Effect of the intermolecular disulfide bond on the conformation and stability of glial cell line-derived neurotrophic factor

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Glial cell line-derived neurotrophic factor (GDNF) is a member of the TGF-β superfamily of proteins. It exists as a covalent dimer in solution, with the 15 kDa monomers linked by an interchain disulfide bond through the Cys101 residues. Sedimentation equilibrium and velocity experiments demonstrated that, after removal of the interchain disulfide bond, GDNF remains as a non-covalent dimer and is stable at pH 7.0. To investigate the effect of the intermolecular disulfide on the structure and stability of GDNF, we compared the solution structures of the wild-type protein and a cysteine-101 to alanine (C101A) mutant using Fourier transform infrared (FTIR), FT-Raman and circular dichroism (CD) spectroscopy and sedimentation analysis. The elimination of the intermolecular disulfide bond causes only minor changes (~4%) in the secondary structures of GDNF. The far- and near-UV CD spectra demonstrated that the secondary and tertiary structures were similar for both wild-type and C101A GDNF. Heparin binding and sedimentation velocity experiments also indicated that the folded structure of the wild-type and C101A GDNF are indistinguishable. The thermal stability of GDNF does not appear to be affected by the absence of the interchain disulfide bond and the biological activity of the C101A mutant is identical with that of the wild-type protein. However, small but significant changes in side chain conformations of tyrosine and aliphatic residues were observed by FT-Raman spectroscopy upon removal of the intermolecular disulfide bond, which may reflect structural changes in the area of dimeric contact. By comparing the Raman spectrum of wild-type GDNF with that of the C101A analog, we identified the conformation of the intermolecular disulfide as trans–gauche–trans geometry. These results indicate that GDNF is an active, properly folded molecule in the absence of the interchain disulfide bond.

Keywords: disulfide geometry/Fourier transform infrared/ Fourier transform Raman/glial cell line-derived factor/ sedimentation/spectroscopy

Introduction

Glial cell line-derived neurotrophic factor (GDNF) belongs to the superfamily of transforming growth factor-β (TGF-β), which includes a variety of multifunctional homodimeric proteins that can be divided into subfamilies based on their sequence homology and activity in cell proliferation and differentiation (Lin et al., 1993; Cunningham et al., 1995; Kriegstein et al., 1995). One of the characteristic structural features of the TGF-β superfamily is the conserved disulfide structure, which consists of an intramolecular disulfide knot and an intermolecular disulfide bond linking the two monomers (Daopin et al., 1992; Schlunegger and Grutter, 1992; McDonald and Hendrickson, 1993; Griffith et al., 1996; Massague, 1996). The crystal structure of TGF-β2 has been determined by several groups (Daopin et al., 1992, 1993; Schlunegger and Grutter, 1992), which provides insight into the common structural motifs and overall protein folds of members of the TGF-β superfamily. The disulfide structure of GDNF was recently determined (Hui et al., 1996), showing that GDNF contains the seven conserved cysteine residues that form the intramolecular disulfide knot characteristic of the TGF-β superfamily, although it shares very little sequence homology with TGF-β2 (Lin et al., 1993). In addition, Cys101 of GDNF was shown to be involved in the intermolecular disulfide bond (Hui et al., 1996, 1999). The three-dimensional structure of GDNF, determined by X-ray crystallography (Eigenbrot and Gerger, 1997), revealed that the dimeric contact in GDNF is significantly different from that found in TGF-β. It has been shown that elimination of the intermolecular disulfide in some members of the TGF-β superfamily can lead to a marked reduction in biological activity. For instance, activin A was reported to be only 1% active compared with the wild-type protein while TGF-β1 showed only 20% activity when the intermolecular disulfide bond was removed (Amatayakul-Chantler et al., 1994; Huesken-Hindi et al., 1994). However, bioactivity appeared to remain unchanged following reduction of the disulfide bond in GDNF (Hui et al., 1999). For most of the homodimeric proteins of the TGF-β family it is unclear whether they remain as non-covalent dimers or become monomeric after the removal of the intermolecular disulfide bond. For platelet-derived growth factor (PDGF), it has been shown that a mutant lacking the intermolecular disulfide bond remains as a non-covalent dimer and is fully active (Kenney et al., 1994). However, structural information on how the intermolecular disulfide may affect the conformations and stabilities of proteins in the TGF-β superfamily is so far very limited (Prestrelski et al., 1992, 1993; Schlunegger and Grutter, 1992; McDonald et al., 1996). To gain insight into the structures of this neurotrophic subfamily and to determine further the impact of the conserved intermolecular disulfide bond on the conformation and stability of GDNF, we investigated the solution structures of GDNF and its analog without the intermolecular disulfide bond by employing various biophysical techniques including sedimentation velocity and equilibrium, Fourier transform infrared (FTIR), FT-Raman and circular dichroism (CD) spectroscopy.

