Heterodimers of the Neurotrophic Factors: Formation, Isolation, and Differential Stability

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ABSTRACT: We have determined that all four known members of the neurotrophin family, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4 (NT-4), are capable of forming noncovalent heterodimers. The formation of these heterodimers was accomplished by homodimer subunit exchange promoted by treatment with guanidine hydrochloride, urea, low pH, or acetonitrile. In some cases (BDNF and mouse NGF; BDNF and NT-4), generation of the heterodimers was achieved by incubating homodimer mixtures in a neutral buffer at ambient temperature. The formation of heterodimers was in each case detected by nondenaturing gel electrophoresis at pH 7.4. High-performance cation-exchange chromatography was used to separate neurotrophin heterodimers from their parental homodimers. Heterodimers between BDNF and NT-3, BDNF and NT-4, and NT-3 and NT-4 are stable and show only a very small increase in homodimer content after 24 h of incubation at 37 °C. In contrast, heterodimers containing NGF subunits undergo gradual rearrangement to the homodimers. Our studies indicate that low pH, acetonitrile, and urea merely increase the neurotrophin subunit exchange rate and decrease the time needed to reach an equilibrium between a heterodimer and its two parental homodimers.

The neurotrophins are small, basic, disulfide-linked protein factors which have been implicated in the growth and maintenance of neuronal cells (Glass & Yancopoulos, 1993; Lindsay, 1993; Snider & Johnson, 1989). The neurotrophins are currently considered as potential drug candidates in the treatment of neurological disorders such as Parkinson's disease, Alzheimer's disease, and epilepsy (Snyder, 1991; Phillips et al., 1991; Enfors et al., 1991; Lindsay et al., 1992; Lindsay, 1992). At the present time, four structurally related proteins, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) (Leibrock et al., 1989; Barde et al., 1982; Hofer & Barde, 1988), neurotrophin 3 (NT-3) (Maisonpierre et al., 1990; Hohn et al., 1990; Rosenthal et al., 1990), and neurotrophin 4 (NT-4) (Ip et al., 1992; Halböök et al., 1991), constitute the family of neurotrophins. Biological activity of the neurotrophins is mediated by binding to cell surface receptors. Two classes of receptors have been identified: the transmembrane protein *p75* and the members of the *trk* family of integral tyrosine kinases (Lo, 1992; Anderson, 1992; Glass & Yancopoulos, 1993). Neuronal growth, differentiation, and survival effects of neurotrophins are elicited through binding to *trk* receptors, possibly in conjunction with binding to *p75*.

Mouse NGF, under physiological conditions, exists as a noncovalent homodimer (Bothwell & Shooter, 1977). Dimeric growth factor structure may play a functional role in signal transduction. By dimerizing cell surface receptors upon binding, activation of cytoplasmic kinase domains may be accomplished without transmission of conformational changes through a transmembrane domain (Ullrich & Schlessinger, 1991; Li & Schlessinger, 1991).

The three-dimensional structure of mouse NGF has only recently been elucidated by X-ray crystallography (McDonald et al., 1991). Elongated molecules with three, closely spaced disulfide bonds, were found to possess a novel fold comprised exclusively of β-sheet and loop structures. The X-ray diffraction studies established that the amino acid residues involved in the hydrophobic dimer interface are conserved within the amino acid sequences of the neurotrophins. We have recently reported that, at subnanomolar concentrations, human BDNF and human NT-3 also exist as tightly associated homodimers (Radziejewski et al., 1992). Deconvolution analysis of BDNF and NT-3 circular dichroism spectra led us to conclude that both proteins possess a predominantly β-sheet structure similar to, but also somewhat different from, the structure of NGF. We speculated that the changes in BDNF and NT-3 circular dichroism and fluorescence spectra brought about by denaturation reflected both dimer dissociation and protomer unfolding. Fluorescence depolarization studies of NGF unfolding equilibria in guanidine hydrochloride (Timm & Neet, 1992) indeed suggested that NGF homodimers undergo dissociation concomitant with unfolding of the monomers. Other denaturing agents also appear to promote dissociation: Moore and Shooter (1975) utilized low pH to form heterodimers between truncated and full-length mouse NGF. Recently, Burton et al. (1992) provided evidence that dimeric combination of mouse and human NGF could be obtained by a transient exposure of their mixture to acidic pH (Burton et al., 1992).

Recent X-ray diffraction studies of human transforming growth factor β-2 revealed topologies partially resembling that of NGF despite there being little amino acid sequence similarity (Daopin et al., 1992; Schlunegger & Grütter, 1992; Oefner et al., 1992; Swindells, 1992; Murzin & Chothia, 1992). In both cases, the dimer geometry was shown to be vastly different (McDonald et al., 1991; Daopin et al., 1992; Schlunegger & Grütter, 1992; Murzin & Chothia, 1992; McDonald & Hendrickson, 1993).

