Sodium Chloride Enhances the Storage and Conformational Stability of BDNF and PEG-BDNF

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Purpose. BDNF, a noncovalent homodimer, was modified by covalently attaching polyethylene glycol (PEG) with an average molecular weight of 20kDa to the N-terminal methionine. Stability of modified BDNF (PEG-BDNF) in aqueous solution was compared to BDNF after storage at elevated temperature in the presence and absence of NaCl.

Methods. SDS-PAGE, Light Scattering and Size Exclusion Chromatography were used to assess conformational stability and chemical degradation. In addition, CD spectroscopy was used to follow changes in secondary and tertiary structures upon thermal stress of the protein.

Results. NaCl containing formulations are more stable than NaCl-free formulations. In NaCl-free formulations, the main degradation product of BDNF and PEG-BDNF had a molecular weight of monomer that was more chemically degraded than the dimer. Additionally, the degradation of PEG-BDNF occurred at an accelerated rate compared to BDNF in NaCl-free environments.

Conclusions. The addition of NaCl to formulations enhances the shelf-life and conformational stability of both BDNF and PEG-BDNF.

KEY WORDS: BDNF; PEG-BDNF; stability.

INTRODUCTION

Brain derived neurotrophic factor (BDNF) is a noncovalent homodimer with a theoretical molecular weight of 27.2kDa (1). The protein exists as a dimer under physiologically relevant conditions (2) and thus the integrity of the dimer may be important in binding to its receptor (3). Enhancement of neuronal survival is associated with BDNF for specific nerve cell populations, including central nervous system connected motorneurons and retinal neurons (4,5). BDNF is a member of the neurotrophin family of growth factors that have the potential for treating a variety of neurological disorders.

The covalent attachment of polyethylene glycol (PEG) has been employed to extend the circulation time of a variety of proteins. For example, the 5 hour plasma circulation time of Interleukin-2 was improved to 16 hours after PEG modification (6). The thrombopoietic effect of IL-6 was enhanced ten-fold and the blood half-life increased from 3 minutes to 48 hours after linking PEG with approximately 50% of the available lysines (7). Since increasing the circulating half-life has been attributed to differences in size upon PEG conjugation (8), modification of a single lysine improves the clearance time through the covalent attachment of a high molecular weight PEG group. This has been demonstrated with Granulocyte Colony Stimulating Factor and other proteins, in which a single large PEG molecule attached to the N-terminal residue increases the circulation time with an accompanying prolongation of activity (9,10). The same technology has been applied to modify the N-terminal residue of each monomer subunit of BDNF (9). However, the effects on the solution stability of the native versus the modified (PEGylated) protein have not been determined.

In the current study, evidence is shown that NaCl appears to be a conformational stabilizer for both PEG-BDNF and BDNF. The mechanism for salt stabilizing the protein's conformation is not understood, but data suggests that dissociation is driven in the absence of salt and that the monomer state is less stable than the dimer state.

MATERIALS AND METHODS

All buffer salts were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma Co. (St. Louis, MO). The reagents were used without further purification. Recombinant human brain-derived neurotrophic factor was prepared as previously described (11). PEG-BDNF was prepared by the same procedure used to prepare BDNF, with the subsequent covalent attachment of PEG with an average molecular weight of 20 kDa at the N-terminus according to Kinstler, et.al. (9). HPLC grade solvents were obtained from Burdick and Jackson (Muskegon, MI). Trifluoroacetic acid (TFA) was obtained from Baker (Rockford, IL).

Size-Exclusion Chromatography and Kinetics

BDNF and PEG-BDNF were each dialyzed into three formulation buffers, all containing histidine at pH 7. The buffers were 10 mM histidine, (H7), 10 mM histidine with 5% sorbitol, (H7S) and 10 mM histidine with 150 mM NaCl (H7N). Samples were diluted with the appropriate buffer to the same final protein concentration of 2 mg/ml. All samples were then sterile filtered and aliquots set aside for kinetic timepoints. Sample aliquots were stored at 52°C and analyzed after 2, 5, 8, 11, 14, 17, and 21 days. Size-exclusion HPLC (SEC) was used to assess degradation on a Waters (Milford, MA) 625 system equipped with a single wavelenth detector (Model 486). A G2000SW$_{XL}$ column (7.8mm × 30cm, TosoHaas, Montgomeryville, PA) was used to analyze BDNF and a G3000SW$_{XL}$ column (7.8 mm × 30 cm) was used for PEG-BDNF. The SEC mobile-phase consisted of 100 mM sodium phosphate, 0.5 M NaCl, pH 6.9 for non-PEGylated BDNF and the same mobile-phase with the addition of 10% (v/v) ethanol for PEGylated-BDNF. Ethanol has previously been shown to enhance the resolution and recovery of PEGylated proteins during size-exclusion chromatography (12). Both

