Comparison of the Biophysical Characteristics of Human Brain-derived Neurotrophic Factor, Neurotrophin-3, and Nerve Growth Factor*

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The structural properties of human brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) were studied using sedimentation equilibrium and circular dichroism (CD), fluorescence and Fourier-transform infrared spectroscopies, and compared with those of human nerve growth factor (NGF). Both the far UV CD and infrared spectra indicate that these three proteins have similar, but not identical, secondary structures which contain primarily \( \beta \)-sheet and irregular structures. NGF appears to contain the most \( \beta \)-sheet while NT-3 contains a small fraction of \( \alpha \)-helix. The near UV CD spectra appear to indicate that the three proteins contain disulfide bonds in similar environments, suggesting a resemblance in tertiary structure. The fluorescent tryptophans found in the molecules are relatively solvent exposed, while Trp\(^{122} \) found only in NT-3 is possibly quenched. The fluorescent Trp(s) in NGF are significantly quenched relative to those in the other two neurotrophic factors. Both NT-3 and BDNF have very hydrophilic surfaces at neutral pH, as indicated by a low binding affinity to a hydrophobic probe, anilinonaphthalenesulfonate. Sedimentation equilibrium showed that BDNF, NT-3, and NGF exist as strongly associated dimers in phosphate-buffered saline, pH 7.1. Fits of the observed fringe displacements to various association models suggested that the BDNF, NT-3, and NGF samples contain, in addition to the principal dimeric species, some oligomers, and that NT-3 contains a small fraction of incompetent monomer.

Nerve growth factor (NGF)\(^1 \) is the parent member of a family of neurotrophic factors that is now known to have at least three other members. The first member to be discovered after NGF was brain-derived neurotrophic factor (BDNF), which was isolated by Barde et al. (1982) and recently cloned (Leibrock et al., 1989). BDNF is 55% homologous to NGF, and comparison of the two sequences demonstrates that the regions around the 6 cysteines, all of which form intrachain disulfides in NGF, are strongly conserved.

Primer sequences constructed from sequences in the conserved regions led to the discovery, by polymerase chain reaction techniques, of a third member of the family, neurotrophin-3 (NT-3) (Maisonpierre et al., 1990; Hohn et al., 1990; Rosenthal et al., 1990; Ernfors et al., 1990; Jones and Reichardt, 1990). NT-3 was also discovered by low stringency hybridization using the NGF sequence as a probe (Kaisho et al., 1990). This protein is 58% homologous to BDNF and 57% homologous to NGF, again with strong conservation of the residues about the cysteines.

NGF is a dimer in solution (Angeletti et al., 1971), and this dimerization has an unusually high affinity constant such that at physiologically active concentrations NGF is a dimer (Bothwell and Shooter, 1977). The recent structure of murine \( \beta \) NGF obtained by x-ray crystallography (McDonald et al., 1991) revealed the existence of a hydrophobic face involved in dimerization and also a secondary structure rich in \( \beta \)-sheet but containing no \( \alpha \)-helix. The conserved hydrophobic regions of the protein could be involved in this contact site.

The high degree of sequence homology, particularly around the residues involved in the disulfide bonds in NGF, raises questions as to the degree of similarity of the higher order structures between NGF and the more recently discovered neurotrophic factors. With this in mind, we undertook the structural characterization of recombinant human BDNF and NT-3 using CD, fluorescence, and FTIR spectroscopic techniques and sedimentation equilibrium and compared the results with the structural properties of human NGF. Since NGF was shown to form non-covalent dimers, we have examined in detail the association behavior of the recombinant BDNF, NT-3, and NGF. In addition, because NGF is a protein rich in \( \beta \)-structure and CD does not reliably quantitate \( \beta \)-structure, we have used FTIR to compare the secondary structures of the proteins. A partial comparison of the properties of human BDNF, and NT-3, with murine NGF was recently reported (Radziejewski et al., 1992). However, this is the first report in which the three human molecules were compared.

