thrombin. It appears that Asp(7) may be involved in a long-range interaction with a remote residue of fibrinogen not included in the synthetic peptide. Alternatively, thrombin may have altered reactivity at its primary (active) site, when bound to fibrinogen at its secondary binding site.

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Dimeric Structure and Conformational Stability of Brain-Derived Neurotrophic Factor and Neurotrophin-3

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ABSTRACT: We have examined the molecular structure of the related neurotrophic factors brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) by physical methods, including gel filtration, velocity sedimentation, sedimentation equilibrium, urea gel electrophoresis, fluorescence spectroscopy, and far-ultraviolet circular dichroism. The results of these studies indicate that at physiologically relevant concentrations both recombinant proteins exist as tightly associated dimers. The dimers are stable even in 8 M solutions of urea. In solutions of guanidine hydrochloride, BDNF and NT-3 undergo slow unfolding between 3 and 5 M concentration of denaturant. Circular dichroism spectroscopy revealed approximately 70% β-sheet and 20% β-turn content in the native structure of both neurotrophic factors. In this respect, BDNF and NT-3 resemble other polypeptide growth factors whose receptors are also integral protein-tyrosine kinases.

Brain-derived neurotrophic factor (BDNF) (Leibrock et al., 1989; Barde et al., 1982; Hofer & Barde, 1988) and neurotrophin-3 (NT-3) (Maisonpierre et al., 1990; Hohn et al., 1990; Rosenthal et al., 1990) in addition to nerve growth factor (NGF) and the recently discovered NT-4 (Hallböök et al., 1991) constitute a family of structurally related neurotrophic factors termed neurotrophins. Mature BDNF and NT-3 are 119 amino acid residue polypeptides with NGF being shorter by 1 amino acid residue. The neurotrophins display about 55% sequence identity including six conserved cysteine residues that,

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in NGF (Angletti & Bradshaw, 1971), were determined to form three disulfide bridges. Despite similar primary structures, BDNF and NT-3 display neuronal specificities different from those of NGF (Thoenen, 1991).

Under physiological conditions, NGF exists as a noncovalently associated homodimer (Bothwell & Shooter, 1977). The dimeric nature of the ligand may be important in promoting receptor dimerization, thought to be a critical step in signal transduction mediated by several receptor protein-tyrosine kinases (Cochet et al., 1988; Li & Schlessinger, 1991). NGF was crystallized (McDonald et al., 1990), and its three-dimensional structure was only recently solved (McDonald et al., 1991). Prior information available about the secondary structure was derived principally from Raman spectroscopy measurements (Williams et al., 1982). Using the intensity distribution of the amide I region of the Raman spectrum of mouse NGF, its secondary structure was estimated to contain between 53 and 67% antiparallel β-strand and between 0 and 11% helix. The aforementioned X-ray diffraction studies revealed an elongated structure consisting of three antiparallel pairs of β-strands with no contribution from the α-helix. Three disulfide bridges are located at the base of the NGF molecule. The availability of recombinant BDNF and NT-3 prompted us to investigate their molecular structures in comparison to that of NGF. The goal of this study was to gain a better understanding of the overall molecular architecture and conformational stability of these important proteins.

Materials and Methods

Proteins. Mouse NGF (ultra-pure) was purchased from Bioproducts for Science, Inc. Recombinant human BDNF and NT-3 were isolated from the conditioned media of Chinese hamster ovary cells expressing the individual proteins using a two-step purification procedure consisting of cation exchange (S-Sepharose Fast Flow) and gel filtration (Sephacryl S-100 HR) chromatography. Purified BDNF was provided by Dr. James Miller (Amgen Corp.). Purity of the proteins was assessed by silver staining of samples subjected to electrophoresis on reducing polyacrylamide gels. N-Terminal sequencing using the Porton integrated microsequencing system revealed the presence of intact as well as proteolytically altered proteins. In the case of BDNF, approximately two-thirds of the molecules had the expected N-terminal residue (His), and one-third was truncated by six residues at the N-terminus. In the case of NT-3, about half of the molecules were full-length, and half were truncated by five residues at the N-terminus. Biological activity was routinely assayed in a dorsal root ganglia (DRG) neurite outgrowth assay using embryonic day eight chick tissue (Lindsay & Peters, 1984; Lindsay et al., 1985). Protein concentration was determined both spectrophotometrically and by quantitative amino acid analysis. The value of the molar absorbance coefficient was 26 800 M^{-1} cm^{-1} (monomer) for BDNF and 27 400 M^{-1} cm^{-1} (monomer) for NT-3. Proteins were iodinated using the lactoperoxidase enzymatic method (Marchalonis, 1969). Labeled proteins were assayed for bioactivity in the DRG assay. The biological activity of the iodinated proteins was at least 90% that of the unlabeled factors.

