Glial Cell Line-Derived Neurotrophic Factor: Selective Reduction of the Intermolecular Disulfide Linkage and Characterization of Its Disulfide Structure

Mitsuru Haniu,* John Hui, Yunjen Young, John Le, Viswanatham Katta, Richard Lee, Grant Shimamoto, and Michael F. Rohde

Departments of Protein Structure and Process Development, Amgen, Inc., 1840 DeHavilland Drive, Thousand Oaks, California 91320-1789

Received March 6, 1996; Revised Manuscript Received August 8, 1996[⊗]

ABSTRACT: Glial cell line-derived neurotrophic factor is a protein known to enhance the survival of dopaminergic neurons against several neurotoxins. It has been shown to have therapeutic potential in the treatment of Parkinson's disease and other neurodegenerative diseases. We have determined the inter- and intramolecular disulfide linkages of the dimeric molecule by a combination of direct peptide analysis and peptide analysis after either partial reduction or partial oxidation of the protein. Under an acidic condition, the interchain disulfide bond was selectively cleaved with tris(2-carboxyethyl)phosphine, revealing that Cys101 was involved in the intermolecular disulfide linkage. Three other disulfides, Cys68−Cys131, Cys72−Cys133, and Cys41−Cys102, were identified as intramolecular linkages. The determined disulfide structure is highly homologous to that of transforming growth factor β2. Since one intramolecular disulfide points through a ring consisting of eight amino acid residues based on the similarity with transforming growth factor β2, the disulfide-linked peptides were not purified by conventional methods. Only the peptides from an N-terminal region (residues −1 to 37) were liberated by proteolytic treatment with trypsin or endoproteinase Lys-C, resulting in a stable cystine-knot protein.

Glial cell line-derived neurotrophic factor (GDNF)[1] is a member of the superfamily of transforming growth factor β (TGF-β) which comprises an expanding list of multifunctional proteins serving as regulators of cell proliferation and differentiation (Krieglstein *et al*., 1995; Cunningham *et al*., 1995). GDNF has been demonstrated to increase the survival of tyrosine hydroxylase-immunoreactive dopaminergic neurons in the substantia nigra. These neurons are involved in motor functions known to degenerate during the progression of Parkinson's disease (Kopin & Markey, 1988; Otto & Unsicker, 1990; Forno *et al*., 1993). GDNF represents a major therapeutic candidate for protecting dopaminergic neurons and also has the reported capacity to promote the survival of embryonic neurons *in vitro* (Martinou *et al*., 1990; Schubert *et al*., 1990; Chalazonitis *et al*., 1992; Lin *et al*., 1993; Prehn *et al*., 1993).

GDNF was first isolated from a rat glial cell line (Lin *et al*., 1993). The primary structure of GDNF has only 20% sequence homology with that of TGF-β2; however, the location of cysteine residues in GDNF is homologous to that of TGF-β2 except for two extra cysteine residues in the latter protein (Lin *et al*., 1993). The tertiary structures of TGF-β2 and osteogenic protein 1 have been successfully elucidated by X-ray crystallography (Schlunegger & Grutter, 1992; Daopin *et al*., 1992; Griffith *et al*., 1996), both revealing that one disulfide bond points through a ring consisting of eight amino acid residues. The determined disulfide structure is further complicated because each cystine cluster is associated with an intermolecular disulfide bond. Although GDNF is assumed to have a disulfide structure similar to that of TGF-β2, it has not been confirmed by either X-ray study or direct peptide analysis. In this paper, we report the chemical determination of the inter- and intramolecular disulfide linkages of GDNF by characterization of the partially reduced or partially oxidized proteins.

MATERIALS AND METHODS

Materials. Sodium iodate was purchased from Aldrich Co. (Milwaukee, WI), and 4-vinyl pyridine was obtained from Sigma Chemical Co. (St. Louis, MO). Pepsin was obtained from Sigma Chemical Co., and thermolysin was from Boehringer/Mannheim (Indianapolis, IN). TCEP was purchased from Molecular Probes (Eugene, OR). Other reagents were of HPLC grade as described (Haniu *et al*., 1995).

Purification of GDNF. Recombinant human GDNF was expressed in *Escherichia coli* cells and was purified using gel permeation chromatography, cation-exchange chromatography, and high-performance liquid chromatography procedures. Cation-exchange chromatography was performed with a Hewlett-Packard 1050 liquid chromatography system using a TosoHaas ion-exchange column (4.6 × 50 mm). Protein was eluted with a linear gradient from 0.3 to 0.6 M NaCl in 0.1 M Tris-HCl (pH 8.0) over 60 min at a flow rate of 1.0 mL/min. The protein sample was desalted by reversed phase HPLC using a Vydac C18 column (4.6 × 250 mm) prior to further analysis. The final product was shown to be approximately 95% pure on the basis of SDS−PAGE, and the major protein analyzed in this study contained an additional Met at the N-terminal end of each subunit (Lin *et al*., 1993).

* To whom correspondence should be addressed. Telephone: (805) 447-3117. Fax: (805) 499-7464.

⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1996.