**EXPERIMENTAL PROCEDURES**

**Preparation of Proteins**—Recombinant human BDNF and NT-3 were expressed in Chinese hamster ovary cells and purified from conditioned media by sequential column chromatography involving size exclusion, ion-exchange, and reverse-phase columns. NGF was...
expressed in Escherichia coli and after extraction and oxidation was purified in a similar fashion. Protein concentrations were determined assuming an extinction coefficient of ε_{280} = 2.0 for BDNF, ε_{280} = 2.17 for NT-3, and ε_{280} = 1.6 for NGF. All analyses were performed in PBS, pH 7.1, unless otherwise noted.

Analytical Ultracentrifugation—Sedimentation equilibrium experiments were carried out using a Beckman model E ultracentrifuge equipped with an automated real-time data acquisition system for the Rayleigh interference optics. Fringe displacements were obtained using a solid-state television camera and a frame grabber connected to an IBM PC compatible computer (Zenith Z-386/20) (Lary et al., 1990). A near-infrared (785 nm) laser diode, pulsed in synchrony with rotor rotation (Lary et al., 1990), was used as the interferometer light source. Typically about 300 data points were measured and used for each loading concentration in the 2.6-mm column height experiments and about 60 data points for each loading concentration in the short-column experiments.

Experimental observations were fitted to several assumed models using a non-linear least squares approach (Johnson et al., 1981). The FORTRAN programs used were based on a version of NONLIN, written for a PDP-11. All programs were run under VAX/VMS systems. The indicated ranges of the fitting parameters from the least-squares fittings correspond to confidence ranges of 68% probability. (For Gaussian error distributions these confidence regions are essentially equivalent to ranges of the mean plus or minus two standard deviations.) Apparent weight average molecular weights were estimated as a function of position using the BIOSPIN program (Roark and Yphantis, 1988) using the meniscus concentrations estimated by the non-linear least squares programs.

Short column equilibrium experiments were carried out in 12-mm thick multichannel centerpiece (Yphantis, 1960) loaded with enough solution (about 18 μl) to provide column heights of about 0.75 mm. Loading concentrations ranged from 0.5 to 2.35 g/liter. These experiments were run at speeds of 26,000 and 32,000 rpm for a minimum of 120 min at each speed, well over the time interval estimated (van Holde and Baldwin, 1959) to attain equilibrium within epsilon = 0.001. Actual attainment of equilibrium was verified by a direct least-squares comparison of fringe displacements at various time intervals using a procedure that fits one set of displacements in terms of another, allowing for linear displacements both in the direction of fringe displacement (to take care of differences in fringe reference level), and in the radial direction (to account for instantaneous differences in apparent radial position between two data sets caused by rotor precession). All comparisons (for times over about 50 min of running time) showed root-mean-square (r.m.s.) differences of less than 0.01 fringe between pairs of measurements, with no systematic deviations.

"Long column" experiments used external loading cells (Ansevin et al., 1970) with three pairs of observation channels and either 12- or 20-cm optical pathlengths at speeds of 26,000-40,000 rpm for a minimum of 120 min at each speed, well over the time interval estimated (van Holde and Baldwin, 1959) to attain equilibrium within epsilon = 0.001. Actual attainment of equilibrium was verified by a direct least-squares comparison of fringe displacements at various time intervals using a procedure that fits one set of displacements in terms of another, allowing for linear displacements both in the direction of fringe displacement (to take care of differences in fringe reference level), and in the radial direction (to account for instantaneous differences in apparent radial position between two data sets caused by rotor precession). All comparisons (for times over about 50 min of running time) showed root-mean-square (r.m.s.) differences of less than 0.01 fringe between pairs of measurements, with no systematic deviations.

RESULTS AND DISCUSSION

Equilibrium Sedimentation of NGF—Recombinant human NGF was examined at loading concentrations of 0.4, 0.29, and 0.14 g/liter in pH 7.1 PBS buffer at 19.4 °C using a 12-mm thick centerpiece at speeds of 30,000 rpm and 36,000 rpm. Estimates were made of the average molecular weights for each channel by fitting the observed fringe displacements to the model of a single ideal component (Correa, 1980, 1983; Correa and Yphantis, 1992). These average estimates (data not shown) ranged from about 29.6 to 34.2 kg mol⁻¹ and increased with loading concentration, suggesting that under these conditions NGF exists primarily as a self-associating dimer.