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ABBREVIATIONS: BDNF, Brain Derived Neurotrophic Factor; PEG, polyethylene glycol; NaCl, salt; TFA, trifluoroacetic acid; H7, 10 mM histidine; H7S, 10 mM histidine, 5% sorbitol; H7N, 10 mM histidine, 150 mM NaCl; H7N500, 10 mM histidine, 500 mM NaCl.

columns were run at a flow rate of 0.7 ml/min for 30 min and monitored for absorbance at 230 nm.

The initial rate of degradation for both BDNF and PEGylated BDNF was pseudo-first-order for the first eleven days. This was determined by plotting the natural log of the ratio of the percent dimer at time zero to the percent dimer at a given timepoint, versus time in days. Exponential curve fits were then used to calculate degradation rate constants.

SDS-PAGE

Fractions of PEG-BDNF that had been incubated at elevated temperatures were collected from size-exclusion and analyzed without dilution on SDS-PAGE gels. The gels were run using a discontinuous buffer system and stained with Coomassie Blue (13). PEG-BDNF starting material was diluted for a sample load of 5 μg per lane. BDNF fractions were collected from size exclusion and also loaded without dilution, while BDNF starting material was loaded at 15 μg per lane. SDS-PAGE was performed using Novex (San Diego, CA) 10–20% Tris-tricine gradient gels for BDNF and 4–20% Novex Tris-glycine gradient gels for PEG-BDNF.

Thermal-Induced Conformational Stability as Measured by Circular Dichroism (CD)

The thermal stability was assessed by following changes in the near- and far-UV CD spectra with temperature. Protein samples were diluted to 0.25 mg/ml with the appropriate buffer, and the change in ellipticity at 231 nm was followed as the sample was heated from 25°C to 95°C at a heating rate of 100°C/hour, using a Jasco J-720 spectropolarimeter (Spectroscopic Co., Ltd., Tokyo, Japan) equipped with a Peltier thermal cuvette holder. Rectangular thermal cuvettes with a path length of 0.1 cm were used. Samples in H7, H7S and H7N formulation buffers were tested, along with samples in a formulation buffer consisting of 10 mM histidine, 500 mM NaCl at pH 7 (H7N500).

Reversed-Phase HPLC

Reversed-phase HPLC was performed on a Hewlett-Packard (Wilmington, DE) 1090 HPLC system equipped with a diode-array detector and controlled by Chemstation Software. BDNF samples were analyzed on a YMC (Wilmington, NC) butyl-column (3mm × 15cm) equilibrated with 72% buffer A (HPLC grade water containing 0.1% TFA). The samples were eluted with a linear gradient of 0.24% buffer B/min (90% acetonitrile, 10% HPLC grade water and 0.1% TFA) from 5–30 minutes starting at a buffer B concentration of 28%. PEG-BDNF was analyzed on the same type of column used for BDNF equilibrated with 75% buffer B. Samples were eluted with a linear gradient of 1% buffer B/min, for 20 minutes. Elution was monitored at 215 nm at a flow rate of 0.2 ml/min.

