Sites of iodination in recombinant human brain-derived neurotrophic factor and its effect on neurotrophic activity

ROBERT ROSENFELD, JOHN S. PHILO, MITSURU HANIU, KENDALL STONEY,
MICHAEL F. ROHDE, GAY-MAY WU, LINDA O. NARHI, CAROLINE WONG,
TOM BOONE, NESSA N. HAWKINS, JAMES M. MILLER, AND TSUTOMU ARAKAWA

Amgen Inc., Amgen Center, Thousand Oaks, California 91320-1789

(RECEIVED April 15, 1993; ACCEPTED July 12, 1993)

Abstract

Recombinant human brain-derived neurotrophic factor (BDNF) is now under extensive investigation because of its potential clinical applications. Radioactively labeled proteins are usually required to study receptor binding and pharmacokinetic properties of proteins. This study was undertaken to see if iodination affects the biological and conformational properties of a recombinant BDNF. BDNF was iodinated using a stoichiometric amount of nonradioactive cold NaI to minimize multiple iodinations. Of the four tyrosines present in BDNF—Tyr-52, Tyr-54, Tyr-63, and Tyr-86—only Tyr-63 and Tyr-86 were iodinated under the experimental conditions used. Iodination of Tyr-63 resulted in modification without alteration of the biological activity, whereas iodination of Tyr-86 resulted in a molecule with highly compromised biological activity. Similar inactivation was observed if both Tyr-63 and Tyr-86 were iodinated. These modified proteins exhibited conformation and dimerization apparently identical to those of the native protein, as demonstrated by analytical ultracentrifugation, gel filtration, light scattering, and circular dichroism. From these results, we concluded that Tyr-52 and Tyr-54 are not accessible to the reagent and are probably buried in the hydrophobic core, whereas Tyr-63 and Tyr-86 are exposed on the surface of the molecule; of the two exposed residues, only Tyr-86 contributes to the biological activity.

Keywords: BDNF; iodination; refolding; sedimentation

Neurotrophic factors are believed to play an essential role in the growth, survival, and differentiation of neurons in the nervous system (Barde, 1989). So far, six neurotrophic proteins have been identified: NGF, BDNF, NT-3, NT-4, NT-5, and CNTF, of which NGF was the first to be isolated and extensively characterized (reviewed by Levi-Montalcini [1987]). Although all but CNTF are structurally related to each other, they show distinctly different neuronal specificities (reviewed by Barde [1989] and Thoenen [1991]). These molecules have a potential

clinical application for the treatment of neurological disorders such as Alzheimer's disease (Yankner et al., 1990) and Parkinson's disease (Hyman et al., 1991).

We have generated a recombinant *Escherichia coli*-derived form of BDNF that shares 55% sequence homology with NGF, whose crystal structure was recently reported (McDonald et al., 1992). BDNF and NGF have similar spectroscopic markers for secondary and tertiary structure (Radziejewski et al., 1992; Narhi et al., 1993). Therefore, like NGF, BDNF is thought to be stabilized by three intrachain disulfides and to consist primarily of β -sheet and irregular secondary structure. The disulfide structure of BDNF was determined by our groups (Acklin et al., 1993). In solution, the neurotrophins are strongly associated to form dimers. For NGF the dissociation constant of the native dimer was reported as 10^{-13} M (Bothwell & Shooter, 1977). The dissociation constant for BDNF is probably similar, since equilibrium ultracentrifugation

Reprint requests to: Tsutomu Arakawa, Amgen Inc., Amgen Center, 1840 Dehavilland Drive, Thousand Oaks, California 91320-1789.

Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT, neurotrophin; CNTF, ciliary neurotrophic factor; rp-HPLC, reverse-phase high performance liquid chromatography; TFA, trifluoroacetic acid; GPC, gel permeation chromatography; PTH, phenylthiohydantoin; LALLS, low angle laser light scattering; PBS, phosphate buffered saline.

studies show no evidence for BDNF monomers even at protein concentrations near 10^{-10} M (Radziejewski et al., 1992; Narhi et al., 1993).