Transforming growth factors β and members of the platelet-derived growth factor family, platelet-derived growth factor and vascular endothelial growth factors, exist as homo- and

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heterodimers (Cheifetz et al., 1987; Cleason-Welsh et al., 1989; Ogawa et al., 1992). Unlike the noncovalently bound neurotrophin dimers, the dimers of these factors involve an intersubunit disulfide bridge. The experimental indications of subunit exchange in NGF and the conservation of the amino acid residues comprising the dimer interface within the neurotrophin family prompted us to examine the ability of different neurotrophic factors to enter into heterodimeric combinations. Here, we report that *in vitro* all known neurotrophic factors are capable of forming relatively stable noncovalent heterodimers.

MATERIALS AND METHODS

Proteins. Ultrapure mouse NGF (mNGF) was purchased from Harlan Bioproducts for Science, Inc. Recombinant human BDNF and NT-3 were obtained from Dr. James Miller of Amgen Corp. Recombinant human NT-4 was provided by the Process Development and Manufacturing Department of Regeneron Pharmaceuticals, Inc., and was a mixture of authentic and N-terminal methionine forms. Protein concentrations were calculated from their optical densities at 280 nm. The extinction coefficients of neurotrophin monomers at 280 nm were 18 600 M^{-1} cm^{-1} for mNGF, 26 800 M^{-1} cm^{-1} for BDNF, 27 400 M^{-1} cm^{-1} for NT-3, and 24 900 M^{-1} cm^{-1} for NT-4. The extinction coefficient of mNGF was based on the value provided by the manufacturer. The extinction coefficients of the recombinant BDNF, NT-3, and NT-4 were calculated from their amino acid sequences (Gill & Hippel, 1989).

Formation of Neurotrophin Heterodimers. Neurotrophin heterodimers were generated by four different approaches. In each case equimolar amounts of two neurotrophins at a final concentration of approximately 1 mg/mL were mixed and subjected to a treatment promoting dissociation followed by dialysis against neutral buffer to promote reassociation.

In the first method, two neurotrophins were mixed and dialyzed against 6 M guanidine hydrochloride buffered with Tris·HCl (20 mM, pH 8.0) at room temperature for 6 h. The mixture was then dialyzed at 4 °C against two changes of 20 mM Tris·HCl buffer, pH 8.0, over a 24-h period.

In the second method, heterodimers were made by dialyzing proteins against 8 M urea buffered with Tris·HCl (20 mM, pH 8.0) at room temperature for 8 h and then twice against 20 mM phosphate buffer, pH 7.0, at 4 °C.

In the third method, a mixture of two neurotrophins was first dialyzed against 135 mM HCl buffer, pH 1.0, containing 50 mM KCl at room temperature for 6 h, then at 4 °C, against two changes of 20 mM phosphate buffer, pH 7.0, over a 24-h period. An alternative approach was to incubate 3 volumes of the same HCl/KCl buffer with 1 volume of premixed stock neurotrophins for 6 h (all the native neurotrophins were in PBS, and the final pH was 1.3), and to dialyze the mixture against 20 mM phosphate buffer, pH 7.0.

In the fourth approach, heterodimers were generated with the aid of an organic solvent. Acetonitrile was added to a neurotrophin mixture to reach a final concentration of 45% (v/v). Samples were incubated at room temperature for 6 h and then dialyzed at 4 °C against Tris·HCl buffer. Higher percentages of acetonitrile were found to cause the neurotrophins to precipitate.

Finally, the formation of some heterodimers was accomplished by incubating an equimolar mixture of the homodimers in 20 mM Tris·HCl buffer, pH 8.0, at room temperature for a period of at least 24 h.

Nondenaturing Gel Electrophoresis. Heterodimer formation was monitored by running samples on nondenaturing 11% polyacrylamide gels. Gels (10 cm × 8 cm) without a stacking gel were cast according to an established procedure (McLellan, 1990). Gels cast in 43 mM imidazole and 35 mM HEPES, pH 7.4, were found to be optimal for the separation of the neurotrophins and their heterodimers. The electrophoretic separation was run for 120 min at 150 V toward the cathode, and the gels were stained with Coomassie Blue.

