monomer (Van Holde, 1975). Thus, the BDNF monomer in acid assumes a compact structure, and has hydrodynamic properties similar to those expected for half of the native dimer.

GdnHCl-Induced Denaturation. The GdnHCl-induced denaturation of BDNF proceeds by a different mechanism. CD spectroscopy indicates that tertiary and secondary structures are lost immediately (within 1 min) upon the addition of 6 M GdnHCl, resulting in the spectrum of a disordered molecule. No changes are observed between the initial near- and far-UV CD spectra and those obtained up to 2 h later. However, when the GdnHCl-denatured BDNF is analyzed by SEC, there is a difference between the early stage denatured state and the later stage denatured state. At the earlier stages of GdnHCl-induced denaturation, the BDNF renatures to dimer during the SEC analysis, while at later times it remains as monomer over the course of the analysis. This suggests that there are two monomeric forms of BDNF in 6 M GdnHCl; the initial unfolded monomer can dimerize rapidly, while the end stage unfolded monomer has reached a state where the contact surface required for dimerization has been rearranged.

DISCUSSION

The near-UV CD and fluorescence signals of BDNF decrease slowly with time when the protein is acidified, indicating a slow conversion of the native form (N) to the denatured form (D). It is unlikely that the protein undergoes a gradual loss of tertiary structure via different intermediate stages, especially as the unfolding kinetics at a given temperature and protein concentration are the same regardless of the technique employed to follow the reaction. This denaturation may be described simply as

$$N_2 \rightleftarrows 2D$$

since the light scattering experiments demonstrated that the change in the weight average molecular weight parallels the observed loss of the tertiary structure measured at the same protein concentration. However, the denaturation also depends strongly on the protein concentration, becoming much slower at higher protein concentrations; hence, the observed denaturation may be more accurately described as

$$N_2 \rightleftarrows 2N \rightleftarrows 2D$$

in which the conversion of native dimer to native monomer is unfavorable, either from thermodynamic or from kinetic control. After conversion to monomer, unfolding takes place rapidly, compared to the dissociation step.

According to this mechanism, the native monomer does not accumulate during the course of denaturation, consistent with the experiments described in this report. Similarly, native monomer also does not accumulate during the refolding reaction (Philo *et al.*, 1993). Thus, a native monomer is a relatively unstable species relative to the collapsed intermediate and native dimer states in both acid and neutral pH.

Increasing temperature accelerates the overall denaturation, although which reaction is more strongly affected by temperature has not been determined. This acid-induced denaturation was independent of the type of acid used, and hence appears to be due to an increase in positive charges on the protein, but not to any specific ionic effects. BDNF is a very basic protein and would carry positive charges on 11 arginine residues, 11 lysine residues, and 2 histidine residues at pH 2.0.

From these results, one can conclude that the slow conformational change during rp-HPLC observed by Benedek and Rosenfeld (1993) is primarily induced by acid, with the organic solvent and nonpolar column material acting as catalysts. The reason for slow denaturation appears to be the strong association between the two native monomers to form the native dimer. The crystal structure of NGF, a protein highly homologous to BDNF, indicates that three strands of antiparallel β-sheet form a large flat hydrophobic surface for contact between the subunits in the dimer (McDonald *et al.*, 1992). It appears that the acid-denatured form has completely lost the surface necessary for subunit contact.

This loss of potential intersubunit contacts in the acid-denatured form is also supported by the SEC experiments. When the acid-denatured protein was injected onto the SEC column at neutral pH, the predominant form observed is a collapsed form with the monomeric molecular weight. It thus appears that the acid-denatured monomer is stable at neutral pH. This suggests that the acid-denatured form must undergo some rearrangement prior to dimer formation. The hydrophobic interface present in the native protein is absent, as it is in the initial refolding intermediate seen following long-term exposure to GdnHCl (Philo *et al.*, 1993).

The possibility that the acid-denatured form is an A state, or molten globule, was examined by CD, FTIR, sedimentation velocity, and SEC. It was difficult to evaluate the secondary structure of the protein by CD for the reasons described previously. Some β-sheet structure is present and remains during the unfolding. FTIR analysis of the acid-denatured form was consistent with a more flexible molecule which still has a significant amount of β-sheet secondary structure. Sedimentation velocity and SEC suggested that the acid-denatured form is relatively compact. Thus, the acid-denatured form appears to be a compact molecule with secondary structure but no detectable tertiary structure, satisfying the criteria for its characterization as an A state and also a molten globule (Kuwajima, 1977, 1989; Goto & Fink, 1989; Christensen & Pain, 1991). The formation of this compact intermediate is a reversible reaction; upon titration to neutral pH, it refolds into the native dimer in a manner similar to that of the compact folding intermediate obtained following dilution from GdnHCl (Philo *et al.*, 1993).

The denaturation is much faster in 6 M GdnHCl, as expected from the stronger denaturation action of GdnHCl relative to TFA. The far- and near-UV CD spectra are lost almost immediately upon exposure to 6 M GdnHCl, suggesting that, unlike denaturation in acid, BDNF rapidly denatures and dissociates under these conditions. However, it is puzzling that the renaturation of the GdnHCl-denatured form depends on the length of exposure to GdnHCl. Renaturation after short lengths of exposure to GdnHCl resulted in more of the dimeric form while renaturation after longer exposure generated more of the monomeric collapsed structure. One possible explanation is that the GdnHCl-denatured states are different from those in acid and comprise two monomeric forms, D_1 and D_2, in which the conversion from D_1 to D_2 is a relatively slow process. The D_1 form retains the dimer contact surface more than the D_2 form and leads directly to dimer under refolding conditions, while the D_2 form generates the molten globular structure. The D_1 to D_2 transition may involve proline isomerization. However, if this is true, the fact that the equilibrium unfolded state appears to be 100%

D_2 would require that it has 100% of the nonnative isomer. Refolding of the D_2 form and characterization of this molecule have been described in the preceding paper (Philo *et al.*, 1993).

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