Materials and methods

Materials

The wild-type and C101A mutant of glial-derived neurotrophic factor (GDNF) were produced in Escherichia coli and purified and refolded from inclusion bodies by a proprietary procedure.

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Activity assay

GDNF activity was measured by its ability to promote the proliferation/survival of a 32D cell line (32D) expressing a Ret/EPOR chimera and GFRA-1 (Xu et al., 1998). Serial dilutions of protein were added to the culture media and cell growth was plotted as a function of GDNF concentration. The experiment was repeated three times, using slightly different protein and cell concentrations. The activity is reported as a percentage of control activity.

Size exclusion chromatography (SEC)

SEC was performed on a Superdex 75 column connected to an FPLC system (Pharmacia). Approximately 200 μl of protein sample were loaded on to the column and eluted with PBS at a flow-rate of 0.5 ml/min.

Fourier transform infrared (FTIR) spectroscopy

Stock GDNF solutions were concentrated and hydrogen-deuterium exchanged into 10 mM sodium phosphate, 50 mM NaCl, pH 7.0 buffer using a Centricon (Amicon) cartridge with a 10 kDa molecular weight cutoff membrane. Diafiltration of GDNF into D2O buffers was repeated several times to ensure complete H–D exchange. The final protein concentrations were ~10 mg/ml before IR data collection and the protein solution was injected into a sample holder consisting of a pair of CaF2 windows with a 25 μm spacer to form a uniformly thin film. The sample temperature was maintained at 20°C during IR data collection using a thermal jacket controlled by an electronic thermal controller (Boulder Nonlinear). Typically, 1024 interferograms were co-added and Fourier-transformed to generate an absorbance spectrum at 4 cm⁻¹ resolution by employing a Mattson Research Series Model 1000 spectrometer with an MCT detector cooled by liquid nitrogen. The spectrometer was continuously purged with a Model 75-60 dry air system (Balston Filter System, Whatman) to eliminate the spectral interference from atmospheric water vapor. Residual contributions from atmospheric vapor were digitally subtracted from the protein spectra. The broad buffer background of D2O was subtracted from protein spectra to ensure a flat baseline in the spectral region 1400–1800 cm⁻¹. IR spectral analyses, including spectral deconvolution and calculation of second-derivative spectra, were done by employing Grams/368 and Spectral Calc software (Galactic Industries).

FT-Raman spectroscopy

Stock solutions of GDNF were buffer exchanged into 10 mM sodium phosphate, 50 mM NaCl buffer at pH 7.0. Approximately 20 μl of the GDNF solutions, with a protein concentration of about 50 mg/ml, were placed in quartz capillaries (1.5 mm o.d.), which were thermostated at 10°C during data collection. A Nicolet Raman 950 spectrometer equipped with a continuous Nd:YAG laser source (1024 nm) was employed. The laser power was 300–400 mW at the sample. Spectra were recorded at 8 cm⁻¹ resolution with data spacing at 1 cm⁻¹.