Detailed analyses of these experiments were carried out by fitting the six sets of fringe displacements jointly to several models. The joint fit as a single ideal component was inadequate, with r.m.s. deviations of 0.0354 fringes; it returned the value of 32.7 ± 0.6 kg mol⁻¹ for the size of the "best-fitting" single ideal component. The model of a self-associating dimer/tetramer equilibrium was significantly better; assuming the formula weight of 27.02 kg mol⁻¹ for the dimer, the r.m.s. deviations of the joint fit were 0.0151 fringes. Convergence on dimer size gave the value of 26.33 ± 0.65 kg mol⁻¹ with no significant improvement of the fit (r.m.s. deviations = 0.150 fringes) over the assumption of the formula weight.

The fits were improved significantly by the inclusion of further self-association of the dimers to form small numbers of larger oligomers. Our best fits indicate the presence of traces (less than 0.3 fringe at the highest observable concentration (7.8 fringes)) of an apparent dodecamer (converging on the degree of association of the larger oligomer gave n = 12.1 ± 4.4). The assumption of our best model (reversible dimer/tetramer/dodecamer association) and the formula weight for the dimer gave an r.m.s. fitting deviation of 0.0131 fringes; convergence on the dimer size provided no decrease in the fitting r.m.s. deviations and returned the value of 27.42 ± 0.55 kg mol⁻¹ for the dimer molecular weight. There were no significant improvements in the fits (the change in the r.m.s. fitting deviations was less than 0.0002 fringes) on the assumption of heterogeneity in association behavior of either component.

2 Convergence on the degree of association of the smaller oligomer gave n = 3.98 ± 0.27.
of the two equilibria. There was no improvement of the fits on the further addition of a monomer-dimer equilibrium (either homogenous or heterogeneous) to the fitting model and no evidence for any detectable monomer. The inclusion of a second virial coefficient gave no significant improvement in the least-squares fits and returned (small) negative values, reflecting self-association (Stafford and Yphantis, 1972) rather than any repulsive non-ideality, such as would be expected from excluded volume or charge effects.

The associations to form tetramer and dodecamer from dimer appear to be in rapid equilibrium (compared to the time course of the experiments, about 20 h) and to be homogeneous, i.e. the protein self-associates as a single thermodynamic component. We estimate the association constants at 19.4 °C (from the 2:4:12 fits assuming the formula weight for the dimer) as: $K_{12} = 3.300 \pm 300 M^{-1}$ and $K_{32} = 5.5 \pm 1.5 \times 10^{18} M^{-4}$. The corresponding standard free energies of association are $-4.71 \pm 0.05$ kcal mol$^{-1}$ of tetramer and $-25.1 \pm 0.14$ kcal mol$^{-1}$ of dodecamer.

Equilibrium Sedimentation of NT-3—Sedimentation equilibrium experiments were carried out on two preparations of human NT-3 at 20.0 ± 1.2°C: the first preparation was examined in short columns with loading concentrations of 2.35, 1.17, 0.71, and 0.35 g/liter at 26,000 and 3,000 rpm. The apparent $r$ average molecular weights (data not shown) ranged from slightly below to slightly above the dimer formula weight, suggesting that the dimer is the predominant molecular species under these conditions. The observed molecular weights increased with loading concentration and were independent of speed, suggesting reversible association.