[1] Abbreviations: GDNF, glial cell line-derived neurotrophic factor; GDF, growth/differentiation factor; BMP, bone morphogenetic protein; DTT, dithiothreitol; TCEP, tris(2-carboxyethyl)phosphine; IAM, iodoacetamide; PBS, phosphate-buffered saline; MALDI, matrix-assisted laser desorption/ionization; SDS−PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TGF, transforming growth factor; diPTH-Cys, diphenylthiohydantoin of cystine; CAMC, *S*-[(carboxamido)methyl]cysteine; PEC, *S*-[(pyridyl)ethyl]cysteine; TNM, tetranitromethane; 4-HCCA, α-cyano-4-hydroxycinnamic acid.

S0006-2960(96)00555-7 CCC: $12.00

Enzymatic Fragmentation. Native GDNF (250 μg) was digested with pepsin (10 μg) in 0.02 N HCl (pH 2) for 24 h at 37 °C. The digest was separated by reversed phase HPLC using a Vydac C18 column (4.6 × 250 mm) to isolate the Cys-containing core peptides. Further digestion of the core material was achieved with either thermolysin or endoproteinase Lys-C after the pH had been raised to approximately 7.2−7.5 with 1 M Tris base. Trypsin and endoproteinase Lys-C digestions of GDNF were performed overnight (18−20 h) in 0.1 M Tris-HCl buffer (pH 7.5) with an enzyme/substrate ratio of 1/50 (w/w). All digests were directly analyzed on a Vydac C18 column (4.6 × 250 mm) equilibrated with 2% solvent B. Peptide elution employed three separate gradients using solvent A (0.1% TFA) and solvent B (0.1% TFA/90% acetonitrile): from 2 to 20% B over 20 min, from 20 to 40% B over 40 min, and, finally, from 40 to 60% B over 10 min at a flow rate of 0.7 mL/min unless otherwise noted.

Partial Reduction of GDNF. Native GDNF (100 μg) was reduced with 20 mM TCEP under an acidic condition. The reaction was allowed to proceed for 60 min at 45 °C in 0.1% TFA (pH 2). To prevent or minimize thiol−disulfide exchange reaction during partial reduction, the reaction was performed at acidic pH and the reduced forms were immediately treated with excess iodoacetamide (1 M), followed by neutralization (pH 7.0−7.2) with 1 M Tris base (Gray, 1993). After 60 min of alkylation in the dark, the sample was purified by reversed phase HPLC using a Vydac C18 column (4.6 × 250 mm). The reduced/alkylated proteins were analyzed by SDS−PAGE and MALDI or ESI mass spectrometers. For further enzymatic digestion and peptide mapping, the remaining disulfide bonds in the proteins were fully reduced and alkylated with 4-vinylpyridine under denaturing conditions.

Partial Oxidation of Disulfide Linkages in GDNF. We attempted oxidative cleavage of disulfide bonds using sodium iodate to assist in the determination of intramolecular disulfide linkages or to verify the results from the partial reduction. The protein was treated with 0.1 M sodium iodate in 0.1 N HCl for various times (0−60 min) to selectively cleave any susceptible disulfide bond(s) (Gorin & Godwin, 1966; Lundblad, 1991). Although oxidation reaction using periodate or performic acid results in complete cleavage of disulfide linkages, sodium iodate mildly reacts with proteins, resulting in selective cleavage of the particular disulfide bonds. The resultant samples from iodate oxidation were analyzed with a reversed phase HPLC using a Vydac C18 column (4.6 × 250 mm) in order to remove the excess reagents. An aliqout of each sample was subjected to SDS−PAGE under nonreducing conditions to determine the molecular weight of the modified proteins. The oxidized GDNF samples were further digested with pepsin (1/ 25, w/w) in 0.02 N HCl for 24 h at 37 °C. The peptides were isolated by reversed phase HPLC as described above.

Sequence Analysis of Peptides. Sequence analysis of peptides was performed using ABI sequencer model 470A or 477A (Haniu *et al*., 1993). For detection of diPTH-Cys, ABI model 470A was always used as described previously (Haniu *et al*., 1994). Prior to sequence analysis, the Tyr-containing peptides were modified with 2% tetranitromethane (TNM) for 60 min at 25 °C in 0.1 M Tris-HCl buffer (pH 7.5), and then washed twice with ethyl acetate and purified by reversed phase HPLC. This treatment was necessary to distinguish diPTH-Cys from PTH-Tyr.

SDS−PAGE Analysis. Proteins or large peptide fragments (5−10 μg) were analyzed by SDS−PAGE according to the methods described by Laemmli (1970) under reducing or nonreducing conditions using 7.5% polyacrylamide gels.

Mass Spectrometry of Proteins and Peptides. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) was performed using a Voyager mass spectrometer (PerSeptive Biosystems). The samples dissolved in 0.1% TFA were spotted on the sample cartridge with a matrix, 4-HCCA, dried, and then washed with cold water. Cys-containing peptides were analyzed using a Sciex API triple quadrupole mass spectrometer with an ion-spray interface. The dried sample was redissolved in 0.1% TFA/50% acetonitrile/water and flow injected into the ion-spray interface using a Michrom Biosource Ulrafast Microprotein Analyzer. The carrier solvent was 50% acetonitrile/water with 0.1% TFA flowing at 5 μL/min. The scan range was 300−2400 amu with a step of 0.5 amu. The mass units and standard deviation were calculated using Sciex hypermass software.