We are interested in understanding the biological functions of BDNF *in vivo* and its clinical applications, in which receptor binding and pharmacokinetics play a critical role. Such studies are ordinarily carried out using a radioactive protein and, hence, we have prepared iodinated BDNF using cold NaI, in order to see if iodination has any effect on the conformational and biological properties of the protein.

Results

As described in the Materials and methods section, we chose to use a stoichiometric amount of NaI to minimize multiple iodinations per molecule and to mimic the conditions usually used to generate a radioactive iodinated protein.

In order to characterize the iodinated BDNF, the sample was analyzed by rp-HPLC; the results are shown in Figure 1. There are four major peaks observed, labeled P1, P2, P3, and P4. Nearly half of the protein elutes in

Fig. 1. rp-HPLC profile of iodinated BDNF. The iodinated and PD-10 desalted sample was loaded onto a Vydac C4 column equilibrated in 20% buffer B. The column was washed for 45 min with the above solvent, followed by a wash of a linear gradient from 20% to 30% B in 5 min. The column was then developed by a shallow linear gradient from 30% to 35% B in 25 min. The extensive wash with 20% buffer B at room temperature before starting elution is essential to unfold the protein completely. Rosenfeld and Benedek (1993) have shown that BDNF unfolds slowly under rp-HPLC conditions, and insufficient unfolding gives rise to two peaks. **Inset:** Rechromatography of rp-HPLC isolated P1, P2, P3, and P4. rp-HPLC purified samples were diluted with one volume of H_2O and loaded onto a Vydac C4 column in 20% buffer B. A linear gradient from 20% to 30% B in 10 min, sufficient to unfold the protein, was followed by a shallow gradient to 35% B for an additional 30 min.

P1, which is identical in elution time to unmodified BDNF, whereas P2, P3, and P4 are new peaks generated by iodination. When a twofold higher concentration of NaI was used, the amount of P1 decreased to 40% while the amounts of P2–P4 slightly increased. From the absorbance intensity, formation of P3 appears to be more favorable than the formation of P2 and P4, the latter two being generated nearly equally. All four peaks were isolated and collected for further characterization.

Aliquots of the isolated peaks were examined for purity by rechromatographing them on rp-HPLC. These samples were diluted with one volume of water and subjected to rp-HPLC analysis. An overlay of the individually isolated peaks is displayed in Figure 1 (inset), showing that they are homogeneous. Therefore, the isolated peaks were further analyzed as described below.

Mass spectral results

Mass spectral analysis of the P1 material showed a mass of 13,634 (SD = 2.8 mass units), corresponding to the methionyl BDNF, in agreement with the elution position in rp-HPLC analysis. Both P2 and P3 showed a mass of 13,760 (SD = 1.1 mass units), approximately 126 mass units larger than the noniodinated BDNF. Thus, the P2 and P3 materials contain one iodine per polypeptide; their different elution positions suggest that different tyrosines were iodinated. Peak 4 has a mass of 13,886 (SD = 3.4 mass units), which corresponds approximately to the mass of two iodine molecules plus the mass of BDNF polypeptide.

Isolation and analysis of iodinated peptides

In order to locate sites of iodination, the peptides were digested with pepsin; their peptide maps are shown in Figure 2. Comparison of the maps of P1 (Fig. 2A) and P2 (Fig. 2B) indicates that peaks at 41 and 47 min in P1 are also present in P2, whereas peaks at 42 and 43 min, designated 2a and 2b, appear to be slightly shifted relative to the peaks in P1, suggesting that these peptides contain iodinated tyrosine.