Experiments at much lower loading and observation concentrations were carried out in 30-mm thick centerpieces using 2.6-mm high solution columns to provide further description of this system. The first preparation was examined at 32,000 rpm using loading concentrations of 0.29, 0.15, and 0.073 g/liter, and the second preparation was run at 36,000 rpm. In one run we used the same three loading concentrations as above and in another we examined the intermediate concentration (0.15 g/liter) in triplicate. The apparent weight-average molecular weights observed for the first preparation at each observation position are presented in Fig. 1 (upper panel). The lack of overlap of the values of the apparent molecular weight moments at each observation concentration, $c(r)$, for the different loading concentrations indicates this sample to be heterogeneous (Squire and Li, 1961; Yphantis, 1964) so that this sample cannot be described as a single thermodynamic component. Again, as in the short column experiment, the observed molecular weights range from somewhat below to somewhat above the dimer formula weight, indicating the presence of molecules both smaller and larger than dimer. In contrast, the second preparation (Fig. 1, lower panel) gave overlapping curves for the apparent weight-average molecular weight. This lack of dependence on the loading concentrations indicates the sample to be homogeneous, associating as a single thermodynamic component.

Replicate observations of the fringe displacements from these two sets of experiments were fitted exhaustively to various models, ranging from dimer alone to combinations of the dimer with monomer and/or individual oligomers ranging from trimer to dodecamer. Most of these models were fitted both with and without the assumption of non-ideality with no evidence of any positive second virial coefficient; only negative second virial coefficients were seen in such analyses, reflecting association.

An abbreviated summary of some of the ideal fits that were obtained by fixing the dimer size at the formula weight is presented in Table I for some pertinent models. There was no significant improvement of these fits (with decreases of less than 0.6003 fringe in the r.m.s. deviations of the fits) when the dimer size was also converged upon. The dimer sizes thus found varied systematically with the fitting model used and ranged from 25.1 to 29.4 kg mol$^{-1}$; models assuming homogeneous association behavior (with common values of association constants rather than separate association constants for each solution channel) gave dimer sizes ranging from 26.3 to 28.2 kg mol$^{-1}$, corroborating our estimate of the partial specific volume of NT-3 to within about 0.01 cc g$^{-1}$.

The best fits (assuming both homogeneous association and the dimer formula weight) correspond to equilibria between monomers, dimers, and tetramers for the first preparation and to a dimer-tetramer equilibrium for the second preparation. It was not possible to discriminate among trimer, tetramer, or pentamer at the 95% confidence level since only relatively small amounts of the oligomer were visible (less than 0.4 fringe at the most centrifugal point of the most concentrated observation channel).

The inclusion of heterogeneity in either the monomer-dimer or the dimer-tetramer association by fitting with separate association constants for each loading concentration (Yphantis et al., 1979) gave significant (at the 95% confidence level) improvement in the fits (Table I) for the first preparation, indicating heterogeneity in both these association stages. In contrast, the second preparation appeared to be homogeneous in its association behavior. Precise estimation of heterogeneity is difficult; it depends on the assumption of mass conservation, on the species assumed to be present, and on precise localization of the bases of the individual channels.

The individual (for each loading concentration) apparent monomer-dimer association constants returned by the fitting for the first preparation (data not shown) suggest (and can be approximately accounted for by) the presence of monomer incapable of associating to dimer. Estimates of the amounts of such "incompetent monomer" (Teller et al., 1969) depend on the specific model used for the fitting; assuming the higher oligomer to be a tetramer, we find that the first preparation of NT-3 contained about 4-5% of such incompetent monomer. The heterogeneity in the first preparation evident in the association of the dimers to form higher oligomers could be accounted for by assuming the presence of about 3% of the

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4 Correspondingly, when each observation channel was fitted to its own individual apparent association constant, these association constants were indistinguishable within experimental error.

6 During analyses of the second preparation, we found that the initial meniscus region for the most concentrated channel of this experiment appeared to be perturbed, probably from refractive index gradients in the air space between the light source and the lower collimating lens that were induced by the (usual) cooling of the vacuum chamber. (W. F. Stafford initially recognized and tracked this effect.) The region of perturbation extended from the meniscus up to a fringe displacement of 0.5 fringe and corresponded to the concentration range below 70 μg ml$^{-1}$. We omitted observations from the perturbed region of this loading concentration in the final analyses.