Sucrose Density Gradient Centrifugation. Sucrose gradients (5–20%) in 20 mM Tris-HCl (pH 7.8) or acetate buffer (pH 4) containing 150 mM NaCl were formed with a Hoeffer gradient maker equipped with a Pharmacia low-pulsation peristaltic pump. One hundred micrograms of a protein or 10 ng of its radiolabeled counterpart in 100 μL was applied to the top of the gradient. Sedimentation was carried out in 10-mL plastic tubes at 5 °C in a TH-641 Sorvall rotor at 41 000 rpm for 20 h. Gradient displacement and fractionation was performed with an ISCO tube piercer fitted with a syringe pump and connected to a fraction collector. Fractions of 150 μL were collected and analyzed either for radioactivity with a Packard Cobra gamma counting system or for optical absorbance with a Shimadzu UV 160U recording spectrophotometer. The peak of protein concentration was found either in fraction 13 or in fraction 14.

Gel Filtration Chromatography. Gel filtration was performed in a 1 × 50 cm glass column packed with Biogel P-60 from Bio-Rad. The column was equilibrated with 20 mM Tris-HCl (pH 7.8) containing 1 mg/mL protamine sulfate. The column was run at 0.2 mL/min at room temperature. In a typical experiment, 50 μL of radioiodinated neurotrophin (10 ng/mL initial concentration) or 30 μL of unlabeled neurotrophin (1 mg/mL initial concentration) was applied onto the column. Protein elution data were expressed as distribution coefficients: $K_{av} = (V_{el} - V_0)/(V_t - V_0)$.

Sedimentation Equilibrium. We used an approach similar to that of Pollet et al. (1979), utilizing a table-top, preparative ultracentrifuge TL 100 from Beckman. The advantage of this instrument over the commonly used Beckman Airfuge is that rotational velocity and run temperature are controlled much more precisely. The centrifugation was performed at 5 °C in 20 mM Tris-HCl (pH 7.8), 150 mM NaCl, 5 mg/mL BSA, and 1 mg/mL cytochrome *c* in a total volume of 150 μL in a TLA 100.1 rotor; 30 000 rpm was chosen as a rotational velocity to achieve a sufficient protein concentration gradient. It was estimated (Chatelier, 1988) that 40 h was needed to attain sedimentation equilibrium. The resulting gradient was fractionated with a Hamilton syringe fitted with a micropipet, mounted on a micromanipulator. Ten-microliter fractions were withdrawn and counted in a Packard gamma counter. To obtain the radial distribution of each 10-μL fraction, equation A 1.3 (Attri & Minton, 1983) was numerically integrated using dimensions provided by Beckman Instruments Inc. Partial specific volumes of BDNF and NT-3 were calculated from the contribution of individual amino acid residues (Fish, 1975). These calculated values were as follows: BDNF, 0.725 cm^3/g; NT-3, 0.721 cm^3/g; mouse NGF, 0.716 cm^3/g. Nonsedimentable radioactivity was determined in each run by centrifuging a control tube for an additional 6 h at 100 000 rpm and counting the first fraction. Data were plotted as a function of ln *c* vs r^2, where *c* was the number of counts per minute adjusted for the nonsedimentable contribution and *r* the radial distance of a given fraction from the axis of rotation. At least 10 points were utilized in each calculation. Least-squares analysis was used to determine the slope d ln c/d r^2. Error analysis was carried out at 95% confidence level. Radioiodinated mouse NGF was used to verify the validity of this approach.

Urea Transverse Gradient Gels. Gradient gels of 14–11% polyacrylamide/0–8 M urea were used to assess dimer stability and protein unfolding under denaturing conditions. Gels were prepared according to published procedures (Goldenberg, 1990). Proteins were either preincubated with urea or applied directly to the gel and run at pH 6 toward the cathode. Cytochrome *c* was used as an indicator of protein migration. Following electrophoresis at 80 V for 240 min, and gels were stained with Coomassie Blue.