RESULTS

Structural Characterization of GDNF. Purified GDNF was analyzed on SDS−PAGE, exhibiting a single band that migrated at approximately 30 kDa (data not shown). After reduction, the 30 kDa band shifted to a lower band corresponding to 15 kda on SDS−PAGE analysis, consistent with the interpretation that GDNF is composed of two identical subunits linked through disulfide bonds. Mass spectrometry of GDNF using electrospray ionization mass spectrometry (ESI-MS) showed one major signal corresponding to 30 385 amu which was nearly identical to the theoretical averaged mass units (30 386.8 amu) on the basis of the reported sequence (residues −1 to 134), assuming that GDNF forms a homo dimer through seven disulfides and contains N-terminal methionines (Lin *et al*., 1993). Some minor signals corresponding to des-met-GDNF or oxidized GDNF were also observed on the mass spectrum.

Determination of an Interchain Disulfide Linkage, Cys101−Cys101. Partial reduction/alkylation of GDNF was examined using 20 mM TCEP under several acidic conditions (pH 2−5), followed by treatment with iodoacetamide. Figure 1 indicates that the reduction of GDNF with 20 mM TCEP at pH 2 resulted in several products; the peak R-I was initially formed, and then further reduction or raising of the pH increased another peak R-III. The mass spectral analysis of product R-I showed a mass of 15 251 amu corresponding to the monomeric GDNF, in which only one cysteine per subunit was reduced and alkylated with iodoacetamide (IAM). SDS−PAGE showed that two products, R-I and R-III, were monomeric and R-II was a dimer (data not shown). In order to identify the IAM-labeled cysteine in the monomer R-I, the remaining disulfide linkages were fully reduced and modified with another SH reagent, 4-vinylpyridine. The final GDNF product (1-CAMC+6-PEC) was further digested with trypsin, and the IAM-labeled peptides were isolated by reversed phase HPLC (Figure 2). Peptide T-1 represented the sequence corresponding to VGQAC-(101)C(102)RPIAFDDDLSFLDDNLVYHILR (residues 97−124), in which Cys101 was detected as *S*-[(carboxamido)-

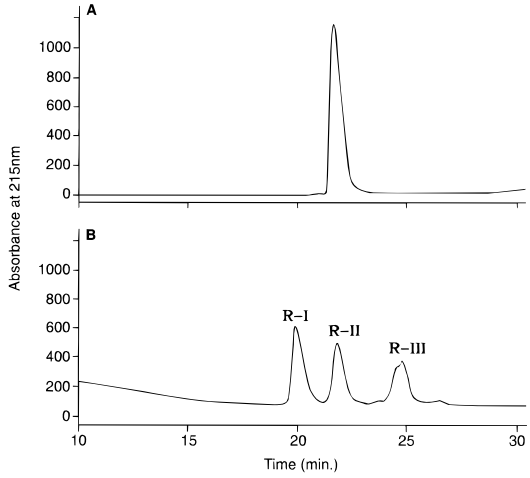

FIGURE 1: HPLC isolation of partially reduced and alkylated GDNF: (A) native GDNF and (B) reduced GDNF with 20 mM TCEP at 45 °C for 60 min in 0.1% TFA (pH 2). The samples were alkylated with excess iodoacetamide (1 M). The proteins were purified by reversed phase HPLC using a Vydac C18 column (4.6 × 250 mm) with a linear gradient from 30 to 45% solvent B over 30 min. R-I−III indicate the partially reduced/alkylated monomer, intact GDNF, and the fully reduced/alkylated GDNF, respectively.

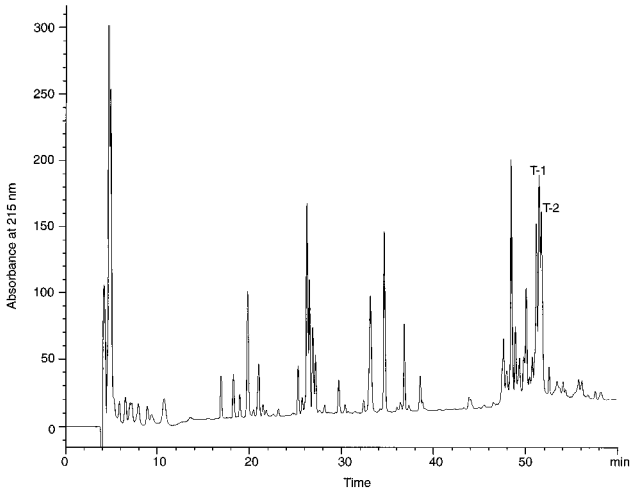

FIGURE 2: Tryptic peptide map of the partially reduced and alkylated form, R-I. The tryptic digest of peak R-I was subjected to reversed phase HPLC using a Vydac C18 column (4.6 × 250 mm), and the peptides were separated with a linear gradient from 2 to 55% solvent B over 60 min at a flow rate of 0.7 mL/min.

methyl] cysteine (CAMC), while Cys102 was *S*-[(pyridyl)-ethyl]cysteine (PEC) (Table 1). Peptide T-2 had the same sequence as that of peptide T-1 except for additional sequences at the N terminus of the latter peptide, namely residues 92−124. Sequence analyses of Cys-containing peptides revealed that all cysteines except Cys101 were detected as PEC (data not shown). Thus, only Cys101 was determined to be involved in intermolecular disulfide linkage.