Purification of the Heterodimers. Cation-exchange chromatography on a Pharmacia 5 × 5 Mono S column was found to be the method of choice to separate heterodimers from homodimers. Eluent buffer A was 20 mM phosphate, pH 7.0, and buffer B was 20 mM phosphate buffer containing 1 M NaCl. Chromatography was performed at room temperature with a typical loading of 0.1–2 mg of protein and a flow rate of 0.75 mL/min. Our elution method was similar to that of Timm and Neet (1992): 0–15 mL 0% buffer B; 27% buffer B at 20 mL; 37% buffer B at 30 mL; 100% buffer B at 60 mL. The heterodimer peaks were always found to elute between the two corresponding homodimer peaks. Fractions were combined and concentrated by ultrafiltration (Amicon ultrafiltration stirred cell Model 3, Filtron Omega 3K membrane). Two changes of 20 mM phosphate buffer, pH 7.0, were applied to decrease the salt concentration arising from buffer B.

Circular Dichroism Spectroscopy. A Jasco J-710 spectropolarimeter equipped with a computer-based data acquisition system was used to acquire CD spectra. Spectra were obtained at 25 °C with a 0.5-mm quartz cell in 20 mM phosphate buffer, pH 7.0. In order to get satisfactory spectra, the protein concentrations were within the range of 0.2–1 mg/mL. For each spectrum, 20 scans were acquired between 200 and 260 nm at 2-nm wavelength intervals. The results were corrected for background and expressed as a mean residue ellipticity. For the heterodimers, the combined molecular weight of two protomers was divided by the combined number of the amino acid residues to give the mean residue mass used in these calculations.

Protein Sequencing. Heterodimer bands were transferred electrophoretically from nondenaturing polyacrylamide gels onto a Porton Hyperbond poly(vinylidene difluoride) (PVDF) membrane. Electroblotting was performed at 90 V for 30 min in a 30 mM histidine and 30 mM MES buffer, pH 6, containing 10% methanol. After being stained with 0.1% Ponceau S in 5% trichloroacetic acid and destained in water, protein bands were excised and directly sequenced using a Porton integrated microsequencing system.

Stability of the Heterodimers. Purified heterodimers were diluted in 20 mM phosphate buffer containing 0.01% Tween 20 to the final concentration of 10 μg/mL, incubated at 37 °C in Nuncimmuno minisorp test tubes for 24 h, and analyzed by cation-exchange chromatography on a Pharmacia Mono S 5 × 5 column. The column was eluted using a sodium chloride gradient in 20 mM phosphate buffer, pH 7 (as above). A sample of 10 μg of protein was applied onto the column, and the proportion of the homodimers was estimated by peak integration.

Gel Filtration Chromatography. Gel filtration chromatography was carried out at room temperature on two Pharmacia Superose 12 columns connected in series. The excluded and included volumes were 15.5 and 40.9 mL, respectively. Protein (50–100 μg) was injected and eluted at 0.5 mL/min with 20 mM phosphate buffer, pH 7, containing 250 mM sodium chloride.

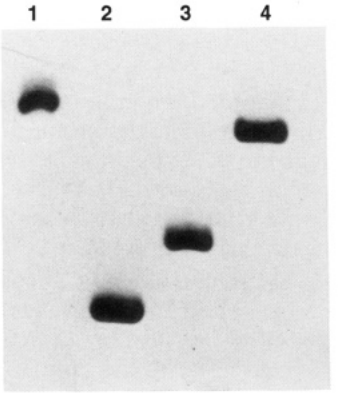

FIGURE 1: Nondenaturing 11% polyacrylamide gel electrophoresis at pH 7.4 of the neurotrophins as described in Materials and Methods. Lane 1, mNGF; lane 2, BDNF; lane 3, NT-3; lane 4, NT-4.

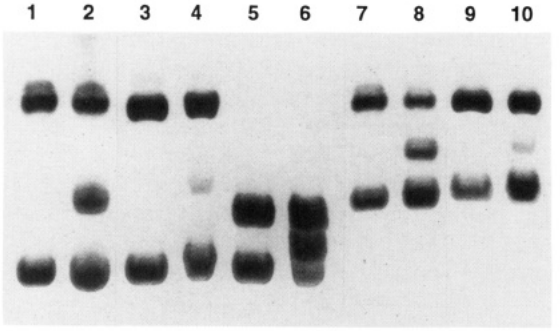

FIGURE 2: Nondenaturing gel electrophoresis at pH 7.4 of the neurotrophins treated either separately (odd-numbered lanes) or jointly (even-numbered lanes) with 6 M guanidine hydrochloride followed by dialysis against neutral buffer. Samples that were treated separately with GuHCl, dialyzed, and combined before the gel was run are shown in lanes 1, 3, 5, 7, and 9. Samples that were treated with 6 M guanidine hydrochloride and dialyzed jointly before the gel was run are shown in lanes 2, 4, 6, 8, and 10. Lanes 1 and 2, mNGF and BDNF; lanes 3 and 4, NT-4 and BDNF; lanes 5 and 6, NT-3 and BDNF; lanes 7 and 8, mNGF and NT-3; lanes 9 and 10, NT-4 and NT-3. A third band with an intermediate electrophoretic mobility is seen in every even-numbered lane.