Circular Dichroism Spectroscopy and Fluorescence Spectroscopy. Circular dichroism spectroscopy was performed on an Aviv 62 DS spectropolarimeter using 0.1-mm and 1-mm quartz cells. The spectra were recorded at 25 °C in 10 mM phosphate buffer, pH 7.1, at a concentration of approximately

Table I: Sedimentation Coefficients, Stokes Radii, and Calculated Molecular Weights of the Neurotrophins[a]

protein	$s_{w,20}$ (S)	R_{St} (Å)	MW[b]
BDNF	2.78	24.0 ● 3.2	27700 ● 3200
NT-3	2.78	23.5 ± 3.0	26700 ± 3300
NGF	2.78	24.0 ± 3.2	26800 ± 3400

[a] These parameters were determined for radioiodinated proteins at physiological concentrations. Values for unlabeled counterparts were identical within experimental error. [b] Calculated from the Svedberg equation.

0.5 mg/mL. Measurements were taken at 1-nm intervals with a 4-s time constant and were averaged over 10 scans. Data were collected and processed with a computer-based data acquisition system and expressed as mean residue ellipticity. Protein secondary structure was estimated by Provencher analysis of the data collected at 1-nm intervals over the wavelength range of 190–240 nm (Provencher & Glöcker, 1981). In order to investigate guanidine hydrochloride induced unfolding of the neurotrophins, solutions of individual proteins were prepared at various concentrations of denaturant and incubated for 24 h at 25 °C. To assess the reversibility of unfolding, either the denaturant was removed by dialysis or its concentration was reduced by dilution. Thermal stability of the neurotrophins was examined in 2-cm water-jacketed quartz cells. The temperature was increased in 5 °C increments. Samples were equilibrated at each temperature for 20 min before CD spectra were recorded. Only at 75 °C did spectral changes become rapid enough to follow, but even at this temperature after 30 min, BDNF (but not NT-3) failed to attain a stable CD spectrum. Fluorescence spectra were obtained at 25 °C using a Shimadzu RF 5000U spectrofluorophotometer and 1-cm quartz cells in 10 mM phosphate buffer, pH 7.1. Excitation wavelengths were 280 or 295 nm. During unfolding/refolding studies, samples were incubated at 25 °C for up to 200 h.

Results

Estimation of Stokes Radii by Biogel P60 Gel Filtration Chromatography. Radioiodinated BDNF, NT-3, and NGF eluted as single, symmetrical peaks under the experimental conditions used for gel filtration. The elution pattern was unaffected by preincubation for 24 h in the elution buffer. Using several control proteins, a good linear correlation was established between the Stokes radii and the distribution coefficient K_{av}. The estimated Stokes radii (Table I) determined at initial concentrations of 10 ng/mL (radiolabeled neurotrophins) and 1 mg/mL (unlabeled neurotrophins) were essentially identical, leading to the conclusion that the apparent size of these proteins did not depend upon the concentrations used in the experiment.

Sedimentation Coefficients Determined by Centrifugation in a Sucrose Density Gradient. A sucrose gradient (5–20%) was calibrated with the set of standard proteins; an excellent linear relationship was found between sedimentation coefficients and the numeral of the peak fraction. Sedimentation coefficients, $s_{20,w}$, were calculated according to McEwen (1967) and were subject to less than 10% error (see Materials and Methods). The sedimentation velocities of the radioiodinated proteins, preincubated at pH 4 at 4 °C in a sucrose gradient at the same pH, were identical to those at pH 7.8, suggesting a lack of any pH effect on the dissociation equilibrium. Molecular weights calculated from the Svedberg equation are listed in Table I. The results of gel filtration and sedimentation experiments together point clearly to the conclusion that, like NGF, both BDNF and NT-3, at concentrations close to physiological exist as homodimers. The estimated Stokes radii (approximately 25 Å) are larger than the radius calculated for an anhydrous sphere of a dimer (20 Å).