7 It is difficult to see the actual base of a solution column when the refractive index gradient is large, as it is with all but the lowest loading concentration of these "high speed" equilibrium experiments (Yphantis, 1964). We are currently trying to devise procedures for routine estimation of such "base" positions so as to make possible improved estimates of heterogeneity.
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Fig. 1. Two preparations of neurotrophin-3 (NT-3) in PBS buffer, pH 7.1, were examined in a 30-mm thick centerpiece: upper panel, preparation 1 at 32,000 rpm; lower panel, preparation 2 at 36,000 rpm. The loading concentrations were: 0.29 g/liter (circles), 0.15 g/liter (squares), and 0.073 g/liter (diamonds). The apparent weight-average molecular weights estimated at the radius r are presented as a function of \( c(r) \), the concentrations at these observation points. The dashed lines indicate the dimer formula weight of 27.29 kg mol\(^{-1}\). Upper panel, the divergence of the curves for preparation 1 indicates heterogeneity in association behavior. Lower panel, the effective superposition of these curves shows that preparation 2 behaves as a single thermodynamic component. In both cases, the increase of the apparent weight-average molecular weight with observation concentration, \( c(r) \), indicates a weak oligomerization of the dimers (see text for details).

**TABLE I**

Fitting of NT-3 sedimentation equilibrium experiments, assuming the formula weight, 27.287 kg mol\(^{-1}\), for the dimer

| Fitting model                     | First preparation | Second preparation |
|-----------------------------------|-------------------|--------------------|
|                                   | r.m.s. residuals  | (in fringes)       |
| Dimer only                        | 0.0149            | 0.0158             |
| Monomer-dimer                     | 0.0142            | 0.0159             |
| Dimer-tetramer                    | 0.0150            | 0.0117             |
| Monomer-dimer-trimer              | 0.0134            | 0.0116             |
| Monomer-dimer-tetramer            | 0.0134            | 0.0116             |
| Monomer-dimer-pentamer            | 0.0134            | 0.0117             |
| Monomer-dimer-hexamer             | 0.0134            | 0.0119             |
| Monomer-(HET)-dimer-tetramer\(^a\)| 0.0127            | 0.0118             |
| Monomer-dimer-tetramer (HET)      | 0.0121            | 0.0110             |

\(^a\) A significant difference (at the 95% confidence level) in the least-squares fits corresponds approximately to values of 1.043 and 1.036 for the ratios of the r.m.s. residuals from the fits for the experiments of preparation 1 and 2, respectively.

The symbol (HET) indicates that this species was assumed to be heterogeneous in its association behavior so that the fitting model used separate apparent equilibrium (association) constants for each set of observations.

**Sedimentation Equilibrium of BDNF**—Short column equilibrium experiments were run with loading concentrations of 0.9, 0.7, 0.5, and 0.3 g/liter of BDNF in pH 7.1 PBS buffer at 19.9 °C. The apparent \( z \) average molecular weights found depended on loading concentration, giving estimates of about 2.1 times monomer size at the lowest initial concentration and from 3.3 to 3.5 times the monomer size at the other three loading concentrations. These preliminary observations indicated some concentration-dependent association but were insufficient to describe this preparation adequately.

Accordingly, we ran long column equilibrium experiments at loading concentrations of 0.1, 0.3, and 0.9 g/liter at a speed of 26,000 rpm in the same solvent at 20.3 °C in 12-mm thick centerpieces. These concentration distributions could be fit, roughly (with an r.m.s. deviation of 0.024 fringes), as a single component system with an apparent molecular weight of 29.1
assuming that the solutions were not homogeneous in their association behavior. The best fits (with r.m.s. deviations = 0.0065 fringes) were to models describing heterogeneous association of a dimer and two higher oligomers. This fitting converged to degrees of association of the oligomers of 3.92 ± 0.06 and 11.8 ± 0.7 monomers/oligomer, and yielded systematically varying and non-overlapping values of the apparent association constants. Simultaneous fitting assuming both a reversible homogeneous equilibrium between dimers and tetramers and the presence of heterogeneous association gave estimates for the upper limit of the association constant, $K_{d,4}$, as 2700 ± 400 M$^{-1}$. The maximum amount of dodecamer in the visible part of the concentration distributions was less than 0.4 fringe. Our estimates for the upper limit of the association constant for formation of dodecamer from dimer are $K_{d,12} = 5.6 \pm 1.3 \times 10^{18}$ M$^{-6}$. There was no indication of monomer in any of the least-square fits.