RESULTS

Formation of Heterodimers. Initial experiments to create heterodimers were performed by treating neurotrophin mixtures with 6 M guanidine hydrochloride. The homodimers were first allowed to dissociate/unfold in the guanidine hydrochloride solution and then to refold/dimerize at pH 8 in Tris·HCl buffer. The products were examined by pH 7.4 native 11% polyacrylamide gel electrophoresis. Figure 1 demonstrates the migration pattern of all four native neurotrophic factors on the nondenaturing 11% polyacrylamide gel. Migration distance of each protein on such gel is dependent upon both their p*I* values (charges) and their sizes. Despite evident similarities between the neurotrophic factors, their electrophoretic mobilities on nondenaturing 11% polyacrylamide gels diverge quite clearly (with the exception of mNGF and NT-4). This figure shows the utility of the nondenaturing gel electrophoresis in the biochemical characterization of the neurotrophins. In Figure 2, even-numbered lanes show a third species that resulted from the guanidine hydrochloride treatment of the neurotrophin mixtures. The heterodimeric species migrate halfway between the two native proteins, a behavior expected from a molecule having attributes of both parental neurotrophins. In control experiments, as illustrated in odd-numbered lanes, the two neurotrophins were treated separately by the same method and combined before the gel was run. These lanes showed no new band of an intermediate mobility. Sequencing of protein bands transferred to Hybond PVDF membrane indicated that the bands of intermediate electrophoretic mobility indeed included amino acid sequences of the two starting homodimers, therefore providing unequivocal evidence of heterodimer formation. Figure 3 demonstrates the formation of the heterodimers induced by 8-h incubation of a mixture of two homodimers in an 8 M solution of urea, followed by dialysis against neutral buffer. Unlike the incubation in 6 M guanidine hydrochloride, 8-h incubation in 8 M urea induces no apparent change in the circular dichroism spectra of the neurotrophins, but at the same time, it is sufficient to generate the heterodimers. It would appear, therefore, that the dimer–monomer equilibrium is perturbed in the solution of 8 M urea and the subunit exchange rate is fast enough to result in the formation of heterodimeric species without substantial unfolding. The monomer/dimer equilibrium can also be perturbed by exposing neurotrophic factors to acidic pH. In an acidic environment, protonation of the basic amino acid residues and the resulting electrostatic repulsion between the subunits will counteract the hydrophobic interactions responsible for the stability of the dimeric structure. As demonstrated in Figure 4, heterodimers can be generated by exposing a pair of neurotrophins to pH 1 for 6 h. Similarly, an organic solvent would be expected to weaken intersubunit hydrophobic interactions and consequently to accelerate the subunit exchange. Figure 5 shows the formation of the heterodimers induced by the presence of acetonitrile. The yield of heterodimer formation, estimated

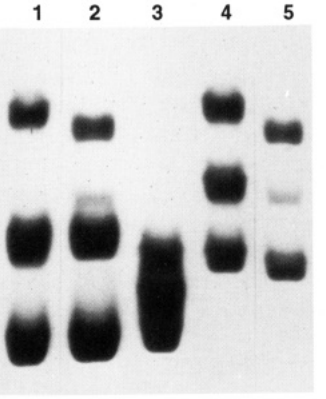

FIGURE 3: Nondenaturing gel electrophoresis at pH 7.4 of the neurotrophins treated with 8 M urea and dialyzed against neutral buffer. Lane 1, mNGF and BDNF; lane 2, NT-4 and BDNF; lane 3, NT-3 and BDNF; lane 4, mNGF and NT-3; lane 5, NT-3 and NT-4.

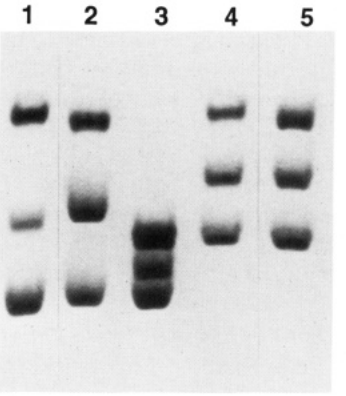

FIGURE 4: Nondenaturing gel electrophoresis at pH 7.4 of the neurotrophins treated with low pH and dialyzed against neutral buffer as described in Materials and Methods. Lane 1, mNGF and BDNF; lane 2, NT-4 and BDNF; lane 3, NT-3 and BDNF; lane 4, mNGF and NT-3; lane 5, NT-3 and NT-4.