Sedimentation analysis

Sedimentation equilibrium and velocity studies were done in a Beckman Optima XL-1 centrifuge using absorbance scans at 229 or 280 nm. Velocity data were analyzed using the program SVEDBERG (Philo, 1997). Equilibrium data were fitted as ideal single species or monomer–dimer association schemes through global analysis of 16–18 data sets using the program KDALTON (Philo et al., 1994).

Circular dichroism (CD)

CD spectra were obtained using protein samples of ~1 mg/ml on a Jasco J-715 spectropolarimeter. Cylindrical cuvettes with a pathlength of 1 cm for the near-UV CD region (350–250 nm) and 0.01 cm for the far-UV CD region (250–190 nm) were used, with scan rates of 10 and 20 nm/min and response times of 4 and 2 s, respectively, averaging 15 spectra. Mean residue ellipticity was calculated using an extinction coefficient at 280 nm of 0.39 for a 0.1% protein solution and a mean residue weight of 117.3. This extinction coefficient was determined empirically from the absorbance of protein samples that were quantitated by amino acid analysis. Protein was dialyzed into 10 mM sodium citrate, 150 mM NaCl, pH 7.0 prior to analysis.

Heparin binding

The heparin binding affinity was assessed by applying the protein samples to a heparin HiTrap column (Pharmacia). The column was equilibrated with 10 mM sodium phosphate at a flow-rate of 0.5 ml/min. The proteins in phosphate-buffered saline (PBS) were applied to the column which had been equilibrated in 10 mM sodium phosphate (buffer A), washed with buffer A and then eluted with a linear NaCl gradient from 0 to 1 M in buffer A. A Biologic chromatography station (Bio-Rad) was used and both the absorbance at 280 nm and the ionic strength of the eluate were monitored.

Results

Biological activity

The bioactivity of the C101A mutant GDNF was compared with that of the wild-type protein in an assay that measures binding to receptor and consequent survival of a factor-dependent cell line in which the GDNF is expressed (Figure 1). C101A and WT proteins were assayed side-by-side three times. The C101A was as active as the native protein in this assay (95 ± 15% native activity), indicating that the interchain disulfide is not involved in the bioactivity of GDNF.
Role of intermolecular disulfide bond of GDNF

Fig. 2. Sedimentation data for Cys101–Ala GDNF at pH 7.0. Sedimentation equilibrium data for C101A GDNF at pH 7 and 20 000 r.p.m. The data are plotted as the natural logarithm of the net absorbance (after subtracting the baseline offset) versus (r^2 - r_0^2)/2, where r is the radial position in the centrifuge and r_0 is a reference position. In such a plot a single species will give a straight line with a slope proportional to buoyant molecular weight. Experimental data points are shown for samples loaded at concentrations of ~600 µg/ml (squares, scanned at 280 nm) or ~30 µg/ml (circles, scanned at 229 nm). The two solid lines each indicate the slope predicted for a dimer of molecular weight 30 385 and a calculated partial specific volume of 0.724 ml/g. The dashed line shows the slope predicted for a monomer.

Size exclusion chromatography
SEC was carried out on a Superdex-75 column. Both the wild-type and C101A GDNF eluted at the identical position, suggesting that the C101A GDNF is also a dimer (data not shown). However, if C101A is monomeric and unfolded, it could elute in a position corresponding to a folded dimer using this technique. Comparison with standard proteins indicates a molecular weight for both GDNF species of 60 000–70 000. This apparent molecular weight is considerably higher than the actual molecular weight of the dimer (30 385) and could be ascribed to either further self-association of the dimers or to the dimers having an extended shape. In order to clarify this, sedimentation equilibrium and velocity experiments were carried out for both proteins.

