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Conformational changes of brain-derived neurotrophic factor during reversed-phase high-performance liquid chromatography

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ABSTRACT

Recombinant human brain-derived neurotrophic factor (r-HuBDNF) is eluted as two peaks under reversed-phase liquid chromatographic conditions with gradient elution. Sodium dodecyl sulfate polyacrylamide gel electrophoresis confirmed identical molecular weights in the two peaks, while rechromatography of the separated peaks showed interconvertibility. The two peaks are identified as the monomeric forms of the parent molecules. The molecular weight of the components in the peaks was determined by on-line 90° light scattering using a fluorescence detector as a scatterograph. The early eluted peak is a folded form of the r-HuBDNF monomer, while the later eluted peak is an unfolded form of the BDNF monomer. The conformational states were established using a fluorescence detector both at a fixed wavelength and in the scanning mode.

INTRODUCTION

The discovery of a diffusible factor(s) responsible for the survival and enhanced growth of sympathetic sensory ganglia was demonstrated in the early 1950s [1]. The first discovered protein in this family to be purified and sequenced was nerve growth factor (NGF) [2].

Brain-derived neurotrophic factor (BDNF), the second member in the NGF family to be discovered, is a protein containing 119 amino acids. It has a molecular weight of 13.5 kD and a p*I* of 10.3 [3–5]. This molecule is 55% homologous to NGF. Both NGF and BDNF support the survival of distinct neuronal populations *in vivo*. NGF supports sympathetic and sensory neurons in the peripheral nervous system as well as cholinergic neurons in the basal forebrain [6,7]. BDNF also supports sensory neurons from embryonic peripheral sensory ganglia *in vitro*, just like NGF, but has activities on neurons that are unresponsive to NGF, such as neurons originating from the neural crest, ectodermal placoid and retinal ganglion [8].

A third member of the NGF family, neurotrophin-3 (NT-3), was discovered utilizing primer sequences constructed from conserved regions of BDNF and NGF by means of polymerase chain reaction (PCR) techniques [9,10]. NT-3 is 58% homologous with BDNF and 57% homologous with NGF. Recently, a fourth member of this family has been identified and named neurotrophin-4 (NT-4) [11]. With the help of genetic engineering techniques some of these factors are now cloned, expressed, and purified in sufficient quantities for their analytical and biochemical characterization as well as their potential pharmaceutical application.

In this study r-HuBDNF, derived from Chinese Hamster Ovary cells (CHO) was examined. r-HuBDNF shows one band by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and one peak in size-exclusion and ion-exchange chromatography. The same sample exhib-

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ited two peaks in reversed-phase high-performance liquid chromatographic (RP-HPLC) analyses. The peaks were of identical molecular weight when analyzed by SDS-PAGE. The early eluted peak converts into later eluted peak as shallower gradients were applied. Similar chromatographic behavior has been reported before and was attributed to conformational changes induced by RP-HPLC conditions [12–14]. This publication describes an experimental pathway for peak identification and describes the relationship between the observed peaks.

EXPERIMENTAL

Materials and methods

r-HuBDNF expressed in CHO cells was purified as described previously [15]. Ribonuclease (RNase), lysozyme (LYS), ovalbumin (OVA) and bovine serum albumin (BSA) were from Sigma (St. Louis, MO, USA). HPLC solvents were purchased from Fisher. Two different VYDAC Protein C4 reversed-phase columns (Separation Groups, Hesperia, CA, USA) were used in this work. The column parameters will be specified in the figure captions.

HPLC instrumentation and conditions

The basic HPLC system consisted of a gradient pump, UV detector, integrator and data station (Spectra Physics, San Jose, CA, USA). The temperature control of chromatography was provided by immersing the column in a thermostated water bath (Neslab Instruments). The temperature was kept at 25°C in all experiments reported here. In general a 1 ml/min flow-rate has been used, unless it is stated otherwise. The mobile phase system used is as follows: mobile phase A, 0.1% trifluoroacetic acid (TFA)–distilled water (0.1:99.9); mobile phase B, TFA–acetonitrile (0.1:99.9) or TFA–acetonitrile–water (0.1:90:9.9). The descriptions of the gradients are provided in the text.