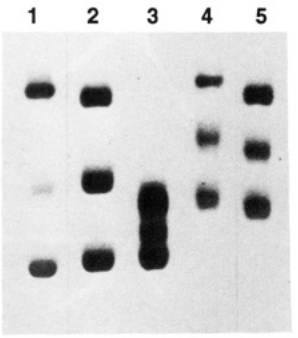

FIGURE 5: Nondenaturing gel electrophoresis at pH 7.4 of the neurotrophins treated with acetonitrile and dialyzed against neutral buffer as described in Materials and Methods. Lane 1, mNGF and BDNF; lane 2, NT-4 and BDNF; lane 3, NT-3 and BDNF; lane 4, mNGF and NT-3; lane 5, NT-3 and NT-4.

Table I: Yield of Heterodimer Formation by Four Different Methods, Estimated by Gel Scanning[a,b]

	6 M GuHCl	8 M urea	pH 1	40% CH_3CN
mNGF/BDNF	27.1	38.8	14.7	4.6
NT-4/BDNF	4.3	39.6	38.7	38.2
mNGF/NT-3	27.5	36.1	36.0	31.4
NT-3/NT-4	4.8	6.2	31.7	29.4

[a] Percentage of each heterodimer in a mixture applied onto 11% polyacrylamide, pH 7.4, nondenaturing gel. [b] For BDNF/NT-3 heterodimer, electrophoretic separation was insufficient for the gel scanning approach. However, as evident from Figures 2–5, BDNF/NT-3 heterodimer constituted approximately one-third of the mixture in each method used to generate the heterodimers.

by gel scanning (Table I), was to some extent dependent upon the method used to generate that heterodimer. The guanidine hydrochloride method produced high yields of mNGF/BDNF, mNGF/NT-3, and NT-3/BDNF heterodimers but was less suitable for BDNF/NT-4 and NT-3/NT-4. The urea method resulted in high yields of BDNF/mNGF, BDNF/NT-4, BDNF/NT-3, and mNGF/NT-3 but was less efficient for making the NT-3/NT-4 heterodimer. The low-pH method resulted in high yields of BDNF/NT-4, BDNF/NT-3, mNGF/NT-3, and NT-3/NT-4 heterodimers but produced lower amounts of BDNF/mNGF. The aqueous acetonitrile method resulted in high yields of BDNF/NT-4, BDNF/NT-3, mNGF/NT-3, and NT-3/NT-4 heterodimers but was less efficient for mNGF/BDNF.

In some cases, the subunit exchange rate was rapid enough to permit the formation of the heterodimers even in the absence of the agents promoting homodimer dissociation. An 8-h incubation at 37 °C results in the formation of two heterodimers: BDNF/NT-4 and BDNF/mNGF (Figure 6). A 24-h incubation of the homodimer mixtures at room temperature also produced the same two heterodimers. A 4-day incubation at room temperature resulted in the formation of even greater amounts of those two heterodimers (data not shown). However, no heterodimer was generated as the result of an 8-h incubation at room temperature or a 24-h incubation at 4 °C (data not shown). Therefore, guanidine hydrochloride, urea, acidic buffer, and aqueous acetonitrile indeed facilitate subunit exchange.

Purification of the Heterodimers. High-pressure cation-exchange chromatography proved to be the most convenient way to separate the heterodimers from the parental homodimers. Figure 7 shows the elution profile for the purification of the BDNF/NT-3 heterodimer. It is worth noting that, even in the case of those two very similar growth factors, excellent resolution was achieved with the aid of a

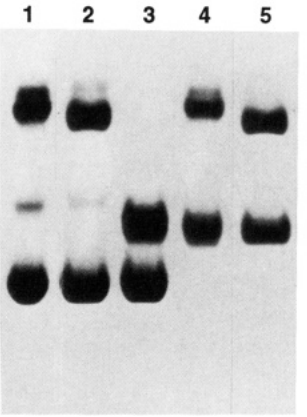

FIGURE 6: Nondenaturing gel electrophoresis at pH 7.4 of the neurotrophins incubated at 37 °C for 8 h in Tris·HCl, pH 8. Lane 1, mNGF and BDNF; lane 2, BDNF and NT-4; lane 3, BDNF and NT-3; lane 4, mNGF and NT-3; lane 5, NT-3 and NT-4.