Determination of an Intramolecular Disulfide Linkage, Cys68−Cys131. In order to isolate Cys-containing peptides from GDNF, several proteolytic digestions of the native protein were examined. However, native GDNF itself was strongly resistant to usual proteases, including trypsin and endoproteinases Lys-C and Glu-C, even in the presence of 2 M guanidine hydrochloride. These proteolytic digests only produced a few peptides from an N-terminal region, resulting in large Cys-containing fragments. This suggests that GDNF had a compact structure due to intermolecular or intramolecular disulfide linkages. To obtain suitable peptide fragments for determination of the disulfide linkages, the native GDNF (250 μg) was digested with pepsin (10 μg) in 0.02 N HCl as described in Materials and Methods. The HPLC peptic map is shown in Figure 3, indicating two major peaks denoted as P-I and -II with a shoulder P-III. These designated peptide peaks are believed to contain multiple disulfide linkages since peaks P-II and -III disappeared from the map upon DTT reduction. Peak P-I did not change apparently after reduction (Figure 3C). However, this peak contained one of the reduced peptides. SDS−PAGE of peptides P-I−III exhibited a common band at approximately 15 kDa (data not shown). These fragments were separated into individual peptides through reversed phase HPLC after complete reduction and alkylation. Sequence analyses of the individual peptides revealed that peptides P-I−III each contained four Cys-containing peptides as shown in Table 2. The difference between these fragments is largely due to differential cleavage in one or two peptides. One of the peptides derived from peak P-I showed the sequence KVGQACCRPIAFDDDL (residues 96−111), whereas the corresponding peptide in peak P-II lacked several residues

Table 1: Sequence Analysis of Peptide T-1[a]

cycle	residue	PTH (pmol)
1	V	23.9
2	G	19.3
3	Q	17.2
4	A	21.9
5	C101	3.9[b]
6	C102	3.4[c]
7	R	1.3
8	P	10.7
9	I	11.6
10	A	12.6
11	F	12.5
12	D	3.5
13	D	2.9
14	D	3.4
15	L	7.0

[a] Although 24 cycles were analyzed, only the results for 15 cycles are presented. [b] Detected as *S*-[(carboxamido)methyl]cysteine. [c] Detected as *S*-[(pyridyl)ethyl]cysteine.

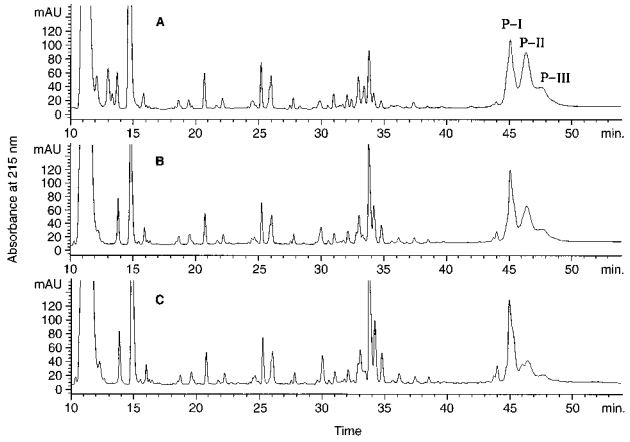

FIGURE 3: HPLC peptide mapping of the peptic digest of GDNF. GDNF was digested with pepsin as described in Materials and Methods. The Cys-containing core fractions (P-I−III) were isolated by reversed phase HPLC using a Vydac C18 column. The digest was reduced with 5 mM DTT at 37 °C in 0.1 M Tris buffer (pH 7.0) for several times: (A) 0 min, (B) 60 min, and (C) 120 min, respectively. HPLC conditions are described in Materials and Methods.

Table 2: Sequences in Pepsin-Generated Disulfide-Containing Peaks P-I and -II[a]

peptide 1, PRRERNRQAAAANPENSRGKGRRGQRGKNR GCVLTA (residues 10−45)
peptide 2, KVGQACCRPIAFDDDL (residues 96−111), P-I KVGQACCRPIAF (residues 96−107), P-II
peptide 3, IFRYCSGSCDAAE (residues 64−76)
peptide 4, VYHILRKHSAKRCGCI (residues 119−134)

[a] Peak P-II lacks the four residues DDDL at the C-terminal portion of peptide 2 due to partial digestion.

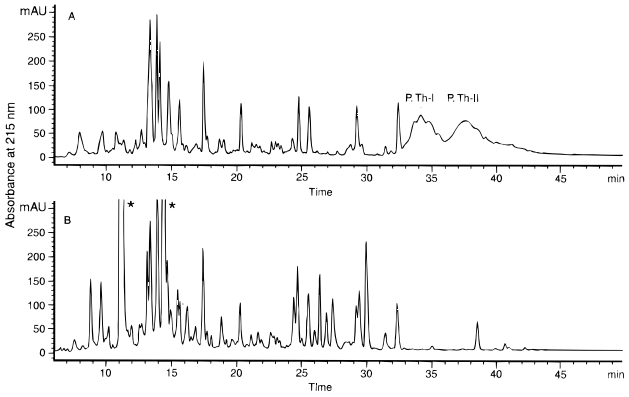

FIGURE 4: Peptic-thermolytic peptide maps of GDNF. Double digest of GDNF using pepsin and thermolysin was subjected to reversed phase HPLC: (A) the whole digest and (B) the digest neutralized with 1 M Tris base and reduced with 5 mM DTT at 37 °C for 60 min. Asterisks show the peaks derived from DTT and oxidized DTT. HPLC conditions are described in Materials and Methods.