Fluorescence detection

Two Shimadzu fluorescence detectors were used, a Model RF-551 scanning fluorescence monitor for the on-line scanning experiments and a Model RF-535 fluorescence detector for the light scattering experiments.

In the scanning mode the fluorescence emission spectra between 300 and 400 nm were collected at the apex of the peaks at an excitation wavelength of 276 nm. A Shimadzu RF-551 fluorescence detector was used to follow the fluorescence trace of the r-HuBDNF elution. The excitation wavelength was 276 nm and the emission wavelength was 385 nm.

Light scattering

The 90° light scattering was measured using a Shimadzu RF-535 fluorescence detector according to the method of Dollinger *et al.* [16]. In essence, the method is based on the fact that selecting a wavelength where no excitation and emission of the sample or buffer can be observed, a fluorescence detector can be used as a 90° scatterometer. Using calibration curves obtained with standard proteins the molecular weight of proteins can be calculated from on line data collection. In this study the excitation and emission wavelength was 465 nm. The elution of proteins was followed by a UV detector at 280 nm and by a scatterometer. The peak areas from both signals were integrated. The molecular weight calculations using the corresponding peak areas and were performed according to Dollinger *et al.* [16]. The extinction coefficients of the standard proteins were obtained from the literature [16], and for r-HuBDNF a value of 1.7 was applied.

SDS-PAGE analysis

SDS-PAGE analysis of r-HuBDNF was performed according to Laemmli [17] using 10–20% (w/v) acrylamide gel. The visualization of the bands was performed by Coomassie Brilliant Blue staining. Under non-reducing conditions, r-HuBDNF migrates as a monomer with an apparent molecular weight of 14.3 kD. Under reduced conditions, the mobility of the r-HuBDNF is slightly decreased, an observation which is characteristic with intramolecular disulphide bonds.

RESULTS AND DISCUSSION

After the purification of r-HuBDNF it was observed that an electrophoretically homogeneous protein sample eluted in two peaks under reversed-phase chromatographic conditions. Fig. 1 displays the reversed-phase chromatographic separation of an r-HuBDNF sample using gradient elution. The chromatogram contains two peaks, which are labeled peak I and peak II.

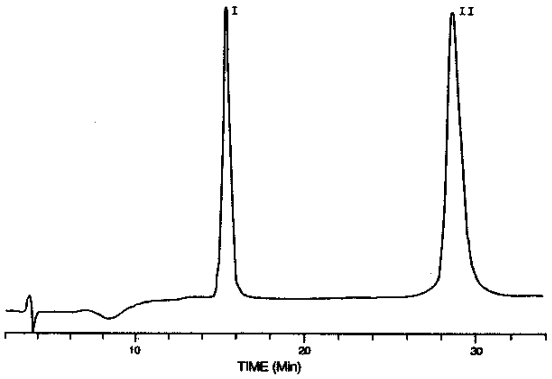

Fig. 1. Reversed-phase chromatographic analysis of r-HuBDNF. Chromatographic conditions: column, 25 cm × 4.6 mm I.D. Vydac Protein C4; mobile phases, A: 0.1% TFA–water (0.1:99.9), B: TFA–acetonitrile–water (0.1:90:9.9); gradient, 20–27% B solvent in 7 min, 10 min hold at 27% B, 27–33% B in 24 min, 3 min hold at 34% B; detection, 214 nm; temperature, 25°C.

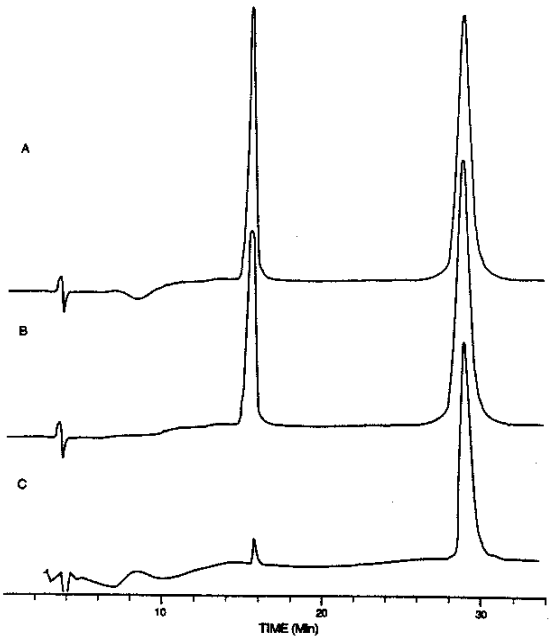

Fig. 2. Reinjection experiments. Peaks I and II were collected and analyzed with the same elution method. (A) Chromatogram of r-HuBDNF, (B) peak I, (C) peak II. Chromatographic conditions: see Fig. 1.