Amino acid sequence analyses of 2a and 2b are shown in Table 1. Figure 3 shows a schematic diagram of locations of the disulfide bonds and pepsin cleavage sites determined in this and the following sequence analyses. Both peaks consist of three peptides joined together by three disulfide bonds; i.e., 2a and 2b represent identical peptide fragments except that 2a lacks the C-terminal tyrosine residue of sequence 1. As shown in Table 1, Tyr-63 is iodinated in both peptides 2a and 2b, whereas Tyr-54 appearing at cycle 1 of sequence 1 is not iodinated. Tyr-86 in peptide 2b is not iodinated because P2 contains only one iodinated tyrosine, which has already been detected at cycle 10. Mass spectral analyses of these two peaks, summarized in Table 1, showed that their observed masses are, within experimental error, equal to the masses cal-

Fig. 2. HPLC peptide maps of the peptide digests of iodinated BDNF samples. The samples (50–100 μ g) were digested with pepsin as described in Materials and methods. The peptides were eluted with a linear gradient from 5% to 40% solvent B over 60 min with a flow rate of 0.7 mL/min.

culated from one iodine plus the amino acid sequences. Sequence cycle 10, corresponding to position 63, showed 76% iodinated tyrosine and 24% unmodified tyrosine, indicating that the iodine may be partially lost during sequence analysis. Comparing the results with the mass spectral analysis demonstrates that P2 contains iodinated tyrosine at residue 63.

The pepsin digest of P3 exhibits four new peaks, in addition to the 41- and 47-min peaks observed in the P1 map, designated 3a, 3b, 3c, and 3d (Fig. 2C). Sequence

analysis of peak 3a showed it to be a tetrapeptide, YVRA (corresponding to residues 86–89), of which Tyr-86 was iodinated (Table 2). Peak 3d also starts with iodinated Tyr-86, but is longer than peak 3a. Mass spectral analysis of these peaks reveals only one iodine in the peptide (Table 2).

Sequence analysis of peptides 3b and 3c showed that they represent peptide fragments joined together by three disulfide bonds, as is the case for peaks 2a and 2b. However, the difference in this case between 3b and 3c is due to an additional N-terminal sequence, MHSDPARRGEL, in peptide 3c. Sequence analyses of these two peptides revealed iodination of only Tyr-86, as confirmed by mass spectral analysis (Table 3). All these results, together with the mass spectral analysis of P3, demonstrate that P3 corresponds to BDNF iodinated at Tyr-86.

The pepsin digest of P4 has more peaks that are not seen in any of the other maps (Fig. 2D). Peaks 4a, 4b, 4c, and 4d were analyzed for amino acid sequence. Sequence analysis showed that 4a and 4d were exactly identical to 3a and 3d, respectively, indicating that Tyr-86 is iodinated in P4. Peptides 4b and 4c again represent peptide fragments joined by three disulfide bonds (Table 4). In this case, both peaks contained two iodinated tyrosines, Tyr-63 and Tyr-86, which appeared at cycles 10 and 33. Mass spectral analysis also indicated two iodinated tyrosines in these peptides. The difference between peaks 4b and 4c is due to an extra N-terminal sequence, MHSDPARRGEL, in peptide 4c. Since the mass spectral analysis of P4 indicated two iodinated tyrosines, the observed peptide map and sequence data demonstrate two iodinations at Tyr-63 and Tyr-86 in P4.

Activity

In order to examine the activity of these proteins, they were refolded as follows. Aliquots of the individual peaks were vacuum dried and resuspended in PBS (pH 7.0) at a concentration of 100 μ g/mL, incubated at 4 $^{\circ}$ C for >12 h, and then assayed in a dorsal root ganglia explant assay. The results are shown in Figure 4. These results indicate that P2 is indistinguishable in activity from the native BDNF and P1, whereas P3 and P4 show an order of magnitude lower activity than the native proteins.

GPC/LALLS

The elution positions in GPC of the untreated BDNF and the unfractionated, iodinated BDNF are shown in Figure 5. It is evident that they elute at identical positions, suggesting that they have similar molecular size in solution. The small peak eluting before the major peak in the iodinated BDNF corresponds to lactoperoxidase. The elution positions of the refolded P1–P4 were also close to the two samples above (data not shown), indicating that the iodination and refolding procedures do not alter their molecular sizes. When the unfractionated,