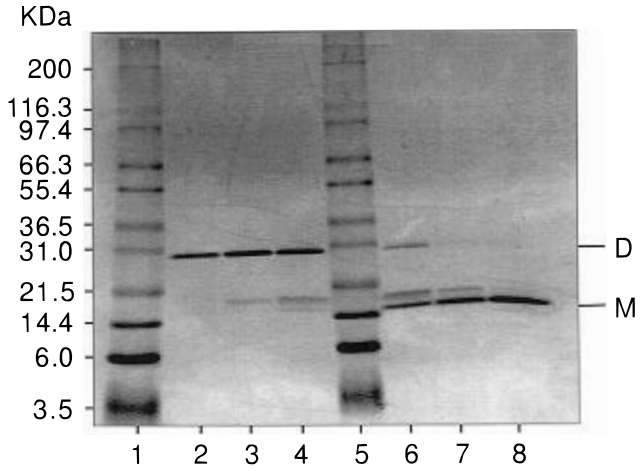

FIGURE 5: SDS−PAGE analysis of iodate-oxidized GDNF. GDNF samples were oxidized with sodium iodate for various time intervals. After removal of the excess reagents by HPLC, one aliquot of each sample was loaded onto a nonreducing gel: lanes 1 and 5, standard markers; lane 2, intact GDNF; lane 3, GDNF oxidized for 5 min; lane 4, GDNF oxidized for 10 min; lane 6, GDNF oxidized for 20 min; lane 7, GDNF oxidized for 30 min; and lane 8, GDNF oxidized for 1 h. D and M denote the positions corresponding to the GDNF dimer and monomer, respectively.

from the C-terminal region, showing the sequence KVGQAC-CRPIAF (residues 96−107). Peptide P-III contained additional residues TTY (residues 77−79) at the C terminus of peptide 3. However, this digest did not provide any additional information on the disulfide linkages.

To determine the disulfide linkages of the cystine knot, the pepsin-generated peptides were further digested with thermolysin. As shown in Figure 4, the double digestion generated two broad peaks, P-Th-I and -II, both containing four Cys-containing peptides: peptide 1 (residues 10−45), peptide 2 (residues 97−107 or 97−111), peptide 3 (residues 64−76 or 64−79), and peptide 4 (residues 127−134). Direct sequence analyses of both peptides P-Th-I and -II showed three major sequences corresponding to IFRYCSGSCDAAE (residues 64−76), SAKRCGCI (residues 127−134), and AANPENSRGKGRRGQRGKNRGCVLTA (residues 20−45), while peptide 2 corresponding to VGQACCRPIAF (residues 97−107) showed a poor PTH yield after cycle 3, perhaps due to the inaccessibilty of Edman reagents. Interestingly, significant amounts of diPTH-Cys at cycle 5 (approximately 20% of the regular PTH) were observed in both peptides P-Th-I and -II, indicating the existence of disulfide linkage Cys68−Cys131 according to the previous observations (Marti *et al*., 1987; Haniu *et al*., 1994). Fraction P-Th-I contained a sequence of VGQACCRPIAFDDDL (residues 97−111), whereas fraction P-Th-II lacked the C-terminal sequence DDDL (residues 108−111) in the corresponding peptide.

Selective Cleavage of Disulfide Bonds with Sodium Iodate. In order to determine other disulfide bonds and to confirm the above assignments, partial oxidation using sodium iodate was attempted under acidic conditions. Sodium iodate oxidations would selectively cleave particular disulfide bond-(s) as described earlier (Gorin & Godwin, 1966). Oxidation time course studies of GDNF examined by SDS−PAGE analysis are shown in Figure 5. The original dimer was predominant after 10 min of oxidation, while at times greater than 10 min, the dimer was gradually converted to the monomer. After 30 min of oxidation, only the monomer was observed. This observation was consistent with the specific cleavage of an interchain disulfide bond by partial reduction described earlier. Double bands at apparently 15 kDa were observed on SDS−PAGE, probably due to the conformational difference.

The oxidation time course samples were desalted through reversed phase HPLC and then further digested with pepsin to obtain Cys-containing peptides (Figure 6A-6D). Samples from 5 and 10 min oxidations did not provide sufficient Cys peptides for disulfide determination. Furthermore, broad peaks corresponding to Cys-core fragments were still observed at 45−54 min of elution time. However, the samples after 20 and 30 min of the reactions yielded several Cys-containing peptides at around 37−45 min of elution time. Sequence analyses of all peptic peptides allowed identification of many disulfide-containing peptides (Figure 6D); those numbered peaks were denoted as P-1−3, etc.