Off-line peak characterization

The resolved peaks were collected and SDS-PAGE of reduced and non-reduced samples revealed that the molecular weights of the proteins in peaks I and II were identical to each other and to the r-HuBDNF standard, confirming that the peaks were r-HuBDNF. Amino terminal sequence analysis showed the first 15 residues to be identical in both peaks (data not shown).

The biological activity of r-HuBDNF from the peaks (I, II) was also measured and the two peaks were equally active. The activity measurement is a biological assay in which the survival of neurons from chick dorsal root ganglia are measured after a 24-h period.

The isolated peaks were rechromatographed and analyzed under the original reversed-phase conditions. Fig. 2A shows the chromatogram of the r-HuBDNF standard. Fig. 2B is the rechromatography of peak I displaying two peaks with retention times matching the original peaks I and II. The rechromatography of peak II, shown in Fig. 2C gave two peaks with retention times matching the original peak I and II. These experiments illustrate that peak I converts into peak II and some portion of peak II converts into peak I. The fact that the peak I/II ratio is significantly smaller than in the original sample indicates that the refolding of peak II in the collection media (TFA–acetonitrile–water) is a slow process, and possibly the equilibrium distribution of the folded and unfolded species is also different as compared to the neutral condition. Nevertheless the appearance of peak I in the chromatogram of peak II is indicative of a reversible conversion mechanism. It has to be mentioned, that protein recovery was always above 95%, based on peak area analysis and the experiments were reproducible.

Because the reversibility of the peaks could be an important factor in explaining their nature, other collection conditions for peak II were explored. Peak II was collected, lyophilized, reconstituted in PBS and then analyzed by RP-HPLC. The chromatograms of the lyophilized peak showed peak I and a second peak with retention times corresponding to peak II. The reversibility hypothesis was further strengthened by an experiment where peak II was collected in PBS, followed by RP-HPLC analysis. Again two peaks appeared in the chromatogram.

Since the peak I/II ratio in all of these refolding experiments was identical, it can be assumed that the conformational state after refolding in PBS is identical or very similar to the original conformation.

Since the amino acid sequence of peak I and peak II is the same, and the biological activity equal in both peaks, we focused our attention on the biophysical characterization of peaks I and II.

Peak conversion

Gradient optimization experiments also revealed that the peak I to peak II ratio decreased with shallower gradients. This observation suggested that the more time r-HuBDNF molecule spent on the reversed-phase column the greater the conversion of peak I to peak II.

The importance of the on-column residence time of r-HuBDNF was substantiated by injecting the sample into the reversed-phase column and washing for 60 min wih the starting mobile phase prior to gradient elution. The result of this extended on-column incubation is shown in Fig. 3B, which demonstrated that peak I had almost completely disappeared. This experiment was repeated several times, with incubation times ranging from 0 to 20 min and identical elution gradients. Plotting the logarithm of the area of peak I as a function of incubation time, shown in Fig. 4, we established that the peak disappearance fits to a first-order kinetic model [18]. The rate constant of conversion at room temperature was calculated as $2.13 \cdot 10^{-3}\ s^{-1}$, which corresponds to a 5.4-min half-life.

Based on the above described experimental results it was established, that (a) peaks I and II represent r-HuBDNF; (b) peak I can be converted into peak II, by varying the RP-HPLC parameters; (c) under appropriate conditions the conversion is reversible.