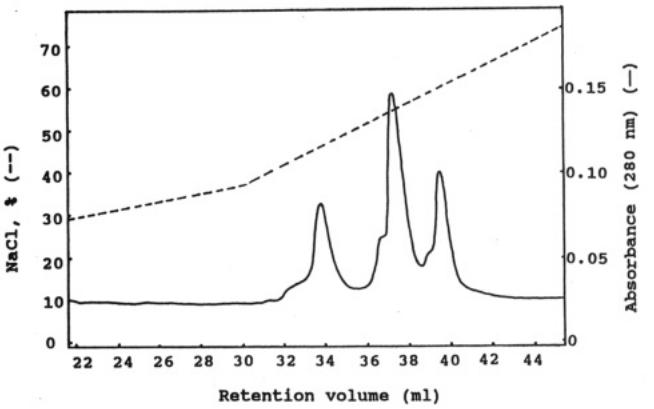

FIGURE 7: Separation of BDNF/NT-3 heterodimer from the parental homodimers on a Mono S 5 × 5 cation-exchange column using a sodium chloride gradient in 20 mM phosphate buffer, pH 7. The heterodimer peak eluted between the peak of NT-3 (lower retention volume) and the peak of BDNF (higher retention volume).

Mono S cation-exchange column. The degree of separation was considerably better for the other heterodimers (data not shown). Neither nondenaturing gel electrophoresis nor high-performance ion-exchange chromatography allowed us to observe the mNGF/NT-4 heterodimer. Purified heterodimers were concentrated and desalted by ultrafiltration and stored at −20 °C. When tested on a nondenaturing polyacrylamide gel, the heterodimers containing NGF showed the presence of their parental homodimers. In contrast, heterodimers without the NGF subunit were essentially homogeneous, indicating their higher stability (Figure 8).

Stability of Heterodimers. Stability of the heterodimers with respect to dissociation and rearrangement to homodimers was examined by reinjecting dilute solutions of heterodimers onto a Pharmacia 5 × 5 Mono S column. A 24-h incubation of the heterodimers BDNF/NT-4, BDNF/NT-3, and NT-3/NT-4 in 20 mM phosphate, pH 7.0, at 37 °C resulted in a small increase of the appropriate precursor homodimers. For the NT-3/NT-4 heterodimer, the homodimer content increased from 2% to 6%. Likewise, for the BDNF/NT-4 heterodimer, the homodimer content upon incubation increased from 2% to 2.2%. Ion-exchange chromatography on a Mono S column failed to detect the presence or any increase in the content of the parental homodimers in the case of BDNF/NT-3 heterodimer. This may be due to a more difficult separation. Most likely, in this case the degree of rearrangement, if any, would be similar to the degree of rearrangement found for BDNF/NT-4 and NT-3/NT-4. In contrast,

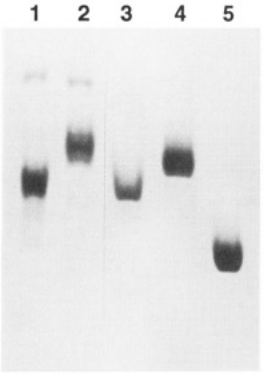

FIGURE 8: Nondenaturing 11% polyacrylamide gel electrophoresis of FPLC-purified samples of the heterodimers. Lane 1, mNGF/BDNF; lane 2, mNGF/NT-3; lane 3, BDNF/NT-4; lane 4, NT-3/NT-4; lane 5, BDNF/NT-3. Heterodimers mNGF/BDNF and mNGF/NT-3 were generated by the guanidine hydrochloride method. Heterodimers BDNF/NT-4, BDNF/NT-3, and NT-3/NT-4 were generated by the low-pH method.

injections of purified heterodimers BDNF/mNBF and mNGF/NT-3, kept at −20 °C, showed the presence of the parental homodimers in addition to the heterodimers. Upon incubation at 37 °C at 10 μg/mL in the same phosphate buffer, the rearrangement process resulted in a significant increase in the content of homodimers. The homodimer content for BDNF/mNGF rose from 12% to 20%. Incubation of the mNGF/NT-3 heterodimer at 37 °C resulted in the formation of multiple peaks in the elution gradient on a Mono S column.

Circular Dichroism Spectra of the Heterodimers. The circular dichroism spectra of the freshly purified heterodimers are distinct from the spectra of the homodimers. The CD spectra of the stable heterodimers display a maximum of positive ellipticity around 230 nm: NT-3/NT-4, λ_{max} 231.2 nm; BDNF/NT-3, λ_{max} 232.7 nm; BDNF/NT-4, λ_{max} 231.0 nm (Figure 9). The maxima of positive ellipticity for the heterodimers fall between the maxima for the parental homodimers. The spectra of each heterodimer were also compared to an average of two spectra of the parental homodimers. The comparison showed that the spectra of NT-3/NT-4 and BDNF/NT-3 heterodimers were nearly identical to the averaged spectra of the parental homodimers. In the case of BDNF/NT-4, the CD spectrum diverges more significantly from an averaged spectrum. Some buried amino acid residues are probably located in a different, asymmetric environment in the heterodimers than in the parental homodimers. Furthermore, the formation of heterodimers may also require some minor structural adjustments in both subunits. The circular dichroism spectra of the neurotrophic factors in 8 M urea at pH 8 and in HCl at pH 1 were examined between 260 and 220 nm. Only very small spectral changes were seen after a 24-h incubation at room temperature (data not shown).