Light Scattering

BDNF and PEG-BDNF were dialyzed into H7 and H7N formulation buffers. Samples were diluted with the appropriate buffer to 2 mg/ml, sterile filtered and stored for two-three weeks at 52°C in order to generate a sufficient amount of resolvable degradation products for light scattering analysis. Protein degradation was characterized using SEC columns as previously described in Materials and Methods, with the modification of two G2000 or G3000 SEC SW_{XL} columns linked in series for BDNF and PEGylated BDNF, respectively. The PEGylated BDNF mobile phase, as described earlier (see Materials and Methods, Size Exclusion Chromatography and Kinetics), was used for both proteins in the light scattering analysis. A Dawn DSP Laser Photometer (wavelength set at 488 nm) and Optilab DSP Interferometric Refractometer (Wyatt Technologies, Santa Barbara, CA) were used in series with a Hewlett Packard (Wilmington, DE) 1050 system equipped with a diode array detector and Chemstation software. The advantage of this approach is that absorbance and refractive index measurements can be made upon elution of the protein from the size-exclusion column. The dn/dc values were obtained through the use of Grams/32 software (Galactic Industries, Salem, NH) by adjusting the chromatograms to account for band broadening and changes in retention time upon delay volume differences between the refractive index and diode-array detector. Molecular weight calculations were obtained through the use of the Astra software package included with the laser light-scattering system. The extinction coefficients for native PEG-BDNF and BDNF were used in the determination of the dn/dc values with the exception of peak 2 (Fig. 1) for PEG-BDNF in H7N buffer. The extinction coefficient used to determine the dn/dc value for peak 2 (H7N

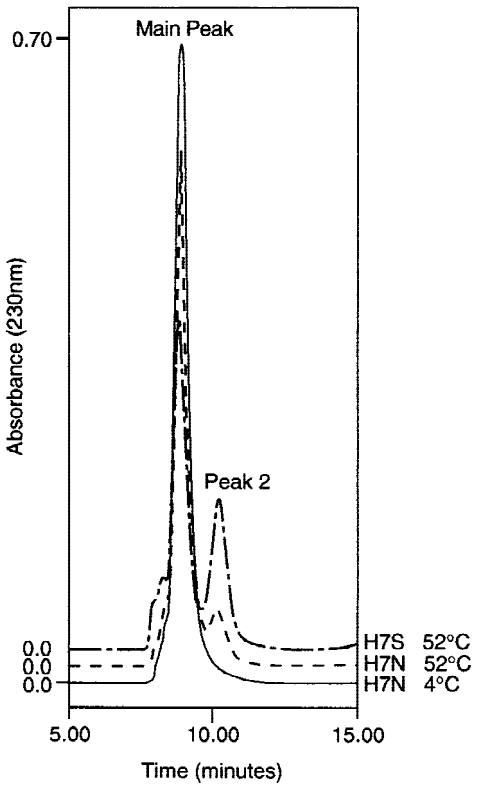

Fig. 1. Size-exclusion chromatography of PEGylated BDNF after storage in histidine buffer with sorbitol or NaCl. (—): Starting material in 10 mM histidine, pH 7.0, containing 150 mM NaCl. (– – –): After storage for 2 weeks at 52°C in 10mM histidine, pH 7.0, and 150 mM NaCl. (— · —): After storage for 2 weeks at 52°C in 10 mM histidine, pH 7.0, and 5% sorbitol.

buffer) was based on assuming that the degradation corresponds to the cleaved mono-PEG form of PEGylated BDNF.

RESULTS AND DISCUSSION

Characterization of PEG-BDNF Degradation Products in NaCl-Free Conditions

PEG-BDNF samples were analyzed by size-exclusion chromatography after storage at elevated temperature. Fig. 1 indicated that upon storage at 52°C, PEG-BDNF predominantly degraded to lower molecular weight species and into large aggregates (in excess of 700kDa, the exclusion limit of the column used). Further, degradation to lower molecular weight forms was greatest in NaCl-free formulations (see Fig. 1).

The PEG-BDNF species labeled as main peak and peak 2 for the H7S 52°C sample, in Fig. 1, were collected and analyzed by SDS-PAGE. The molecular weight of both species, as shown in Fig. 2A, were approximately 50kDa and represent the molecular weight of PEGylated-BDNF monomer (for comparison see PEG-BDNF starting material Fig. 2B, lane 2).

The formation of monomeric species is also indicated by the light scattering results. The PEGylated BDNF apparent monomer (peak 2, Fig. 1) had a molecular weight of approximately 31.9kDa (Table I) which is consistent with the theoretical molecular weight for PEG-BDNF monomer (33.1kDa). These results support the SDS-PAGE data and indicated that the noncovalent dimer of PEG-BDNF degrades to PEGylated BDNF monomer in NaCl-free formulations.