Peaks I and II showed the same monomeric

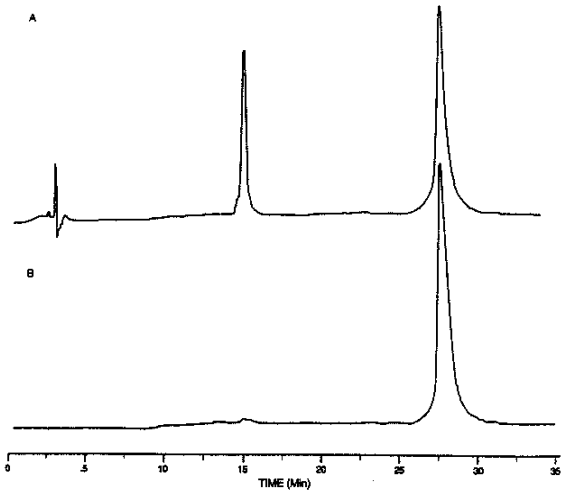

Fig. 3. On-column incubation of r-HuBDNF. The injected sample was incubated for 0 (A) and 60 min (B) on the reversed-phase column prior elution. Chromatographic conditions: see Fig. 1.

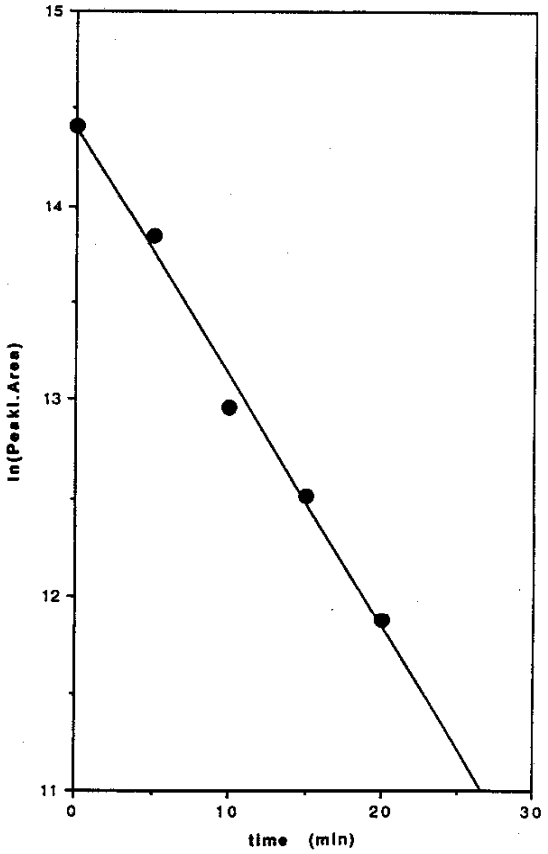

Fig. 4. r-HuBDNF peak conversion kinetics. r-HuBDNF samples were injected onto a reversed-phase column and incubated for 0, 5, 10, 15 and 20 min at constant temperature (25°C) prior elution. $y = 14.400 - 0.12805x$; $R^2 = 0.992$.

molecular weight under reducing and non-reducing SDS-PAGE. It is known that r-HuBDNF exists as a non disulphide linked dimer in PBS, as shown by size exclusion chromatography and sedimentation equilibrium [19], and has been reported for NGF [2]. The question remains whether peaks I and II, observed under RP-HPLC conditions represent a dimer and monomer distribution or both peaks are in the same dimeric or monomeric form. Because of the reversibility and short time scale of conversion the utilization of off-line methods was excluded and instead we employed on-line analytical techniques suitable for detecting size and conformational changes of proteins.

On-line peak characterization by light scattering

An on-line light scattering method, developed by Dollinger *et al.* was selected for characterizing the molecular weight of r-HuBDNF in peak I and II [16]. This novel method based on the Takagi approach [20] but utilizes a fluorescence detector as a 90° scatterometer and a UV detector for concentration determination. The detector signals were calibrated against well defined protein standards such as RNase, LYS, OVA, and BSA. The molecular weight is proportional to the two signals as follows:

$$M_w = \frac{Is\ \varepsilon}{k\ A}$$

where k is an empirical constant, Is and A are the light scattering and ultraviolet signals, respectively, and ε is the extinction coefficient at the UV absorption wavelength. Using standard proteins with known molecular weight (M_w^k) the molecular weight (M_w^u) of unknown proteins can be calculated according to the following equation:

$$M_w^u = M_w^k \frac{Is^u\ A^k\ \varepsilon^u}{Is^k\ A^u\ \varepsilon^k}$$

Fig. 5 displays the UV trace (A) and the scatterogram (B) of r-HuBDNF under reversed-phase elution conditions. In both chromatograms the ratio of peak I to peak II is similar and close to one, implying that the molecules constituting both peaks have very similar mass. The results of the calculations using 4 different standard proteins are listed in Table I. The calculated average molecular weights for peak I and peak II are 14.8 kD and 12.4 kD, respectively. These values are consistent with the monomeric molecular mass of 13.5 kD calculated from the amino acid composition of r-HuBDNF.