Graphs of the weight-average molecular weights, as calculated from the least-squares fitting parameters, versus $\{r^2-b^2\}/2$ (data not shown), where $b$ is the radius of the base of the solution channel, indicate this preparation of BDNF to behave largely (but not completely; see above) as a non-interacting (non-associating) dimer-tetramer system, with about one-quarter of the solute in the form of tetramer. In addition, there appears to be some further higher association (presumably the detected formation of dodecamers) that is strongly concentration-dependent.

Summary of Equilibrium Sedimentation Results—In conclusion, NGF exists under our conditions as dimers that self-associate reversibly with a well-defined association constant to form tetramers (these tetramers made up as much as 18% of the solute experimentally visible in these experiments). There is no evidence of any dissociation to monomers nor of any non-ideality. Some weak reversible association to (probable) dodecamers is apparent, with less than 0.6% of the sample at the highest loading concentrations estimated to be in the form of dodecamers.

Similarly, NT-3 appears to be primarily a dimer under these near physiological conditions. One preparation was

\begin{table}
| Assignment | BDNF $\sigma$ cm$^{-1}$ | NT-3 $\sigma$ cm$^{-1}$ | NGF $\sigma$ cm$^{-1}$ |
|------------|------------------------|------------------------|------------------------|
| $\beta$    | 1.622 0.04             | 1.624 0.04             | 1.622 0.02             |
| $\beta$    | 1.633 0.32             | 1.636 0.37             | 1.635 0.50             |
| Random coil | 1.645 0.31             | 1.646 0.20             | 1.647 0.12             |
| $\alpha$-Helix | 1.662 0.20         | 1.664 0.18             | 1.664 0.09             |
| Turns      | 1.678 0.10             | 1.676 0.05             | 1.675 0.09             |
| $\beta$    | 1.692 0.02             | 1.692 0.02             | 1.691 <0.01             |

Fig. 2. The second derivative infrared spectra in the amide I' region for NGF (A), BDNF (B), and NT-3 (C). The second derivative spectra were calculated as described in Susi and Byler (1983).
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slightly heterogeneous and appeared to contain less than 5% of monomer incapable of association to dimer and roughly 2% of a higher oligomer. The other preparation appeared to associate as a single thermodynamic component. Both preparations showed weak reversible association of the dimer, probably to tetramer, assuming this stoichiometry.

The main component of our BDNF preparation is the dimer, but, in addition, there appears to be some non-associating (or non-interacting) tetramer. Also there seems to be some weak association to higher oligomers, probably decamers. There was no evidence of any monomer in this preparation.

All these proteins are primarily dimers in PBS buffer at pH 7.1 and all exhibit weak association of the dimer to tetramer, with values of $\Delta G^{\text{z,12}}$ estimated to be $-4.7 \pm 0.1, -3.7 \pm 0.2$, and $-4.7 \pm 0.1$ kcal/mol of tetramer for NGF, NT-3, and BDNF, respectively. The NGF and BDNF preparations both appear to exhibit reversible associations of dimers to decamers with $\Delta G^{\text{z,12}}$ estimated as $-25.1 \pm 0.14$ and $-26.5 \pm 0.2$ kcal/mol of tetramer, respectively. No evidence of such decamers was seen in the NT-3 preparations, possibly because the NT-3 preparations were run roughly 2.5 times more dilute than the NGF and BDNF samples. It should be noted that the standard free energies of association per associating unit (dimer) are roughly the same,\(^6\) ranging from $-3.7$ to $-4.4$ kcal/mol, in all the associations observed with these proteins. This suggests the possibility of a common (or similar) region of the protein surface that is involved in these self-associations.