Gel Filtration Chromatography of the Heterodimers. Size-exclusion chromatography on two Superose 12 columns connected in series allowed us to obtain a baseline separation of NT-4 from BDNF and of NT-4 from NT-3, as well as partial separation of NT-3 from mNGF (elution profiles not shown). As expected, the elution volumes for the heterodimers fall between those for the homodimers. The elution volumes for NT-4, BDNF/NT-4 heterodimer, and BDNF were 27.97, 30.5, and 31.28 mL, respectively. The elution volumes for NT-3/NT-4 heterodimer and NT-3 were 29.12 and 30.34 mL, respectively. The observed resolution of the neurotrophins and their heterodimers may not be exclusively due to the differences in their hydrodynamic radii. Selective interactions

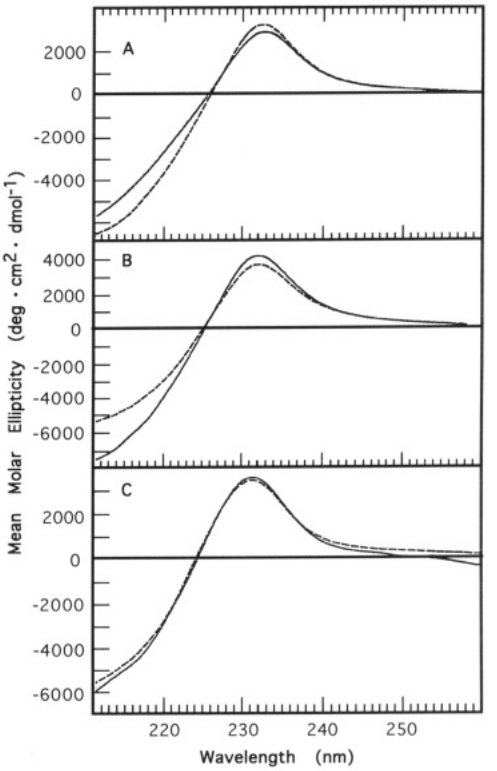

FIGURE 9: Circular dichroism spectra of the purified heterodimers. (a) BDNF/NT-3; (b) BDNF/NT-4; (c) NT-3/NT-4. Solid line, CD spectra of the heterodimers; dashed line, average of the two parental homodimers' CD spectra.

with the column matrix may also be contributing to the separation. These findings provide further evidence that the observed species are indeed heterodimers derived from the starting homodimers and exclude a possibility of new species being, for example, complexes of the homodimers. Furthermore, the stoichiometry of association in the heterodimers must be 1:1. High-performance gel filtration in addition to cation-exchange chromatography may be used to further purify small quantities of neurotrophin heterodimers.

DISCUSSION

The high stability of the neurotrophin homodimers results from the interactions of the hydrophobic amino acid residues comprising the dimer interface (McDonald et al., 1991). Comparison of the amino acid residues involved in the dimer interface reveals a high degree of conservation among the four members of the neurotrophin family (McDonald et al., 1991). The only significant perturbation occurs at position 79: Y in NGF, Q in BDNF and NT-3, and E in NT-4, whereas all the remaining substitutions are much more conservative. That conservation of the residues comprising dimer interface, as well as experimental indications that homodimer dissociation was promoted by the presence of denaturants, prompted us to examine the possibility of heterodimer formation between different neurotrophic factors. We discovered four sets of conditions (guanidine hydrochloride, urea, organic solvent, and acidic pH) under which the neurotrophin homodimers will dissociate and recombine to form heterodimers. Formation of the heterodimers, after treatment with guanidine hydrochloride, almost certainly involves monomer unfolding in addition to the dissociation of the dimer and therefore appears to be mechanistically distinct from the other three methods. However, the presence of a denaturing agent is not absolutely necessary since the generation of BDNF/mNGF