Table 1. Sequence analyses of peptides 2a and 2b^a

Cycle no.	Peptide 2a						Peptide 2b					
	Sequence 1		Sequence 2		Sequence 3		Sequence 1		Sequence 2		Sequence 3	
	Residue	pmol	Residue	pmol	Residue	pmol	Residue	pmol	Residue	pmol	Residue	pmol
1	Y	86	I	105	S	84	Y	62	I	86	S	24
2	E	53	R	15	V	73	E	50	R	+ ^b	V	20
3	T	105	I	86	C	— ^b	T	72	I	85	C	—
4	K	60	D	22	D	22	K	51	D	59	D	59
5	C	—	T	78	S	22	C	—	T	49	S	10
6	N	49	S	51	I	30	N	29	S	20	I	24
7	P	44	C	—	S	27	P	52	C	—	S	18
8	M	46	V	42	E	+	M	28	V	25	E	+
9	G	56	C	—	G	22	C	—				
10	Y	29 ^c	T	44	Y	26 ^c	T	26				
11	T	61	L	44	T	59	L	39				
12	K	29	T	61	K	35	T	46				
13	E	22	I	33	ND ^d	ND						
14	G	32	K	27								
15	C	—	R	17								
16	R	25	G	38								
17	G	40	R	18								
18	I	28										
19	D	11										
20	K	16										
21	R	15										
22	H	5										
23	W	3										
24	N	10										
25	S	6										
26	Q	8										
27	C	—										
28	R	6										
29	T	7										
30	T	10										
31	Q	5										
32	S	2										
Obs. mass units			6,675.0			6,831.8						
Calc. mass units (+iodine)			6,671.8			6,835.0 ^e						

^a Sample amounts analyzed: approximately 200 pmol (peptide 2a) and 160 pmol (peptide 2b).^b +, Present; —, below limits of detection.^c Detected as iodinated Tyr.^d ND, not done after cycle 13.^e Calculated as peptide 2a plus one Tyr residue.

iodinated sample was analyzed by on-line GPC/LALLS, the molecular weight was determined to be 26 kg/mol, indicating that the iodinated protein maintains the dimeric structure. The combination of this result with the similar elution positions observed for the refolded proteins suggests that all these species were refolded into the native dimeric structure.

Sedimentation equilibrium

The sedimentation equilibrium data for the untreated, intact BDNF gave a molecular weight of 27.04 kg/mol and indicated a simple dimeric system. Since the unfrac-

tionated, iodinated BDNF contained residual lactoperoxidase (molecular weight = 77.5 kg/mol), the sedimentation equilibrium data were fitted to a system of BDNF dimers plus a protein species of 77.5×10^3 molecular weight. As shown in Figure 6A, the iodinated BDNF at two different loading concentrations showed reasonable fit to the model without systematic deviations, which gave the expected sedimentation profiles of the enzyme or the BDNF (Fig. 6B, lines 1 and 2). As a control, the result of the untreated BDNF was also fitted to the same model with reasonable residuals, showing no evidence of sedimentation of the enzyme, as expected from the fact that the sample contained no enzyme (Fig. 6B, line 3). These results dem-

Fig. 3. Disulfide structures and pepsin cleavage sites of recombinant human BDNF. The sequence starts with an N-terminal methionine. Major or minor cleavage sites with pepsin are shown by dark or dotted arrows.

onstrate that the iodinated BDNF before rp-HPLC separation retains the full dimeric structure.

Circular dichroism

To determine if iodination interfered with refolding, or affected the structure of the molecule after rp-HPLC, the

structures of P1-P4 as well as unfractionated, iodinated BDNF in PBS (pH 7.0) were analyzed by far-UV CD. The spectra shown in Figure 7 indicate that the unfractionated, iodinated BDNF, as well as P1-P4, has spectra in the far-UV region identical within experimental error to that of the native, unmodified protein. In addition to similarities in the secondary structure, all the spectra have a positive signal of similar magnitude in the 230–235-nm re-

Table 2. Sequence analyses of peptides 3a and 3d^a

Cycle no.	Peptide 3a		Peptide 3d	
	Residue	pmol	Residue	pmol
1	Y	231 ^b	Y	134 ^b
2	V	255	V	117
3	R	99	R	11
4	A	71	A	62
5			L	68
6			T	93
7			M	44
8			D	42
9			S	38
10			K	ND ^c
Obs. mass units	634.0		ND	
Calc. mass units (+iodine)	634.1			

^a Sample amounts analyzed: approximately 400 pmol (peptide 3a) and 300 pmol (peptide 3d).