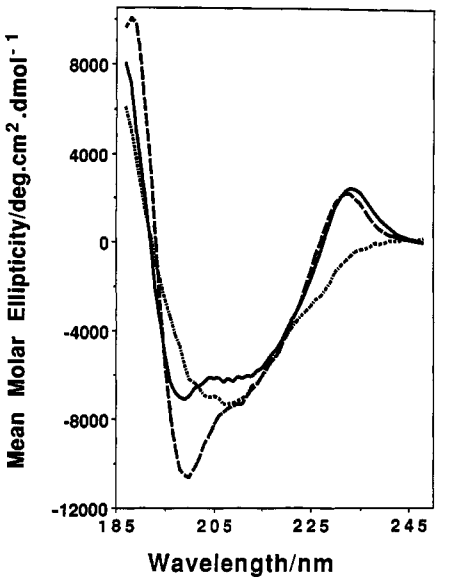

FIGURE 1: Comparison of circular dichroism spectra of the neurotrophins: (···) NGF; (– –) NT-3; (—) BDNF. Spectra were taken in 10 mM phosphate buffer at pH 7.1 at 25 °C.

Sedimentation Equilibrium. Sedimentation equilibrium experiments carried out with radiolabeled proteins in a preparative centrifuge allowed us to accurately determine the molecular weights at very low concentrations. This range of concentrations could not be used in an analytical centrifuge with optical scanning of a gradient. Sedimentation equilibrium studies with radioiodinated NGF (5 ng/mL) gave a calculated molecular weight of 26 150 ± 2300, as expected for a dimer, and in good agreement with previous studies. Sedimentation equilibrium experiments carried out with radioiodinated BDNF at 2 ng/mL (1.4×10^{-10} M) and 5.0 ng/mL (3.5×10^{-10} M) gave calculated molecular weights of 26 900 ± 2600 and 26 950 ± 4000, respectively. Similarly, sedimentation equilibrium experiments for NT-3 at 2 and 5 ng/mL yielded values of 26 300 ± 4000 and 27 300 ± 3500, respectively. Thus the sedimentation equilibrium analysis provided strong additional evidence that these proteins exist in solution as stable homodimers at physiologically relevant concentrations.

Estimation of Protein Secondary Structure from Circular Dichroism Spectroscopy. Circular dichroism spectroscopy, which is sensitive to the contribution of various secondary structure elements, was used in order to evaluate the overall conformation of the neurotrophins. Far-ultraviolet circular dichroism spectra of BDNF and NT-3 but not mouse NGF displayed positive peaks around 233 nm (Figure 1). Both BDNF and NT-3 were, like NGF, observed to have broad negative ellipticity signals in the 195–225-nm wavelength range. However, their spectra differed from that of NGF in having a distinct minimum near 220 nm and a small positive ellipticity signal at 233 nm. The CD minimum for NGF was about 210 nm. Of the three neurotrophins, NT-3 exhibited the strongest CD signal. Deconvolution analysis of the circular dichroism spectra clearly indicated that the β-sheet and β-turn conformation contributes very significantly to the overall structure of these three neurotrophic factors (Table II). The predicted secondary structure contents of the recombinant human BDNF and human NT-3 are quite similar; each appears to contain about 20% more β-sheet conformation than mouse NGF. Furthermore, the resultant fitted spectra successfully reproduced all of the major spectral features described

Table II: Estimation of Secondary Structure Contents of Recombinant Human BDNF, Human NT-3, and Mouse NGF by Circular Dichroism Spectroscopy

protein	helix	sheet	turn	other
BDNF	0.00	0.74	0.21	0.05
BDNF[a]	0.00	0.86	0.00	0.14
NT-3	0.03	0.70	0.15	0.11
NGF	0.09	0.48	0.20	0.24

[a] Estimated secondary structure of BDNF that was incubated at 90 °C for 30 min. The CD spectrum was recorded after the sample was cooled to 25 °C.

above, including the positive peak at 233 nm for BDNF and NT-3 and the lack of this peak for NGF. This suggests that the positive peak at 233 nm in the spectra of BDNF and NT-3 is probably the result of a simple combination of the signals from common secondary structural features, most likely their high β-sheet and β-turn contents, rather than being derived from unusual conformational features that are not represented in the basis set of protein CD spectra used for deconvolution analysis. Accordingly, the lack of a positive peak at 233 nm in the spectrum of NGF is probably a result of relatively lower β-sheet contribution to its structure than to the structures of BDNF and NT-3 (Table II). The positive peak was extremely useful for following protein denaturation at long wavelength, where interference due to denaturant absorbance was minimized. Its disappearance seems to correspond to complete loss of β-turn structure, according to a deconvolution analysis of the spectrum of heat-denatured BDNF. In contrast to the present results, the circular dichroism spectrum of human ciliary neurotrophic factor (CNTF), a protein distinct from these neurotrophins of the NGF family (Lin et al., 1989; Stöckli et al., 1989), is indicative of a structure with large content of α-helix (unpublished observations).