and mNGF/NT-4 heterodimers also takes place in Tris buffer, pH 8. In this case, the yield of heterodimer formation was determined to be a function of time and temperature of incubation. These attributes are typical of a process reaching thermodynamic equilibrium. Prolonged incubation would presumably result in the formation of all heterodimers. This observation also indicates that the rates of dissociation and association vary between the neurotrophic factors. Our findings are reminiscent of the results obtained by Chiappinelli and Wolf (1989) in regard to κ-neurotoxins. κ-Neurotoxins were found to spontaneously form heterodimers. Interestingly, just like neurotrophic factors, κ-neurotoxins are also dimeric, basic proteins containing predominantly β-sheet conformation. Present studies strongly suggest that low pH, acetonitrile, and urea merely increase the neurotrophin subunit exchange rate, and they decrease time needed to reach an equilibrium without grossly affecting the subunit structure, as no significant changes in the circular dichroism spectra of the neurotrophins were observed after 24 h in the presence of either 8 M urea or acidic pH. Among all the heterodimers examined, those involving the mNGF protomer were found to be least stable. Isolated heterodimers containing mNGF protomers undergo gradual rearrangement to the starting homodimers. In contrast, the heterodimers that do not contain NGF withstand 24-h incubation at neutral pH, at 37 °C, with only a small increase in the content of parental homodimers. The differential stability may be a reflection of small structural differences between neurotrophic factors. Indeed, a superposition of the circular dichroism spectra of the four known neurotrophic factor reveals dissimilarity between BDNF, NT-3, and NT-4 on one hand and NGF on the other. In particular, the far-ultraviolet CD spectra of BDNF, NT-3, and NT-4 display positive peaks of ellipticity at 233, 231.5, and 231 nm, respectively, whereas the NGF spectrum displays no positive ellipticity. The secondary structure of NGF estimated from circular dichroism spectroscopy appears to contain a lower proportion of β structures than BDNF or NT-3 (Radziejewski et al., 1992). This is in contrast to FTIR studies (Nahri et al., 1993) of human BDNF, NT-3, and NGF showing the NGF to possess the highest content of β structures. The circular dichroism spectra of the heterodimers, obtained in the present studies, may suggest that some structural alteration of the neurotrophin promoters may be required in order to create their dimeric combination as the spectra diverge to a small extent from the averaged spectra of the two parental homodimers. Our stability results indicate that the structure of the NGF promoter is less compatible with other neurotrophin promoters. Heterodimer stability would then appear to depend not exclusively upon the hydrophobic interactions at the dimer interface but also upon the structure of the entire molecular aggregate. A judicious chemical cross-linking may enhance the stability of the heterodimers containing NGF protomer. Likewise, it may be possible to engineer more stable neurotrophin heterodimers by the introduction of an intermolecular disulfide bond into each heterodimeric molecule. The fact that NT-4 is able to participate in heterodimer formation is particularly interesting as the primary structure of human NT-4 shows a 10 amino acid insert when aligned to other neurotrophins. This also provides strong indirect evidence that NT-4, like all other neurotrophins, exists in solution as a noncovalent homodimer. The three crystal structures reported within the NGF superfamily (NGF, TGF-β2, and PDGF-BB) show that each family forms a different dimer interface (Murzin & Chothia, 1992); this implies that the monomeric fold is intrinsically stable and does not require a particular dimerization. We would propose that the conservation of the dimerization interface within the neurotrophin family, and hence the ability to form heterodimers, has resulted from the absolute need for receptor dimerization in signal transduction. This maxim would be also true for both the PDGF and TGF-β families. Several members of the TGF-β gene superfamily (TGF-β, inhibin, activin, bone morphogenic protein) exist as heterodimers in vivo (Massagué, 1990; Ogawa et al., 1992). By analogy to neurotrophins, we would predict that it should be possible to make many more of these heterodimers *in vitro*. Furthermore, as the TGF-β2 dimer interface is reasonably conserved throughout the TGF-β gene superfamily (Daopin et al., 1992; Schlunegger & Grütter, 1992), it may well be possible to produce heterodimeric examples between the individual families within the TGF-β superfamily, for instance, between a member of the TGF-β2 family and a member of the bone morphogenic protein family. These disulfide-linked heterodimers would be useful tools in unraveling the complex receptor–ligand interactions within these families (Lin & Lodish, 1993; Massagué, 1990). The ability of the neurotrophic factors to form heterodimers, in some cases even spontaneously, brings up the possibility that these species might also exist *in vivo*, although establishing their identities might prove difficult. The heterodimers may prove useful in probing neurotrophin–receptor interactions. For instance, it will be interesting to compare the binding affinities of BDN/NT-3 heterodimer and of the parent homodimers to the NT-3-specific receptor *trkC* (Lamballe et al., 1991). If the homodimeric ligand is required for high-affinity binding, then a significant difference should be seen between the binding affinities of NT-3 and the BDNF/NT-3 heterodimer. It would also be intriguing to determine if the stable heterodimers could elicit biological responses distinct from those of the homodimers. Should this be the case, the heterodimers may themselves become valuable drug candidates.

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