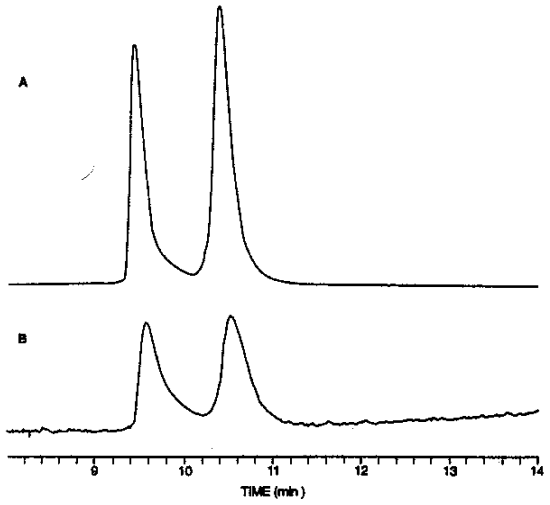

Fig. 5. Molecular weight determination of r-HuBDNF using 90° light scattering. (A) UV; (B) scatterogram. Column, 15 cm × 4.6 mm I.D. Vydac Protein C4; mobile phases, A: TFA–water (0.1:99.9), B: TFA–acetonitrile–water (0.1:90:9.9); gradient, 5–45% B solvent in 30 min; UV detection, 280 nm; light scattering detection, 465 nm; temperature, 25°C.

These light scattering results eliminated the existence of dimers and the dimer-monomer transition model, consequently we focused our attention on further characterizing the peaks. In previous cases involving a two peak phenomena the early eluting

TABLE I

MOLECULAR WEIGHT CALCULATIONS OF BDNF USING STANDARD PROTEINS

Standard proteins	BDNF I	BDNF II
LYS	16 118	13 534
RNase	12 595	10 576
OVA	16 433	13 799
BSA	14 153	11 884
Average	14 825	12 448
Standard deviation[a]	1796	1508

[a] $n = 4$.

peak corresponded to a native and/or a more folded conformation while the late eluting peak represented a denatured and/or a more unfolded conformation of the protein [13]. The conformational state of these molecules was studied by on-line and/or off-line analytical techniques [13,21,22].

Since r-HuBDNF interconverts at neutral pH, the activity cannot be determined on the time scale of chromatography and it cannot be used for determining whether the peaks correspond to the native and/or a more folded conformation or a denatured and/or a more unfolded conformation of the protein. In order to determine the conformational state peak I and II, we used on-line fluorescence spectroscopy at both fixed wavelengths and in the scanning mode.

On-line fluorescence spectroscopic peak analysis

Fluorescence of the eluents was monitored by setting the excitation wavelength at 276 nm and the emission wavelength to 385 nm. Fig. 6 displays the chromatogram of r-HuBDNF by RP-HPLC as monitored by UV (A) and fluorescence detection (B). It is apparent that the ratio of the two peaks (I/II) is about unity on the UV trace (A) while on the fluorescence trace (B) this ratio is much larger than one. This indicates that the fluorescence intensity is lower for the second peak than for the first peak. Decreased fluorescent intensity can occur when (a) the emission spectrum is altered, or (b) by solvent quenching. In order to answer the question, we utilized the detector's on-line spectrum acquisition capability and we took the fluorescence spectra at the apex of both peaks. The collected fluorescence spectra using 280 nm excitation revealed an about 10 nm red-shifted emission maximum for peak II, suggesting that this form is more unfolded than peak I. Similar fluorescence spectral shift was observed in acid induced denaturation of BDNF [23].