Chemical and Heat Denaturation of Neurotrophins. Unfolding of BDNF and NT-3 in guanidine hydrochloride was followed with CD spectroscopy and fluorescence spectroscopy. Samples examined by CD spectroscopy were incubated in denaturant solution for 24 h prior to measurements. An unfolding transition between 3 and 5 M guanidine hydrochloride was observed for BDNF, and a similar transition between 3 and 4 M was observed for NT-3. Removal of the denaturant by overnight dialysis restored the original CD spectra of the proteins. Dilution to reduce the denaturant concentration from 5 to 3 M failed to restore the original spectrum after 24 h, suggesting apparent irreversibility of denaturation under these conditions. The electrophoretic mobility of BDNF and NT-3 was not affected by a 0–8 M gradient of urea. Incubation with 100 mM dithiothreitol for 12 h at pH 6 did not affect mobility either (data not shown), thus indicating that dimer dissociation and unfolding in urea are difficult and not facilitated by a strong reducing agent. Furthermore, circular dichroism spectra recorded after a 24-h incubation period at room temperature in 8 M urea indicated no unfolding induced by the presence of the denaturant.

In order to carry out more detailed studies of unfolding and refolding of BDNF and NT-3, fluorescence spectroscopy was employed. Fluorescence spectra of proteins are primarily governed by the number of tryptophan residues and their environment. Fluorescence spectroscopy is frequently used to study folding transitions since these usually result in changes of some tryptophan environments. All three neurotrophins share three conserved tryptophan residues; in addition, NT-3 possesses a fourth unique residue. Excitation at 280 nm resulted in emission maxima at 340.4, 341.2, and 337.6 nm for native BDNF, NGF, and NT-3, respectively. Upon unfolding

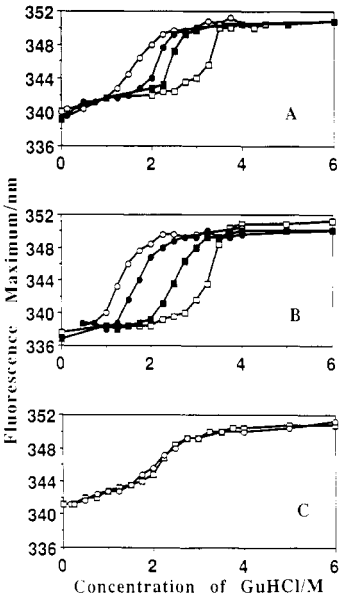

FIGURE 2: Unfolding and refolding of the neurotrophins (5 μg/mL) in guanidine hydrochloride followed by the shift of the fluorescence emission maximum on excitation at 280 nm; (A) BDNF; (B) NT-3; (C) NGF. Open squares, unfolding after 24 h; open circles, refolding after 24 h; solid squares, unfolding after 200 h; solid circles, refolding after 175 h.

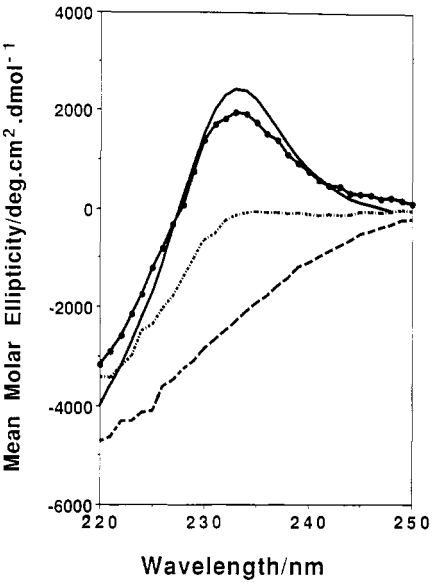

FIGURE 3: Circular dichroism spectra of recombinant human BDNF at various temperatures: (—) 25 °C; (●—●) 50 °C; (– –) 90 °C; (···) sample recooled to 25 °C after incubation at 90 °C.

in guanidine hydrochloride, a decrease in fluorescence intensity as well as a spectral shift of emission maximum toward longer wavelengths was seen for all three proteins, typical for increased solvent exposure of tryptophan.