Sedimentation analysis of wild-type and C101A GDNF
We used both sedimentation equilibrium and sedimentation velocity to establish the oligomeric state and conformation of the C101A GDNF. Figure 2 demonstrates that this analog is a dimer at both high and low protein concentrations, despite the loss of the intermolecular disulfide bond. A more detailed, global analysis of 16 sedimentation equilibrium data sets at loading concentrations from 30 to 900 µg/ml revealed no significant dissociation into monomers even at the lowest concentrations we can study (~10 µg/ml), implying that the dimer–monomer dissociation constant is <1 nM. These data are consistent with our finding that the biological activity of the C101A mutant is identical with that of the wild-type protein. Even if dimer formation were a prerequisite for receptor dimerization, removal of the interchain disulfide bond would not affect this. The sedimentation data at higher concentrations suggest the presence of some tetramer, which is consistent with the tetramers reported in the crystal structure (Eigenbrot and Gerger, 1997). The quantity of this form is too low to be detectable in SEC experiments.

Sedimentation velocity studies also confirmed that C101A GDNF is dimeric, based on the ratio of the sedimentation and diffusion coefficients derived from directly fitting the velocity scans (Philo, 1997). Its sedimentation coefficient s_{20,w} of 2.28 S is very close to the value of 2.25 S for the wild-type under the same conditions, indicating that the overall shapes of wild-type and C101A GDNF are very similar. These sedimentation coefficients are fairly low for a protein of this molecular weight, indicating that both proteins have a very high frictional coefficient (f/f_0 = 1.54–1.58). If we model the GDNF dimer as a prolate ellipsoid, these results imply an axial ratio of ~7, i.e. a very rod-like overall shape. This is consistent with the observed elution volume of these proteins in SEC experiments. A detailed comparison with the sedimentation coefficient as calculated from the X-ray structure (Eigenbrot and Gerger, 1997) is not worthwhile since about 30% of the residues are disordered and therefore missing from the structure. However, these sedimentation results are consistent with a solution conformation matching that in the crystal.

FTIR spectra
The IR spectrum (Figure 3A) of the wild-type GDNF at pD 7.0 displays amide I bands at 1640, 1650, 1664 and 1677 cm⁻¹. The major amide I band at 1640 cm⁻¹ suggests β-strand as the predominant secondary structure in GDNF, while the weak amide I band at 1650 cm⁻¹ indicates the presence of a small amount of α-helix. This is consistent with the X-ray crystal structure of GDNF (Eigenbrot and Gerger, 1997). The FTIR spectrum of C101A GDNF is similar to that of the wild-type protein (Figure 3B), with amide I bands at 1640, 1664 and 1677 cm⁻¹, suggesting that the removal of the intermolecular disulfide has a minimal impact on the secondary structures of GDNF. However, the C101A mutant does display slightly stronger IR intensity near 1645 cm⁻¹ and weaker IR intensity at 1650 cm⁻¹. Also, the IR bands at 1645 and 1640 cm⁻¹ display different intensity ratios in the two spectra. This suggests that the C101A mutant contains slightly more disordered structure than the wild-type does (Li et al., 1997).

Raman spectra
Consistent with the FTIR data, Raman spectra (Figure 4) of the wild-type and C101A GDNF are also similar. The C101 GDNF spectrum minus the wild-type spectrum (the difference spectrum) reveals only minor bands at 542, 1444 and 1663 cm⁻¹. The major amide I band at 1640 cm⁻¹ suggests β-strand as the predominant secondary structure in GDNF, while the weak amide I band at 1650 cm⁻¹ indicates the presence of a small amount of α-helix. This is consistent with the X-ray crystal structure of GDNF (Eigenbrot and Gerger, 1997). The Raman spectrum of C101A GDNF shows a major band at 515 cm⁻¹ and a very

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Fig. 3. FTIR spectra of (A) wild-type GDNF and (B) C101A mutant.

Fig. 4. FT-Raman spectra (300–1800 cm\(^{-1}\)) in PBS, pH 7.0 of wild-type GDNF, C101A GDNF and the difference spectrum obtained by subtracting the wild-type GDNF spectrum from the C101A GDNF spectrum.