^b Detected as iodinated Tyr.

^c ND, not done.

Fig. 4. Dose response of neurotrophic activity of P1-P4 in DRG assay. □, P1; ●, P2; ■, P3; ○, P4.

Table 3. Sequence analyses of peptides 3b and 3c^a

Cycle no.	Peptide 3b						Peptide 3c					
	Sequence 1		Sequence 2		Sequence 3		Sequence 1		Sequence 2		Sequence 3	
	Residue	pmol	Residue	pmol	Residue	pmol	Residue	pmol	Residue	pmol	Residue	pmol
1	Y	486	I	566	S	255	Y	202	I	254	M	215
2	E	401	R	172	V	460	E	128	R	100	H	22
3	T	480	I	493	(C)	— ^b	T	178	I	233	S	61
4	K	933	D	293	D	293	K	408	D	81	D	81
5	(C)	—	T	480	S	138	(C)	—	T	188	P	89
6	N	358	S	277	I	290	N	124	S	82	A	104
7	P	341	(C)	—	S	132	P	152	(C)	—	R	+ ^b
8	M	366	V	333	E	44	M	145	V	140	R	+
9	G	322	(C)	—	G	100	(C)	—	G	207		
10	Y	261	T	303	Y	117	T	132	E	58		
11	T	339	L	409	T	146	L	200	L	52		
12	K	552	T	350	K	107	T	152	S	37		
13	E	152	I	251	E	76	I	109	V	70		
14	G	175	K	447	G	80	K	175	(C)	—		
15	(C)	—	R	176	(C)	—	R	+	D	29		
16	R	188	G	167	R	+	G	70	S	19		
17	G	167	R	140	G	83	R	22	I	41		
18	I	200	I	94	S	19						
19	D	108	D	52	E	7						
20	K	237	K	91								
21	R	128	R	+								
22	H	30	H	12								
23	W	28	W	6								
24	N	62	N	27								
25	S	41	S	15								
26	Q	59	Q	21								
27	(C)	—	(C)	—								
28	R	15	R	+								
29	T	39	T	17								
30	T	60	T	24								
31	Q	24	Q	16								
32	S	11	S	6								
33	Y	13 ^c	Y	5 ^c								
Obs. mass units			6,833.5			8,082.3						
Calc. mass units (+iodine)			6,835.0			8,084.6						

^a Sample amounts analyzed: approximately 900 pmol (peptide 3b) and 500 pmol (peptide 3c).^b +, Present; —, below limits of detection.^c Detected as iodinated Tyr.

gion. We have previously shown that this signal arises from the aromatic chromophores that are folded into a distinct tertiary structure (Narhi et al., 1993). Therefore, we can conclude that these iodinated species were refolded into a similar, distinct tertiary structure. This indicates that they all have the proper structure, and that iodination of Tyr-63 and Tyr-86 does not affect the ability of the protein to refold. The iodination of Tyr-63 and Tyr-86 also does not appear to have directly affected the CD signal. As CD signals usually arise from aromatic chromophores buried in the asymmetric interior of a protein, this is consistent with these amino acids being on the exterior of the protein, exposed to solvent.

Discussion

There are four tyrosine residues—Tyr-52, Tyr-54, Tyr-63, and Tyr-86—in human BDNF and hence eight potential iodination sites in the dimer. Although a stoichiometric amount of NaI (i.e., one molecule per dimer) was used, two tyrosine residues (Tyr-63 and Tyr-86) were iodinated, suggesting that these two tyrosine residues were more solvent-exposed than Tyr-52 and Tyr-54. The X-ray crystal structure of murine NGF shows that Tyr-52, Phe-54, and Phe-86 are packed in the dimeric contact of NGF, while Pro-63 locates in a reverse turn and is solvent-exposed (McDonald et al., 1991). Based on the sequence homol-