The results of fluorescence spectroscopy studies confirmed the unfolding transition seen by CD. However, the apparent irreversibility of the transition for BDNF and NT-3 at intermediate concentrations of guanidine hydrochloride resulted from the slow kinetics of the unfolding and refolding processes (Figure 2A,B). In contrast, mouse NGF did not show such slow kinetics, as unfolding/refolding curves were identical when measured after 24 h (Figure 2C).

Thermal stability studies of BDNF followed by CD spectroscopy demonstrated the protein to be relatively stable up to 60 °C (retaining more than 80% of the ellipticity signal at 233 nm at this temperature), after which a slow, irreversible denaturation took place (Figure 3). The CD spectrum of

heat-treated BDNF (90 °C for 40 min) revealed a decrease in the contribution from ordered structures (Table II). A comparative study of BDNF and NT-3 stability at 75 °C revealed NT-3 to be irreversibly denatured within approximately 10 min while BDNF retained over 50% of its original structure as monitored by CD at 233 nm.

Discussion

Bothwell and Shooter (1977) reported compelling evidence for the dimeric structure of mouse NGF. Because of the primary sequence similarity between members of the neurotrophin family, and especially the absolute conservation of cysteine residues involved in three intrachain disulfide bonds, it was expected that the newly discovered factors BDNF and NT-3 would also exists as homodimers. However, Barde et al. (1982) reported that BDNF purified from pig brain behaved as a monomer in gel filtration experiments. Our own results obtained during the course of purification of recombinant human BDNF (identical in sequence to the porcine protein) also suggested that the protein might indeed exist as a monomer on the basis of Sephacryl S100 gel filtration chromatography. However, we established that dimeric mouse NGF eluted anomalously from the same column, at a position that would be expected for NGF monomer. Incidently, this relative retardation of the neurotrophins on a Sephacryl gel filtration column proved to be useful in purification since it effectively separated the neurotrophins from a number of contaminating proteins. By contrast, the results of gel filtration on Biogel P60 were consistent with a dimeric structure for all three neurotrophins. That the elution patterns of neurotrophins are matrix-dependent is most likely due to the highly basic nature of these proteins (p*I* about 10). Therefore, to accurately determine the molecular weights of BDNF and NT-3, it was important to employ other methods in addition to gel filtration.

The results of sedimentation in sucrose density gradients reported here clearly indicated similar molecular weights as well as similar molecular shapes for all three neurotrophins NGF, BDNF, and NT-3. On the basis of the result of sucrose gradient sedimentation and gel filtration, molecular weights consistent with homodimeric structure were calculated from the Svedberg equation. Sedimentation equilibrium analysis was used to confirm the molecular weights. The results obtained with NGF were consistent with its known dimeric structure. Similarly, the recombinant human BDNF and NT-3 clearly behaved as dimers. No evidence for free monomers was found in any of these studies. Therefore, the dissociation constants for BDNF and NT-3 dimers may be as low as that established previously for NGF (10^{-13} M) (Bothwell & Shooter, 1977). We would predict that neurotrophin-4 exists in solution also as a dimer, based on the homology of the deduced amino acid sequence with the other neurotrophins.

Deconvolution of far-ultraviolet circular dichroism spectra according to Provencher (Provencher & Glöcker, 1981) revealed very substantial contribution of a β-sheet conformation to the secondary structures of dimeric BDNF, NT-3, and NGF. BDNF and NT-3 were estimated to have very similar secondary structures (Table II). These structures are, however, about 20% more abundant in β-sheet content than the structure of mouse NGF. Although these conclusions should be confirmed by other methods such as FTIR, one could expect that the molecular modeling of BDNF and NT-3 on the basis of NGF structure may not be entirely straightforward. The results of our own CD analysis of mouse NGF secondary structure were quite similar to those obtained with Raman spectroscopy (Williams et al., 1982), indicating in both cases a relatively large degree of β-sheet contribution. It is also consistent with the recently determined crystallographic structure of mouse NGF (McDonald et al., 1991). It is worth noting further that the algorithms of Chou and Fasman and of Robson and Garnier, applied to the neurotrophins, predicted structures with substantial β-sheet content.