PEG-BDNF Undergoes Cleavage in NaCl-Containing Formulations

Peak 2 (Fig. 1), from the PEG-BDNF formulation with NaCl, was collected and analyzed by SDS-PAGE. This peak showed a significant amount of a lower molecular weight band at approximately 14kDa (see Fig. 2B, lane 4) that was equivalent in molecular weight to non-PEGylated BDNF monomer. The ratio of the density (as determined from a densitometric scan, data not shown) of the bands at approximately 50kDa versus 14kDa is 50:50.

The SDS-PAGE results suggested that unlike the PEG-BDNF material stored in sorbitol, the samples containing NaCl appeared to remain as a dimer, in which one of the monomer subunits has lost the PEG entity due to peptide cleavage in the N-terminal region. These results were confirmed through N-terminal sequencing, indicating cleavage between histidine-2 and serine-3 (of the linear sequence, data not shown) had ocurred in the 14kDa peptide.

Light scattering results confirmed the presence of a cleaved dimer. The average molecular weight of peak 2 (46.5kDa, Table I) in the H7N formulations is equivalent to the expected molecular weight of cleaved PEG-BDNF dimer (46.9 kDa, the expected molecular weight includes the removal of the Met-His cleavage and the addition of a single 20kDa PEG group to the dimer).

BDNF Degrades to Lower Molecular Weight Species in NaCl-Free Formulations

Figure 3 compares BDNF at time-zero and after two weeks incubation at elevated temperature in H7S buffer. As

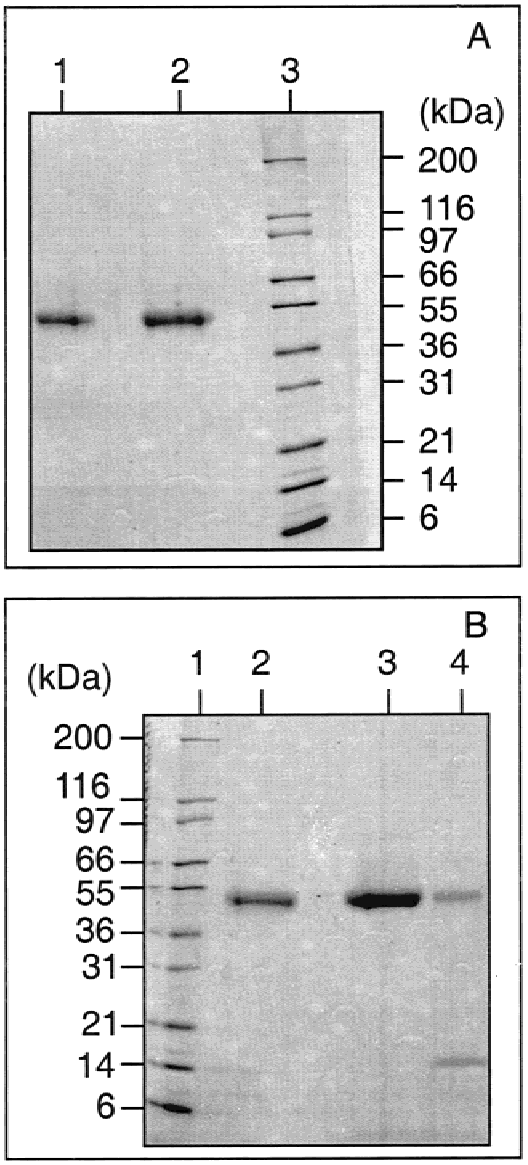

Fig. 2. Panel A, SDS-PAGE analysis of SEC fractions for PEGylated BDNF after elevated temperature storage. Lane 1, SEC main peak (noncovalent dimer, Main Peak in Fig. 1) after storage for 3 weeks at 52°C. Lane 2, SEC peak 2 (monomer, Peak 2 in Fig. 1) after storage for 3 weeks at 52°C in 10 mM histidine, pH 7.0, containing 5% sorbitol. Lane 3, Novex broad-range molecular weight standards. Panel B, SDS-PAGE analysis of SEC fractions for PEGylated BDNF after storage in NaCl. Lane 1, Novex broad-range molecular weight standards. Lane 2, PEGylated BDNF starting material. Lane 3, SEC main peak (noncovalent dimer, Main Peak in Fig. 1) after storage for 3 weeks at 52°C. Lane 4, SEC peak 2 (dimer, Peak 2 in Fig.1) after storage for 3 weeks at 52°C in 10 mM histidine, pH 7.0, containing 150 mM NaCl.