Determination of a Disulfide Linkage, Cys41-Cys102. Peak P-4, shown in Figure 6D, showed two Cys-containing peptides in the sequence analysis; one peptide spans residues 10−45 and the other residues 96−107. Mass spectral analysis of peptide P-4 gave 5328.5 amu, 48 mass units higher than the expected, suggesting that the two peptides might be linked to each other through a disulfide bond, together with oxidation of Cys101. Since the oxidized sample for 30 min showed a monomeric form, the interchain disulfide bond Cys101−Cys101 in peptide P-4 might be oxidized and cleaved. To support this hypothesis, the peptide was further digested with endoproteinase Lys-C, resulted in shorter peptide P-4 K-1 consisting of two fragments: NRGCV-

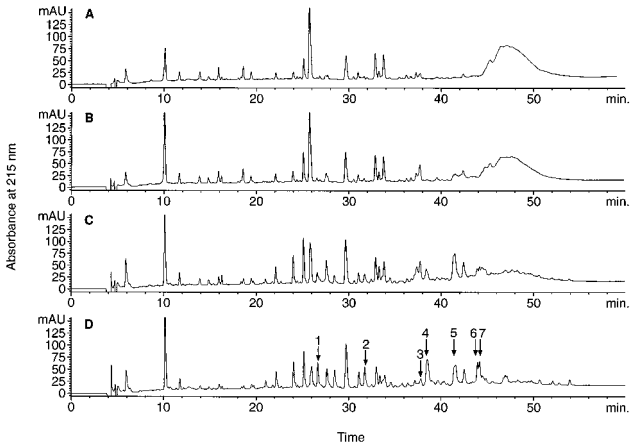

FIGURE 6: Peptic peptide maps of several oxidized forms of GDNF. Each oxidized sample was digested with pepsin and subjected to reversed phase HPLC using a Vydac C18 column as described in the text: (A) intact protein, (B) 5 min oxidation, (C) 10 min oxidation, and (D) 30 min oxidation. HPLC conditions are described in Materials and Methods. Numbered peaks show Cys-containing peptides that were analyzed.

Table 3: Sequence Analysis of peptide P-4 K-1[a]

cycle	peptide 1, PTH (pmol)		peptide 2, PTH (pmol)	
1	N	7.8	V	12.5
2	R	3.6	G	8.6
3	G	12.6	Q	8.3
4	C41	−	A	11.3
5	V	10.0	**C**101	−
6	L	7.4	C102	−
7	T	2.8	R	2.2
8	A	2.6	P	1.9
9			I	1.2
10			A	1.8
11			F	1.1
observed mass		2042.7 amu[b]		
calculated mass		1995 amu (+48 = 2043 amu)		

[a] Sample amount analyzed was approximately 25 pmol. [b] Observed mass is 48 amu higher than the calculated mass due to oxidation of one cysteine residue.

Table 4: Sequence Analysis of Peptide P-4 CM-1

cycle	residue	PTH (pmol)
1	K	61.9
2	V	81.8
3	G	50.0
4	Q	50.7
5	A	54.6
6	C101	−[a]
7	C102	55.2[b]
8	R	13.1
9	P	24.5
10	I	13.9
11	A	18.5
12	F	7.6
observed mass		1399 amu[c]
calculated mass		1292 amu (+48 + 58 = 1398 amu)[c]

[a] This residue was oxidized to cysteic acid. [b] Detected as an *S*-[(carboxy)methyl]cysteine (additional mass = 58 amu). [c] Observed mass was higher than the calculated due to carboxymethylation and oxidation of the cysteines.

Table 5: Sequence Analysis of Peptide P-7[a]

cycle	peptide 1	PTH(pmol)	peptide2	PTH(pmol)
1	I	84.9	V	75.9
2	F	90.7	Y	−[c]
3	R	26.1	H	10.2
4	Y	77.1	I	23.5
5	C68	−[b]	L	22.2
6	S	18.8	R	6.7
7	G	37.1	K	18.7
8	S	14.7	H	8.6
9	C72	−	S	11.8
10	D	10.2	A	29.4
11	A	50.2	K	10.6
12	A	35.4	R	7.3
13	E	2.3	C131	−
14			G	4.7
15			C133	−
16			I	+
observed mass		3314.6 amu		
calculated mass		3300 amu (+16 = 3316 amu)[c]		

[a] Sample amount analyzed was 130 pmol. [b] Not detected. [c] Tyr was not detected at the normal position upon PTH analysis due to oxidation.

LTA (residues 38−45) and VGQACCRPIAF (residues 97−107) (Table 3). The observed mass number (2043 amu) supported the above hypothesis. To identify if Cys101 is oxidized or subsequently involved in an intermolecular disulfide bond, the peptide P-4 was further reduced and alkylated with iodoacetate, resulting in two peptides, P-4 CM-1 and P-4 CM-2. Sequence analysis of the peptide P-4 CM-1 indicates that Cys102 was recovered as an *S*-[(carboxy)methyl]cysteine, while Cys101 did not show any PTH peak, suggesting that it might be cysteic acid (Table 4). On the other hand, Cys41 in peptide P-4 CM-2 was also detected as an *S*-[(carboxy)methyl]cysteine, demonstrating that Cys41 intramolecularly linked Cys102, but Cys101 was fully oxidized. This further confirms that Cys101 is responsible for dimerization, consistent with the fact that the oxidized GDNF used in this study contained only the monomeric form as shown in SDS−PAGE (Figure 5).

Analysis of Other Cys-Containing Peptides. Sequence analysis of peptide P-3 showed two sequences, namely IFRYXSGSXDAAE (residues 64−76) and VYHILRKHSAKRXGXI (residues 119−134), in which cycles 5, 9, 13, and 15, corresponding to Cys68, Cys72, Cys131, and Cys133, remained blank due to the disulfide linkages. ESI-MS of peptide P-3 showed 3332 amu, 32 mass units higher than the expected, suggesting that disulfide bond(s) still linked the two peptides despite the oxidation of some amino acid(s). Since all residues including Tyr67 and Tyr120 except for cysteine residues were detected as normal PTH amino acids, the oxidation might occur at one disulfide bond, resulting in a thiosulfonate intermediate (Griffith & Weistein, 1987). Disulfide linkage Cys72−Cys133 was not directly identified in this study. However, since linkage Cys68−Cys131 was determined earlier, one can assume that the peptide P-3 must contain another disulfide linkage, Cys72−Cys133.