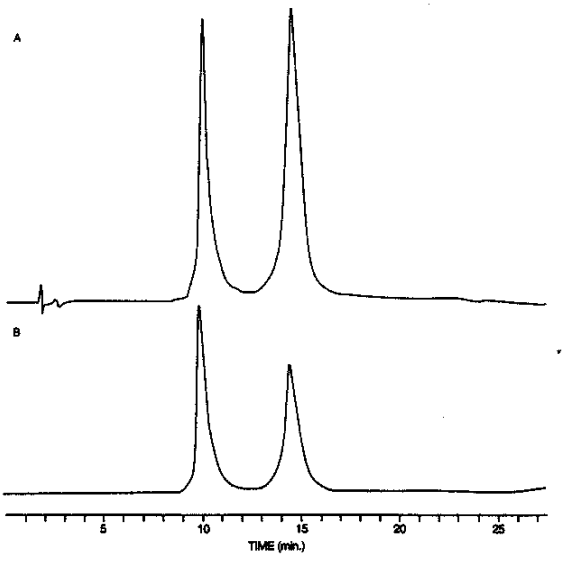

Fig. 6. Analysis of the conformational states of r-HuBDNF during RP-HPLC. (A) UV; (B) fluorescence (excitation wavelength 276 nm; emission wavelength 385 nm). Chromatographic conditions: see Fig. 5.

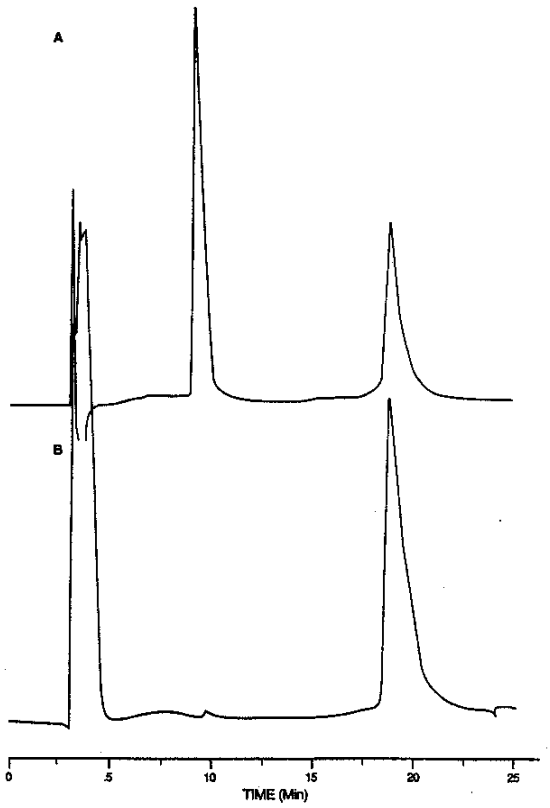

Fig. 7. Chromatogram of guanidine · HCl-denatured r-HuBDNF. (A) Standard r-HuBDNF; (B) guanidine · HCl-treated r-HuBDNF. Chromatographic conditions: see Fig. 1.

Such a red shift is indicative of a greater exposure of a tryptophan residue(s) to an aqueous environment, such as might occur by unfolding of the polypeptide chain. The fluorescence spectroscopic results are consistent with the hypothesis, that peak I represents a less unfolded and peak II a more unfolded BDNF structure.

Effect of guanidine · HCl on the BDNF peaks

Proteins incubated wih guanidine · HCl most often lose all of their tertiary and secondary structure. As a further test of the nature of the peaks, we incubated an aliquot of peak II, which was collected and refolded in PBS, in 6 *M* guanidine · HCl, and then analyzed the sample with RP-HPLC. Fig. 7 displays the chromatograms of BDNF before (A) and after (B) guanidine · HCl treatment. After guanidine · HCl treatment, peak I disappeared from the chromatogram and only peak II was apparent. Collection of peak II, dialysis, lyophilization and resuspension in PBS, followed by a RP-HPLC analysis provided us with a chromatogram similar to our standard BDNF. This experiment confirms two observations: (a) that peak II must represent the denatured structure of r-HuBDNF; and (b) that the denaturation is reversible.

It is important to note that the guanidine · HCl-denatured species elute with the same retention time as the RP-HPLC denatured species, indicating that the final unfolded conformations attained by RP-HPLC alone or guanidine · HCl treatment followed by RP-HPLC are chromatographically identical.

CONCLUSIONS

The two peaks observed in the chromatogram are both the monomeric forms of the r-HuBDNF molecule. The first peak represents a folded form and the second later eluting peak is an unfolded form of the r-HuBDNF monomer. The first peak can be converted into the second by increasing the time the molecule spends on the column. Similar conversion was observed by increasing the temperature of chromatography [24].