Table 4. Sequence analyses of peptides 4b and 4c^a

Cycle no.	Peptide 4b						Peptide 4c					
	Sequence 1		Sequence 2		Sequence 3		Sequence 1		Sequence 2		Sequence 3	
	Residue	pmol	Residue	pmol	Residue	pmol	Residue	pmol	Residue	pmol	Residue	pmol
1	Y	83	I	110	S	49	Y	99	I	136	M	59
2	E	67	R	19	V	83	E	71	R	+ ^b	H	+
3	T	104	I	103	(C)	— ^b	T	83	I	129	S	23
4	K	116	D	47	D	47	K	88	D	36	D	36
5	(C)	—	T	108	S	24	(C)	—	T	98	P	30
6	N	65	S	54	I	42	N	62	S	44	A	27
7	P	65	(C)	—	S	25	P	80	(C)	—	R	+
8	M	66	V	63	E	6	M	75	V	73	R	+
9	G	52	(C)	—			G	39	(C)	—	G	39
10	Y	48 ^c	T	64			Y	37 ^c	T	45	E	17
11	T	72	L	72			T	47	L	50	L	36
12	K	38	T	66			K	43	T	42	S	9
13	E	31	I	43			E	31	I	59	V	19
14	G	31	K	52			G	42	K	62	(C)	—
15	(C)	—	R	33			(C)	—	R	+	D	6
16	R	42	G	33			R	+	G	35	S	6
17	G	35	R	30			G	45	R	+	I	15
18	I	35					I	50			S	6
19	D	26					D	19			E	+
20	K	23					K	32				
21	R	25					R	+				
22	H	8					H	+				
23	W	5					W	6				
24	N	13					N	12				
25	S	8					S	8				
26	Q	11					Q	10				
27	(C)	—					(C)	—				
28	R	12					R	+				
29	T	11					T	6				
30	T	14					T	9				
31	Q	9					Q	7				
32	S	3					S	3				
33	Y	2 ^c					Y	2 ^c				
Obs. mass units			6,960.6						ND ^d			ND
Cal. mass units (+2 iodines)			6,960.9									

^a Sample amounts analyzed: approximately 200 pmol (peptide 4b) and 150 pmol (peptide 4c).^b +, Present; —, below limits of detection.^c Detected as iodinated Tyr.^d ND, not done.

ogy between NGF and BDNF and their similar conformational features, the four tyrosines in the corresponding positions of BDNF may exist in similar environments. The observed pattern of Tyr iodination in BDNF partially agrees with the crystal structure, since the iodination results suggest that Tyr-52 and Tyr-54 are less solvent-exposed, whereas Tyr-63 is more exposed. The observed iodination at Tyr-86 suggests the possibility that the dimeric contact surface is not exactly identical between the BDNF and NGF dimers, in particular around position 86; i.e., Tyr-86 in BDNF is more solvent-exposed than Phe-86 in NGF.

The iodinations at positions 63 and 86 did not appear to affect the conformation and folding of BDNF as shown by CD spectra. They also did not affect the CD signal itself, suggesting that the solvent-exposed tyrosines themselves do not contribute to the CD signal of BDNF. This is consistent with their location in a less asymmetric, more solvent-exposed environment than Tyr-52 and Tyr-54. Neither single nor double iodination affects the secondary, tertiary, or quaternary structure as demonstrated by sedimentation equilibrium, light scattering, and CD measurements on the unfractionated, iodinated BDNF. In addition, each species of the iodinated BDNF isolated by

Fig. 5. GPC profile of untreated BDNF (—) and unfractionated, iodinated BDNF (····).

Fig. 6. Fitting of sedimentation equilibrium data to a model of BDNF dimer plus lactoperoxidase. The results are for the iodinated BDNF at 0.1 (— in panel A) and 0.2 mg/mL (---- in panel A) and for the unmodified protein at 0.2 mg/mL (···· in panel A). **A:** Residuals of the fittings. **B:** Weight fraction of BDNF or lactoperoxidase calculated from the fittings. Line 1, 0.2 mg/mL; line 2, 0.1 mg/mL; line 3, unmodified protein (no lactoperoxidase).