For the wild-type GDNF, the Raman spectrum (D) also displays a major band at 515 cm\(^{-1}\), but the 540 cm\(^{-1}\) band is about twice as intense as that of the C101A GDNF, indicating three S–S bonds in gauche–gauche–gauche conformation, as observed with the mutant, and one in trans–gauche–trans geometry. This confirms the intermolecular disulfide in trans–gauche–trans geometry.

**CD spectra**

The structure of the wild-type and C101A GDNF was also assessed using the near-UV (tertiary structure) and far-UV (secondary structure) CD spectra. The near-UV CD spectra for wild-type and C101A GDNF, shown in Figure 6A, are very similar and are dominated by a doublet at 284 and 280 nm, attributable to tyrosine. The similarity of the spectra for these two proteins in this region demonstrates that the contribution of the intermolecular disulfide bond to the spectrum is minimal, suggesting that the disulfide is in a very flexible or solvent-exposed environment. The slight differences in intensity in the region around 260 nm could be due to the lack of small contributions from the disulfide in the C101A GDNF or to small differences in the environment of the other disulfide bonds or to slight variations in protein concentration.
The far-UV CD spectra, shown in Figure 6B, are also very similar for mutant and wild-type. The spectra for both proteins are characterized by minima at 220 and 208 nm, with maxima at 215 and 200 nm. This interesting spectrum reflects the secondary structure of the protein which consists of β-sheet and cysteine knot. These results suggest that the C101A GDNF has a very similar folded structure to the wild-type protein. The thermal stability of the C101A GDNF, as assessed by changes in the CD and FTIR spectra with increasing temperature, indicated that the structural stability of GDNF is not affected by the removal of the interchain bond (data not shown).

**Heparin binding**

The primary heparin binding site of GDNF is located in the N-terminal 40 amino acids. To determine whether the avidity of heparin binding was influenced by the slight difference in conformation between the wild-type GDNF and C101A GDNF, the affinity of the two proteins for a heparin column was determined as described in Materials and methods. This technique easily differentiates between several N-terminally truncated mutants (L.O. Narhi, unpublished data). Both the C101A and wild-type GDNF elute at an ionic strength of 110 mS/cm. Thus, heparin binding is unaffected by the absence or presence of the interchain disulfide bond. These results are consistent with the data described above and indicate that the dimeric structure of GDNF and its stability are not significantly influenced by the removal of the interchain disulfide bond.

**Discussion**

A number of studies have shown that the intermolecular disulfide may have a significant impact on the activity of TGF-β family members (Amatayakul-Chantler et al., 1994; Huesken-Hindi et al., 1994). However, it is unclear whether the role of the intermolecular disulfide is merely to promote dimeric interactions with the receptor or to maintain the structural integrity of the proteins of the TGF-β family so that they can then form proper interactions with their respective receptors. Since GDNF represents a subfamily of the TGF-β superfamily, it is particularly interesting to know if the intermolecular disulfide affects this protein similarly. Sedimentation velocity and equilibrium data clearly demonstrated that C101A GDNF forms a stable non-covalent dimer in solution with a dissociation constant below 1 nM. This is consistent with the observed biological activity of the mutant, which is indistinguishable from the wild-type protein and is also consistent with the activity of GDNF following reduction of the intermolecular disulfide (Hui et al., 1999). These results suggest that the dimerization is a prerequisite for receptor dimerization. Analysis of other dimeric ligands suggests that the mechanism of receptor dimerization for a dimeric ligand is to simultaneously bind two receptors together (Philo et al., 1994, 1996). A similar result was observed with PDGF; which also forms covalent dimers through two interchain disulfide bonds (Jaumann et al., 1991; Andersson et al., 1992; Haniu et al., 1993). Substitution of the two cysteines responsible for this interchain disulfide bond did not cause a significant change in its ability to form non-covalent dimers (Kenney et al., 1994). An intriguing question is why disulfide bonds are needed, if these proteins can form a stable non-covalent dimer. Activin A and TGF-β1 mutants, however, exhibit different behavior. Their activities are greatly compromised by the removal of the interchain disulfide bond (Amatayakul-Chantler et al., 1994; Huesken-Hindi et al., 1994). This may be due to much weaker non-covalent interactions between the monomers at the dimer interface. Therefore, GDNF may be an exception in that the dimerization contact has improved to such an extent that the interchain disulfide is no longer required to stabilize the dimeric structure. Alternatively, the interchain disulfide may be physiologically essential to maintain the proper association of each family member. TGF-β and PDGF comprise separate
subfamilies, each consisting of two or more family member which are capable of forming homo- and heterodimers (Waterfield et al., 1983; Johnsson et al., 1984; Heldin et al., 1988; Hart et al., 1990; Cunningham et al., 1995; Kriegstein et al., 1995). Intercalated disulides may dictate the assembly of each family member as a homodimer or heterodimer. In this regard, it is interesting to point out that most physiologically observed non-covalent dimeric ligands, including interferon-γ (Yphantis and Arakawa, 1987), stem cell factor (Arakawa et al., 1991, 1992) and macrophage-colony stimulating factor (Das and Stanley, 1982), do not have closely related family members and therefore can exist as homodimers only.