One of the major Cys peptides, P-7, showed the same sequences as in peptide P-3 (Table 5). ESI-MS showed that the peak P-7 contained two peptides linked through a disulfide bond and some residue was oxidized (16 mass units higher than the expected). The sequence analysis indicated that the oxidation might occur at Tyr120 because it was not detected at the normal position on PTH analysis. In conclusion, disulfide linkages in these peptides, P-3 and -7, might be the same as mentioned above. Overall analyses of disulfide linkages are summarized in Table 6, including fully oxidized peptides, P-1 and -2, and cystine-knot peptides,

Table 6: Summary of Iodate Oxidized Cys Peptides[a]

peptide	sequence	observed mass (calcd) (amu)
P-1:	PRRERQAAAANPENSRGKGRRGQRGKNRG^{41}CVLTA *	4038.2 (3988+48)
P-2:	$^{101\ 102}$KVGQACCRPIAF * *	NA
P-3:	^{68}IFRYCSGS^{72}CDAAE $^{131\ 133}$VYHILRKHSAKRCGCI	3332.7 (3300 +32)
P-4:	PRRERQAAAANPENSRGKGRRGQRGKNRG^{41}CVLTA $^{101\ 102}$KVGQACCRPIAF *	5328.5 (5280.7+48)
P-4K-1:	^{41}NRGCVLTA $^{101\ 102}$VGQACCRPIAF *	2043 (1995+48)
P-5: P-6:	^{68}IFRYCSGS^{72}CDAAE $^{131\ 133}$VYHILRKHSAKRCGCI PRRERNRQAAAANPENSRGKGRRGQRGKNRG^{41}CVLTA $^{101\ 102}$KVGQACCRPIAF *	NA
P-7:	^{68}IFRYCSGS^{72}CDAAE $^{131\ 133}$VYHILRKHSAKRCGCI *	3314.6 (3300+16)

[a] Asterisks denote the residues oxidized with sodium iodate. Peptides P-6 and -7 consisted of four peptides linked together through multiple disulfide bonds. NA, Not determined.

P-5 and -6. ESI mass spectral analyses of peptides P-5 and -6 did not show any signals, probably because the four peptides were still associated with each other through multiple disulfide bonds or were further oxidized.

DISCUSSION

Although GDNF is classified as a member of the TGF-β superfamily, the sequence homology is considerably low, compared to the other members; the sequence identity is 18−24%. As mentioned by Cunningham (1994), at least five subfamily members are in the TGF-β superfamily: GDF-10 group, BMP-2 group, BMP-5 group, GDF-5 group, and TGF-β1 group. GDNF contains seven cysteines that are highly conserved in the TGF-β superfamily members. Therefore, a disulfide structure of this family member seemed to be basically the same as that of TGF-β2 (Schlunegger & Grutter, 1992; Daopin *et al*., 1992). However, since inhibin βA, and βB and TGF-β1, -β2, and -β3 contain one or two additonal cysteines, some functional variability may be due to the differences in the primary sequences, the protein conformations, including an extra disulfide bond, or the tertiary structures. Although most TGF-β family members have similar biological functions in bone formation, GDNF has a unique biological function specific to neuronal growth of dopaminergic neurons. Thus, structure−function relationships of GDNF are of interest for understanding the neurotrophic mechanisms.

The determined disulfide structure of GDNF is shown in Figure 7, indicating the similarity with that of TGF-β2 (Daopin *et al*., 1992; Schlunegger & Grutter, 1992). The structure of TGF-β2 as determined by X-ray crystallography indicated that one intramolecular disulfide linkage points through a ring consisting of eight amino acids. GDNF likely has a similar cystine knot since under nonreducing SDS−PAGE the pepsin-generated Cys peptides (P-I−III) showed a band of approximately 15 kDa, which is close to double the sum of the component peptides. However, the existence of disulfide linkages was observed between these covalently separated peptides. Results from partial reduction or oxidation experiments demonstrate that only Cys101 is involved in intermolecular disulfide linkage. Two peptides P-3 and -7 were isolated only after partial oxidation with sodium iodate for 30 min, suggesting that Cys41−Cys102 was initially cleaved, and then peptides P-3 and -7 were released. Therefore, it is consistent that Cys41−Cys102 points through the disulfide ring composed of Cys68−Cys131 and Cys72−Cys133. Since partial reduction using DTT is usually encountered by disulfide scrambling at neutral pH, we have examined partial reduction using TCEP under an acidic condition (pH 2). Disulfide scrambling is largely suppressed at pH 2−3 because its usual mechanism is attack of the disulfide by thiolate anion rather than by un-ionized thiol. The partially reduced form R-I derived from TCEP reduction provided useful information for the assignment of intermolecular disulfide linkage, Cys101−Cys101. The result was also supported by the partial oxidation using sodium iodate. Finally, although iodate oxidation yielded some unknown products as described herein, we suggest that partial