Fig. 7. Far-UV CD spectra of iodinated BDNF species. Spectra were taken in PBS at ambient temperature. **A:** Spectra of original BDNF (—) and the unfractionated, iodinated BDNF (---) and P3 (····). **B:** Spectra of P1 (—), P2 (---), and P4 (····).

rp-HPLC showed folding into a conformation resembling that of the native BDNF.

Having established that neither modification affects protein folding, the observed detrimental effect of iodination at Tyr-86 on the biological activity can most likely be ascribed to the alteration in chemical nature or local conformation around position 86; i.e., Tyr-86 is possibly involved in the receptor binding and/or internalization of BDNF. It is interesting that Phe-86 in NGF appears to be important for packing, while Tyr-86 in BDNF may be more accessible to iodination and receptor binding. This again suggests that BDNF may have a different (at least locally) conformation than NGF.

The results described here indicate that iodination of BDNF produces various derivatives with different modifications. Since they are initially (before rp-HPLC) in the dimeric form, there are a number of combinations to be considered. It would be interesting to know whether the

iodination at Tyr-86 is detrimental to the biological activity only when both subunits in the dimer are iodinated at this position, or if only one modification per dimer is sufficient for loss of activity. Such a study may shed light on the importance of the dimeric structure of the neurotrophins for receptor dimerization.

Finally, the results of this study indicate that care must be exercised when using radioactively iodinated BDNF for receptor binding and pharmacokinetic studies, since each dimeric species with a different combination of modifications may behave differently in such studies.

Materials and methods

Materials

Recombinant human BDNF used in this study was expressed in *E. coli*, refolded, and purified to homogene-

ity using sequential column chromatography involving ion exchange and reverse-phase columns. The disulfide structure was determined to be identical to the mammalian cell-derived material (Acklin et al., 1993). The *E. coli*-derived protein has activity comparable to the mammalian cell-derived material as well. This protein has an extra N-terminal methionine. Lactoperoxidase from bovine milk was purchased from Sigma.

Protein concentration

Protein concentration was spectrophotometrically determined using an extinction coefficient of 1.6 for 0.1% solution at 280 nm and 1 cm pathlength.

Iodination

In order to characterize the BDNF molecule after iodination, we decided to scale the lactoperoxidase labeling 50-fold using cold NaI at a ratio of 1 mole of NaI per mole of BDNF dimer (Marchalonis, 1969). Iodination was performed at room temperature by combining 0.5 mL NaI solution (5.75 $\mu\text{g/mL}$ in 0.1 mM NaOH), 0.5 mL BDNF (2 mg/mL in 20 mM sodium phosphate, 100 mM NaCl, pH 7.4), 0.3 mL of lactoperoxidase solution (0.2 mg/mL in H_2O), and 0.2 mL of 0.015% H_2O_2 in H_2O . After incubation at room temperature for 5 min, the reaction was terminated by adding 1 mL of 20 mM sodium phosphate, 0.1 M NaCl, pH 7.4, and 1% NaN_3 . The reaction mixture was then applied to a PD-10 column equilibrated in phosphate-buffered saline, pH 7.4. The protein fractions were spectrophotometrically monitored and collected.

Reverse-phase HPLC

rp-HPLC purification and analysis of BDNF samples was performed using a Waters 600E solvent delivery module, a 715 autosampler, and a Hewlett Packard 1040 M diode-array detector with an HP Chemstation for data acquisition. The eluents used to develop gradients were buffer A containing 0.1% TFA in water and buffer B containing 90% acetonitrile in 0.1% TFA. A Vydac C4 reverse-phase column 214TP54 (0.46 $\times$ 25 cm or 1 $\times$ 25 cm) was used at a flow rate of 1 mL/min or 2 mL/min.

Gel permeation chromatography

HPLC/GPC of BDNF samples was carried out on a TSK G2000 SWXL column (0.78 $\times$ 30 cm) at a flow rate of 1 mL/min equilibrated in 0.1 M sodium phosphate, 0.5 M NaCl, pH 6.9.

Mass spectral analysis

Molecular weights of BDNF samples were determined using a triple quadrupole mass spectrometer (SCIEX API

III, Toronto, Canada). Samples collected from rp-HPLC were directly infused into the ionspray interface of the mass spectrometer at a flow rate of 3 $\mu\text{L/min}$. Scans were accumulated and their spectra averaged and analyzed using MacSpec version 3.2 software (SCIEX).