was observed with PEG-BDNF, BDNF in NaCl-free conditions degraded predominantly to lower molecular weight species and some higher molecular weight forms. This was illustrated in the inset of Figure 3, in which enhanced aggregation and the lower molecular weight peak (peak 2) was observed upon incubation of the samples. SDS-PAGE results (data not shown) for peak 2 from SEC also indicated a single band equivalent in molecular weight to monomeric BDNF.

Table I. Formulation Conditions (all at pH 7): H7; 10mM Histidine, H7N; 10mM Histidine, 150mM NaCl

Protein	Formulation	Average molecular weight (kDa)[a]	
		Main peak	Peak 2
BDNF	H7	28.5	16.9
	H7N[b]	28.4	—
PEG-BDNF	H7	63.5	31.9
	H7N	66.0	46.5

[a] After storage at 52°C for 2–3 weeks.
[b] Cleaved dimer degradation products were not resolved.

BDNF in NaCl-containing formulations did not degrade to the monomer upon evaluation of size-exclusion results (data not shown). The histidine-2 serine-3 cleavage product was not resolvable from the intact dimer, unlike the PEGylated-BDNF results (in NaCl formulations, see Fig. 1) in which lower molecular weight forms were separated.

Light scattering results (Table I) support the formation of lower molecular weight forms in H7 buffer. The average molecular weight for peak 2, at 16.9kD, is consistent with the theoretical molecular weight of BDNF monomer. As is indicated in Table I, lower molecular weight forms of BDNF in formulation buffer with NaCl were not resolvable upon light scattering analysis.

PEGylated BDNF Degrades Faster than Non-PEGylated BDNF in Salt-Free Conditions

The results of the accelerated degradation studies are presented in Fig. 4 for PEGylated BDNF versus non-PEGylated BDNF in the H7 formulation (the rates of degradation for the remaining formulations are presented in Table II). Fig. 4 represents the decrease in the main peak recovery as analyzed by size-exclusion HPLC.

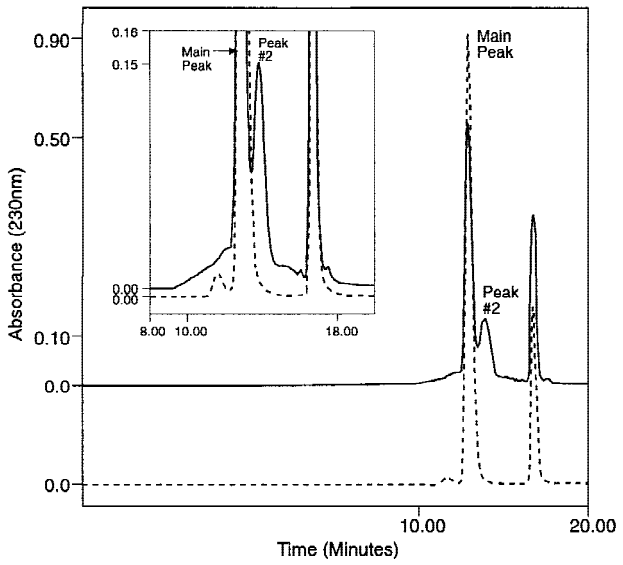

Fig. 3. Size-exclusion chromatography of BDNF after storage in NaCl-free formulation buffer. The dashed line represents BDNF at time zero and the solid line represents BDNF after storage for 2 weeks at 52°C in 10 mM histidine, pH 7.0, containing 5% sorbitol. Insert: Growth in aggregation, observed preceding the main peak, is shown for BDNF upon elevated temperature storage. The peak observed after the main peak at around 17 minutes arises from histidine in the formulation buffer.