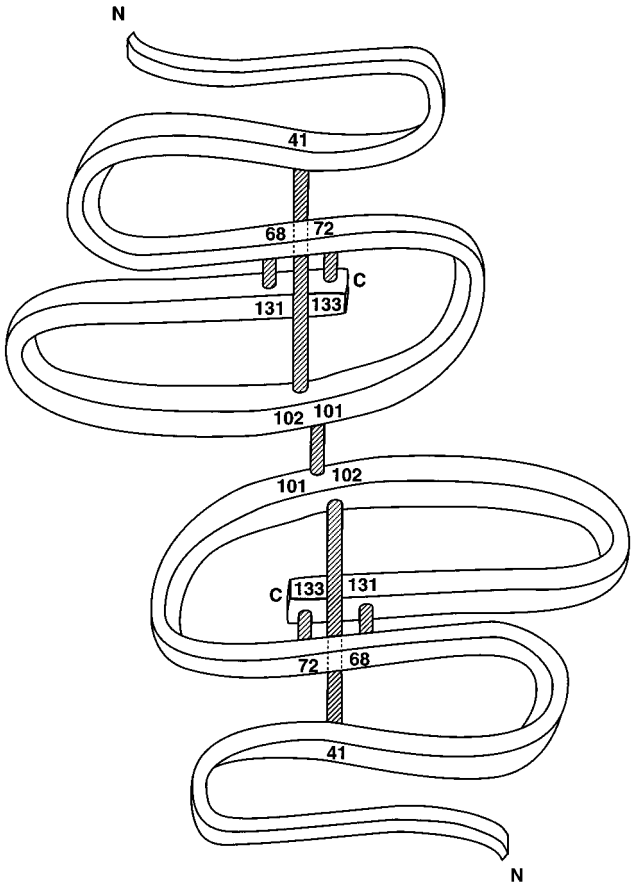

FIGURE 7: Disulfide structure of GDNF. Three intramolecular disulfide linkages, Cys41−Cys102, Cys68−Cys131, and Cys72−Cys133, are shown. One intramolecular disulfide linkage, Cys41−Cys102 points through a ring consisting of four cysteines and four other amino acid residues, which was based on the similarity to TGF-β2 (Schlunegger & Grutter, 1992; Daopin *et al*., 1992). Cys101 is involved in an interchain disulfide linkage between two identical subunits.

oxidation using sodium iodate is also a reliable method for determining the complicated disulfide structure (partial reduction using TCEP is also reliable), if the experiments are performed under controlled conditions.

ACKNOWLEDGMENT

The authors wish to acknowledge Drs. William C. Kenney and Robert Rush for valuable discussions and suggestions for preparation of the manuscript.

REFERENCES

Chalazonitis, A., Kalberg, J., Twardzik, D. R., Morrison, R. S., & Kessler, J. A. (1992) *Dev. Biol. 152*, 121−132.
Cunningham, N. S., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Reddi, A. H., & Lee, S.-J. (1995) *Growth Factors 12*, 99−109.
Daopin, S., Piez, K. A., Ogawa, Y., & Darris, D. R. (1992) *Science 257*, 369−373.
Forno, L. S., DeLanney, L. E., Irwin, I., & Langston, J. W. (1993) *Neurology 60*, 600−608.
Gorin, G., & Godwin, W. E. (1966) *Biochem. Biophys. Res. Commun. 25*, 227−232.
Gray, W. R. (1993) *Protein Sci. 2*, 1732−1748.
Griffith, D. L., Keck, P. C., Sampath, T. K., Rueger, D. C., & Carlson, W. D. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 878−883.
Griffith, O. W., & Weinstein, C. L. (1987) *Methods Enzymol. 143*, 270−273.
Haniu, M., Narhi, L. O., Arakawa, T., Elliott, S., & Rohde, M. F. (1993) *Protein Sci. 2*, 1441−1451.
Haniu, M., Acklin, C., Kenney, W. C., & Rohde, M. F. (1994) *Int. J. Protein Pept. Res. 43*, 81−86.
Haniu, M., Horan, T., Arakawa, T., Le, J., Katta, V., & Rohde, M. F. (1995) *Arch. Biochem. Biophys. 324*, 344−356.
Kopin, I. J., & Markey, S. P. (1988) *Annu. Rev. Neurosci. 11*, 81−96.
Krieglstein, K., Suter-Crazzolara, C., Fischer, W. H., & Unsicker, K. (1995) *EMBO J. 14*, 736−742.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lin, L. F. H., Doherty, D. H., Lile, J., Bektesh, S., & Collins, F. (1993) *Science 260*, 1130−1132.
Lundblad, R. L. (1991) *Chemical Reagents for Protein Modification*, 2nd ed., CRC Press, Boca Raton, FL.
Marti, T., Rosselet, S. J., Titani, K., & Walsh, K. A. (1987) *Biochemistry 26*, 8099−8109.
Martinou, J.-C., Le Van Thai, A., Velette, A., & Weber, M. J. (1990) *Dev. Brain Res. 52*, 175−181.
Otto, D., & Unsicker, K. (1990) *J. Neurosci. 10*, 1912−1921.
Prehn, J. H. M., Peruche, B., Unsicker, K., & Krieglstein, J. (1993) *J. Neurochem. 60*, 1665−1672.
Schlunegger, M. P., & Grutter, M. G. (1992) *Nature 358*, 430−434.
Schubert, D., Kimura, H., LaCorbiere, M., Vaughan, J., Karr, D., & Fischer, W. H. (1990) *Nature 344*, 868−870.

BI9605550