Sequence analysis

Peptide sequence analysis was performed by a gas phase sequencer, ABI (Applied Biosystems, Inc.) model 470A or 477A. PTH derivatives were identified by rp-HPLC in an on-line fashion employing an ABI model 120A PTH analyzer fitted with a Brownlee PTH-C18 column (2.1 $\times$ 150 mm). PTH derivatives of iodinated tyrosine was detected in front of PTH tryptophan.

Proteolytic digestion

BDNF samples (P1, P2, P3, and P4) (50–100 μg) were digested with pepsin (0.5 μg) in 250 μL of 0.02 N HCl (pH 2). Digestion was carried out at 37 $^\circ\text{C}$ for 20–24 h. The digested materials were directly applied to an rp-HPLC Vydac C18 column (4.6 $\times$ 250 mm).

Peptide separation

The peptides were separated by rp-HPLC using a Vydac C18 column (4.6 $\times$ 250 mm) at room temperature. The two-solvent system was used; solvent A contained 0.1% TFA in water and solvent B contained 90% acetonitrile in 0.1% TFA. The peptides were eluted with a linear gradient from 5% B to 40% B and a flow rate of 0.7 mL/min.

Activity

For the chick dorsal root ganglia explant assay, ganglia were dissected from day 8 chick embryos and plated in a collagen matrix in medium containing 10% heat-inactivated horse serum. Five ganglia were plated in each test dish in a final volume of 2 mL. The samples were assayed by adding them directly to the culture medium in the test dish. For control samples, BDNF at 5 and 10 ng/mL, or PBS containing 1 mg/mL bovine serum albumin alone, was added to the test dish. After 24 h at 37 $^\circ\text{C}$, the ganglia were scored from 0 to 5 on the basis of neurite length and density. The score for each sample was the average of five ganglia.

Light scattering

The on-line GPC/LALLS system uses three detectors in series: an absorbance monitor at 280 nm (Knauer A293), a low angle laser light scattering detector (Polymer Laboratories PL-LALLS), and a refractive index detector (Polymer Laboratories PL-RI). An aliquot (100 μL) of BDNF at 1.2 mg/mL in PBS was injected onto a TSK

2000SWXL GPC column with 1 mL/min flow of 0.1 M sodium phosphate, 0.5 M NaCl, pH 6.9, buffer. The system was calibrated using ribonuclease, ovalbumin, and bovine serum albumin as molecular weight standards, following procedures similar to those of Takagi (1990).

Sedimentation equilibrium

Sedimentation equilibrium experiments were conducted using a Beckman Optima XL-A and an An-60 Ti analytical rotor. A six-channel cell was loaded with 0.125 mL each of iodinated or unmodified BDNF solution at 0.1–0.2 mg/mL in PBS and centrifuged at 20,000 and 28,000 rpm at 25 °C. Radial scans of absorbance at 280 nm of each solution were collected at intervals of 0.002 cm per step. For data analysis, an identical value of the partial specific volume was used for iodinated and unmodified BDNF, i.e., 0.723 mL/g, calculated from amino acid composition (Cohn & Edsall, 1943). The program TableCurve (Jandel Scientific) was used for analysis of single data sets, and an in-house nonlinear least squares routine was used to globally fit data sets at different concentrations and rotor speeds to models of ideal or nonideal interacting systems.

Circular dichroism

CD was performed at ambient temperature on a Jasco J-500C spectropolarimeter controlled by a Samtron computer. When possible, a cuvette with a pathlength of 0.1 cm was used; for P2 and P4, a cuvette with a pathlength of 0.02 cm was used due to limited amounts of material. Because of insufficient quantities, protein concentration for CD was determined from the area under the curve of the rp-HPLC peaks and was adjusted to approximately 2 mg/mL after drying and reconstitution in PBS. A mean residue weight of 113 was used for calculating mean residue

ellipticity. The spectra were scanned at 20 nm/min, with three repetitions, from 250 to 200 nm.

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