At the low observation concentrations used for these analyses, we did not expect to see (and did not find) any repulsive non-ideality (especially in the presence of association and/or heterogeneity) since such relatively small molecules usually have only trivial excluded volume effects and since the effective charge under these near physiological conditions is expected to be small.

\(^6\) Making the assumption that the maximum association constants estimated correspond roughly to the true values.

**FTIR**—Having established that the BDNF and NT-3 exist primarily as dimer, as does NGF, we next used FTIR to compare the secondary structure content of the three proteins. It is well established that the amide I region of the IR spectrum of a protein is sensitive to protein secondary structure content (Byler and Susi, 1986; Surewicz and Mantsch, 1988). We applied the band assignments and methods for spectral analysis used in these reports. The second derivative spectra of NGF, BDNF, and NT-3 in the amide I' region are shown in Fig. 2. The infrared spectra of the three proteins are highly similar although not identical. Curve fitting of the deconvoluted spectra was used to quantitate the spectral information, and the results are listed in Table II. An example of the curve fitting is shown in Fig. 3. From this analysis, it is estimated that BDNF contains 47% $\beta$-structure, 31% random coil, and 22% reverse turns. NGF contains 61% $\beta$-structure, 12% random coil, 14% turns, and 12% $\alpha$-helix, and NT-3 contains 46% $\beta$-structure, 22% random coil, 26% turns and 10% $\alpha$-helix.

**CD**—The $\alpha$-helical content determined from the FTIR could also be from loops (Prestrelski et al., 1991a, 1991b). However, we have found that a combination of CD and FTIR gives more reliable determination of both $\beta$-sheet and $\alpha$-helical content. We therefore used CD to analyze the secondary structure of these proteins. Fig. 4 shows the far UV CD spectra of the three human factors. As was reported previously (Radziejewski et al., 1992), both NT-3 and BDNF have a positive maximum at 230-235 nm which is absent in NGF. During denaturation, this feature disappears in concert with the disappearance of tertiary structure, while the rest of the far UV CD spectrum remains unchanged.\(^10\) We therefore attribute this to some characteristic tertiary structure formation. The stacking of aromatic rings has been reported to interfere with far UV CD (Arnold et al., 1992), and this could be the origin of this feature. The disulfide structure and Trp content of BDNF and NGF are identical, but the Tyr and Phe composition differs between these two proteins (Fig. 5):

\(^10\) L. O. Narhi, unpublished data.
therefore this positive peak probably results from ring stacking of either of these aromatics. This provides further evidence that the structures of BDNF and NT-3 are very similar. However, regardless of its origin, this peak interferes with the spectra in the 220-nm region and makes it difficult to fit the spectra with the available model systems. However, the spectra from 220 to 190 nm suggest that BDNF contains primarily β-sheet and irregular structure, NGF appears to be very similar to BDNF, with β-structure but no α-helix, and NT-3 differs slightly from the other two factors, containing β-sheet and some α-helix. Thus, from the CD analysis, these proteins appear to have similar but not identical secondary structures consisting primarily of β-sheet, in agreement with the FTIR analysis. However, only the NT-3 shows evidence of α-helix in the CD spectra, suggesting that the peak at 1655 cm⁻¹ assigned as α-helix in the FTIR spectrum of NGF is attributable to loops or other structures. This ambiguity in the assignment of the peak around 1655 cm⁻¹ has been demonstrated for other growth factors (Prestrelski et al., 1991a, 1991b). CD is the more reliable technique for identifying α-helical content. The spectrum of human NGF appears to differ from that of the murine NGF somewhat, containing less random coil and more closely resembling BDNF (Radziejewski et al., 1992).
To assess tertiary structure, the near UV CD spectra of the three recombinant human neurotrophic factors were taken, as shown in Fig. 4A. All of the spectra contain a broad negative band beginning at 310 nm; for BDNF the minimum is near 280–290 nm, and there is no other distinguishing feature in the spectrum. This type of broad, negative signal, without any fine structure, usually arises from disulfides. The aromatic amino acids are characterized by very sharp CD bands and a considerable amount of fine structure (Strickland, 1974). This feature of the spectra is therefore attributable to disulfides, and in the case of BDNF the disulfide signal is so intense that it appears to mask the fine structure from the aromatics.