In light of the various spectroscopic and biochemical evidences acquired during our work we can establish that the chromatographic behavior of r-HuBDNF under reversed-phase gradient elution can be traced to a conformational change occurring in the molecule as a result of the chromatographic conditions. Similar chromatographic behavior has been observed previously with other proteins [12,13, 21]. Our observationi s in agreement with those examples and serves as another case where the denatured forms of a protein under RP-HPLC conditions elute later than the native and/or folded structures.

r-HuBDNF exists as a dimer at physiological conditions, but under reversed-phase conditions only the monomeric form is present. These results indicate that the chromatographic process has two steps. In step one, the dimer breaks up as the molecule encounters the stationary phase. The dissociation happens rapidly, under RP-HPLC conditions. In step two, the unfolding of the molecule occurs. This second step has a half life, which is comparable with the time scale of chromatography allowing the visualization of both the folded and the unfolded conformers. A detailed analysis of the kinetics and thermodynamics of the observed conversion mechanism will be reported later [24].

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REFERENCES

1 R. Levi-Montalcini, *Ann. NY Acad. Sci.*, 55 (1952) 330.
2 R. H. Angeletti and R. A. Bradshaw, *Proc. Natl. Acad. Sci. USA*, 68 (1971) 2417.
3 Y.-A. Barde, *Neuron*, 2 (1989) 1525.
4 M. M. Hofer and Y.-A. Barde, *Nature (London)*, 331 (1988) 261.
5 J. Leibrock, F. Lottspeich, A. Hohn, M. Hofer, B. Hengerer, P. Masiakowski, H. Thoenen and Y.-A. Barde, *Nature (London)*, 341 (1989) 149.
6 H. Thoenen and Y.-A. Barde, *Physiol. Rev.*, 60 (1980) 1284.
7 H. Thoenen, C. Bandtlow and R. Heuman, *Rev. Physiol. Biochem. Pharmacol.*, 109 (1987) 145.
8 R. M. Lindsay, H. Thooven and Y.-A. Barde, *Develop. Biol.*, 112 (1985) 319.

9 A. Hohn, J. Leibrock, K. Bailey and Y.-A. Barde, *Nature (London)*, 344 (1990) 339.
10 P. C. Maisonpierre, L. Belluscio, S. P. Squinto, H. Y. Ip, M. E. Furth, R. M. Lindsay and G. D. Yancopoulos, *Science (Washington, D.C.)*, 247 (1990) 1446.
11 F. Halböök, C. F. Ibañez and H. Persson, *Neuron*, 6 (1991) 845.
12 S. A. Cohen, S. Dong, K. Benedek and B. L. Karger, in I. M. Chaiken, M. Wilchek and I. Parikh (Editors), *Proceedings of the Fifth International Symposium on Affinity Chromatography and Biological Recognition*, Academic Press, New York, 1983, p. 479.
13 S. A. Cohen, K. P. Benedek, S. Dong, Y. Tapuhi and B. L. Karger, *Anal. Chem.*, 56 (1984) 217.
14 E. Watson and W. C. Kenney, *J. Chromatogr.*, 606 (1992) 165.
15 C. Acklin, K. Stoney, R. Rosenfeld, J. M. Miller and M. Haniu, *Anal. Biochem.*, (1992) in press.
16 G. Dollinger, B. Cunico, M. Kunitani, D. Johnson and R. J. Jones, *J. Chromatogr.*, 592 (1992) 215.
17 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
18 K. Benedek, S. Dong and B. L. Karger, *J. Chromatogr.*, 317 (1984) 227.
19 R. Rosenfeld, G.-M. Wu and K. Benedek, unpublished results.
20 T. Takagi, *J. Chromatogr.*, 506 (1990) 409.
21 S.-L. Wu, K. Benedek and B. L. Karger, *J. Chromatogr.*, 359 (1986) 3.
22 P. Oroszlán, R. Blanco, X.-M. Lu, D. Yarmush and B. L. Karger, *J. Chromatogr.*, 500 (1990) 481.
23 L. Nahri, R. Rosenfeld, T. Arakawa and K. Benedek, in preparation.
24 K. Benedek, *J. Chromatogr.*, submitted for publication.