The intercalated disulfide not only fails to have much effect on the formation of the GDNF dimer, but it also produces only minor effects on protein conformation. Sedimentation velocity, SEC and CD, IR and Raman spectroscopic analyses all indicate that the overall shape and secondary and tertiary structures are very similar for C101A and wild-type GDNF. This is not surprising, as the folded structure of a molecule of a complicated nature such as GDNF is largely dictated by the formation of the appropriate intrachain disulfide bonds [as observed, for example, with epidermal growth factor (Narhi et al., 1992)]. Nevertheless, removal of the intercalated disulfide does slightly affect the secondary and side chain structures, as detected by FTIR, CD and FT-Raman spectroscopy. These types of changes may be due to some conformational adjustments that render the dimer contact stronger. The intercalated disulfide could impose some constraints on the dimer contact and its removal may have caused optimization of side chain packing at the dimer contact. The crystal structure of GDNF shows strong side chain interactions at the dimer contact site (Eigenbrot and Gerger, 1997). The dimerization interface involves a hydrophobic patch where the W80 of one monomer is contacting F66, F108 and Y68 of the other monomer. This hydrophobic patch is surrounded by two salt bridges between R104 from one monomer and D81 from the other and another between D53 and R92 (T.Osslund, unpublished data). This suggests that removal of the intercalated disulfide would not be sufficient to cause dissociation and would cause minimal, if any, changes in conformation at the contact. The dissociation of C101A GDNF that we observe at pH 2.0 (data not shown) may be at least partly due to the protonation of D81, the residue that is responsible for the salt bridges.

Sedimentation velocity and SEC experiments indicated that GDNF, whether covalent or non-covalent, has a very high frictional coefficient, one that is consistent with an axial ratio of ~7. Such a high frictional coefficient could also be observed if the molecule has an extended flexible region. In fact, the first N-terminal 36–39 residues are not identifiable in the electron density (Eigenbrot and Gerger, 1997). However, that sequence is actually located in the center of the dimeric structure, i.e. at the dimer interface, and hence may not be responsible for the high frictional coefficient. It is more likely that the highly elongated dimeric structure that is observed in the crystal structure is responsible for the observed high friction. It is interesting to note that this N-terminal region is responsible for heparin binding (L.O.Narhi, unpublished results).

FTIR spectroscopy indicated a small amount of α-helical structure. Far-UV CD is usually reliable in detecting α-helix, although less reliable for β-structures. However, in this case it was very difficult to judge whether α-helix is in fact present in both wild-type and C101A GDNF, owing to the unusual CD spectral shape observed with these proteins. While the shape of the CD spectra is consistent with the presence of α-helix, the intensity is not, probably owing to the contributions of the cysteine knot signal. This emphasizes the importance of applying two complementary techniques, CD and IR, for studying the secondary structure of proteins.

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