**Fig. 5.** Amino acid sequence of comparison of NT-3, NGF, and BDNF. Shaded areas represent regions of homology.

**Fig. 6.** Fluorescence spectra of BDNF (a, ---), NT-3 (b, - - -), and NGF (c, . . . .) upon excitation at 280 nm.
which might otherwise be detectable. The spectrum of NGF is very similar, although the disulfide band is not quite as deep as that of BDNF. The spectrum of NT-3, in contrast, shows fine structure superimposed on the disulfide band in the 280–290-nm region. This signal could be from Trp104, which has replaced the Phe104 present in BDNF and NGF (Fig. 5). Alternatively, the other two Trp which are conserved in all three proteins could be in a slightly different, possibly more rigid, environment for the NT-3 relative to the BDNF and NGF. The presence of similar disulfide bands in these proteins indicates that the three disulfides are located in similar environments in the molecules, as expected from the conservation of sequence in these regions of the three neurotrophic factors (Fig. 5). The disulfide bond structure of BDNF has recently been determined and is homologous to that of NGF.11

fluorescence—The fluorescence spectra of the three molecules, excited at 280 nm and determined under identical conditions, are shown in Fig. 6. The spectra of both BDNF and NT-3 consist of a single peak at 345 nm with a full width at half-maximum of about 55 nm, and are of equal intensity, although the NT-3 spectrum is slightly blue shifted. This most likely indicates that the fluorescent tryptophans are in similar but not identical environments and are relatively solvent exposed. The extra Trp at position 104 in NT-3 is probably in a slightly more hydrophobic environment, leading to the slightly blue-shifted fluorescence maxima. The overall fluorescence intensity of NT-3 is equivalent to BDNF, in spite of the presence of an extra Trp, indicating that the fluorescent Trp(s) must be in a different environment, reflecting differences in amino acid sequence (Fig. 5) or in conformation or both, resulting in substantial quenching relative to the other two factors. The surface hydrophobicity of the BDNF and NT-3 were examined by 8-anilino-1-naphthalenesulfonic acid binding. Neither of these proteins bound measurable amounts of 8-anilino-1-naphthalenesulfonic acid, even at 100 µM concentrations of the probe, indicating that at neutral pH these neurotrophic factors have very hydrophilic surfaces.

conclusion—In summary, the results reported here indicate that the solution structure of the three neurotrophic factors are very similar, although not identical. Further, both BDNF and NT-3 exist primarily as dimers in solution, as was previously demonstrated for NGF (Angeletti et al., 1971; Green et al., 1971) consistent with the report of Radziejewski (1992). However, both the BDNF and NT-3 as well as the NGF have a tendency to associate into some oligomers, and the NT-3 dimer also appears to contain some incompetent monomer which was formed either before or during purification of the protein. All three molecules contain substantial β-sheet and irregular structure, with little or no α-helix, as was previously reported (McDonald et al., 1991; Radziejewski et al., 1992). Unlike earlier reports (Radziejewski et al., 1992), we find that NT-3 differs from the other two factors in that it contains a small amount of α-helix under physiological conditions. This structure for NT-3 was seen using both the CD, which is well suited for the analysis of α-helix, and the FTIR, a technique particularly well suited for proteins which are primarily β-sheet. NGF appears to have the most β-sheet of the three factors, with a peak in the FTIR spectrum that is probably attributable to loops. The conserved tryptophans appear to be located in similar environments in BDNF and NT-3 but are substantially quenched in NGF. The disulfide bonds appear to be conserved. This has been confirmed for BDNF, where the disulfide bond structure has recently been determined.11 The different biological specificity thus could be due to the conformational differences in structure reported here and/or different surface properties which may affect receptor binding.

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