The intermolecular interactions of β-strands are most likely to be responsible for the formation of the dimer. Homodimers of neurotrophic factors BDNF and NT-3 are relatively stable entities. Circular dichroism spectra recorded in 8 M urea after a 24-h incubation indicated no unfolding induced by the presence of the denaturant. In contrast to the lack of unfolding in urea, CD spectra recorded in guanidine hydrochloride showed an unfolding transition between 3 and 5 M denaturant for BDNF and between 3 and 4 M for NT-3. The observed transition most likely results from the simultaneous shifting of dimer–monomer equilibrium and protomer unfolding. Diluting guanidine hydrochloride from 5 to 3 M did not, after 24 h, restore a CD spectrum characteristic of the native protein, although guanidine hydrochloride at a concentration of 3 M is not sufficient to start the process of unfolding. On the other hand, complete removal of denaturant did result in restoration of the original CD spectrum. Results of fluorescence intensity measurements also indicated that a complicated kinetic mechanism, strongly dependent upon the denaturant concentration, must operate during unfolding and refolding. Only after a prolonged period of time did the unfolding and refolding curves begin to overlap. Kinetics of unfolding and refolding may be additionally complicated by the equilibrium between monomer and dimer. A similar noncoincidence of the unfolding and refolding processes was recently observed for streptavidin, another multisubunit, β-sheet protein (Kurzban et al., 1991). Interestingly, the unfolding/refolding kinetics of mouse NGF are much faster that those for BDNF and NT-3. This difference may be a result of the lesser contribution of the β-sheet conformation to the structure of NGF than to the structures of two other neurotrophins studied (Table II). Because of the complicated and slow kinetics of unfolding and refolding, a detailed comparison of the conformational stabilities of the three neurotrophins is difficult at the present time. However, the observed unfolding induced by guanidine hydrochloride offers an interesting opportunity to create heterodimers between different members of the neurotrophin family.

The functional significance of the dimeric structure of the neurotrophins may be related to the nature of their receptors. The three molecules bind with comparable affinity (10^{-9} M) to the same 75-kDa transmembrane protein (p75) also known as the low-affinity NGF receptor (Rodriguez–Tébar et al., 1990; Squinto et al., 1991). Different members of the *trk* protooncogene family emerged recently as a new class of specific receptors for various neurotrophins. In particular, the protein encoded by the *trk* A gene, a transmembrane tyrosine kinase, functions as an essential component of the NGF receptor (Kaplan et al., 1991; Klein et al., 1991). Similarly, the *trk* B gene product, a related tyrosine kinase, is a functional receptor for BDNF and NT-3 (Squinto et al., 1991; Soppet et al., 1991; Glass et al., 1991). In addition, NT-3 was shown to display preferential affinity to a receptor-like tyrosine kinase encoded by the *trk* C gene (Lamballe et al., 1991). The generation of high-affinity receptors may involve the interaction of neurotrophin homodimers with p75 and $p140^{trk}$, for example, where one monomer interacts with each receptor subtype. Alternatively, cognate *trk* proteins may associate and interact with neurotrophin dimers to elicit high-affinity binding and the subsequent biological response (Bothwell, 1991).

Interesting similarities emerge from comparison of the estimated secondary structures of the neurotrophins versus other ligands that take part in signal transduction mediated by receptor tyrosine kinases. The structures of basic FGF (Prestrelski et al., 1991), PDGF (Prestrelski et al., 1991; Vogel & Hoppe, 1989), and EGF (Campbell et al., 1990), like the members of neurotrophin family, are all rich in β-structures with little or no contribution from α-helix. This may in turn imply general structural similarity of their receptor binding domains.

In conclusion, we established the dimeric composition of recombinant human BDNF and NT-3 at physiological concentrations. Using CD spectroscopy, the secondary structures of the neurotrophins were found to contain large proportions of β-sheet and β-turn and almost complete lack of α-helix. Studies in solutions of denaturing agents indicated conformational stability in urea and a single unfolding transition between 3 and 5 M guanidine hydrochloride. Thermal stability was also assessed in circular dichroism studies, leading to the conclusion that BDNF displays somewhat higher stability than NT-3.

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