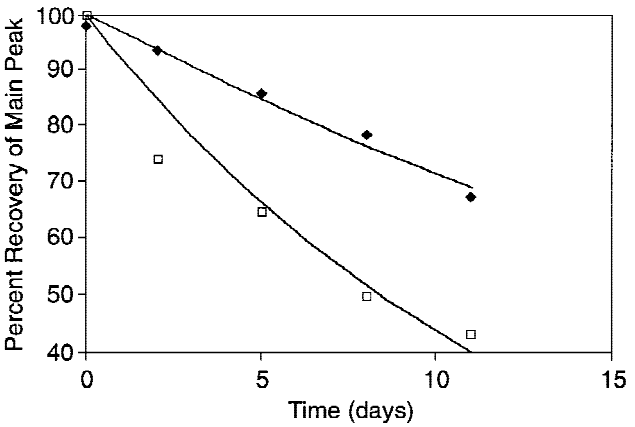

Fig. 4. The degradation kinetics of PEGylated BDNF (□) and BDNF (◆) in H7 buffer (10 mM histidine, pH 7.0) as determined from SEC. An exponential curve fit was used to calculate the rates of degradation using the equation $A = A_0 * e^{-kt}$, where k is the rate constant, A represents the percent dimer remaining at a given time (t) and A_0 is the percent dimer at time zero. Rates of degradation were calculated from nonlinear regression by least squares analysis and resulted in a k value of 0.033 day^{-1} for BDNF ($R^2 = 0.979$) and 0.082 day^{-1} for PEGylated BDNF ($R^2 = 0.986$). See Table II for a complete listing of the rates.

BDNF rates of degradation (Table II) were similar in the non-NaCl formulation buffers H7 and H7S. Inclusion of NaCl at low levels (150 mM) was sufficient to minimize degradation in both BDNF and PEGylated BDNF, although the BDNF cleavage degradation product was not resolvable from the main peak. As shown in Table II, the same trend was observed in the relative ranking of degradation rates for both proteins in NaCl-free conditions; i.e., the H7 formulation was less stable than in H7S. PEGylation does not change the degradation pathway but does have an impact on accelerating

Table II. Formulation Conditions (all at pH 7): H7; 10mM Histidine, H7S; 10mM Histidine, 5% Sorbitol, H7N; 10mM Histidine, 150mM NaCl

Protein	Formulation	Size exclusion degradation rate constant[a] (rate/day) @ 52°C	CD onset of melt (degrees Celsius)
BDNF	H7	0.033	70
	H7S	0.026	70
	H7N[b]	—	84
	H7N500[b]	—	90
PEG-BDNF	H7	0.082	72
	H7S	0.058	70
	H7N	0.014	84
	H7N500[c]	—	90

[a] Degradation was measured as the loss of dimer peak upon incubation at 52°C. The rate constant was determined assuming a pseudo-first order reaction.
[b] Degradation products of BDNF formulations with sodium chloride were not resolvable from the main peak.
[c] The SEC degradation rate constant was not determined.

degradation. This destabilizing effect upon PEGylating BDNF was not expected (14).

Addition of NaCl Increases the Conformational Stability of BDNF

A difference in the effect of NaCl on thermodynamic stability was illustrated by the onset of thermal melts, shown in Table II, as measured by CD spectroscopy. BDNF and PEG-BDNF showed structural changes at a lower temperature in NaCl-free environments. Changes at 231 nm in the BDNF spectrum have previously been demonstrated to correspond to changes in the tertiary structure of the protein (11,15). There is little change in the rest of the far-UV CD spectrum with heat, demonstrating that the majority of the secondary structure remained intact throughout this experiment (data not shown). All of the samples in the H7N and H7N500 (contains 500 mM NaCl instead of 150 mM) formulations had an onset of melt at 84°C and 90°C, respectively, while the H7S and H7 samples began melting at around 70°C (Table II). The addition of NaCl to sample buffers resulted in increased thermal stability, which is postulated to have beneficial consequences in improving the shelf-life stability of BDNF.

An explanation for this salt effect on thermal stability is not obvious. General preferential exclusion of salt from the protein surface, resulting in preferential hydration (originally proposed by Timasheff and Arakawa) could be a possibility (16,17). What is unusual with BDNF is the large increase in the onset of thermal melt (around 14°C) with low salt levels in physiologically relevant environments (150mM NaCl). In an effort to determine if NaCl is bound directly to BDNF, isothermal calorimetry was performed by titrating incremental NaCl into BDNF (data not shown). No specific binding was observed, suggesting the binding energy is weak and too small to measure calorimetrically.

Pegylation of BDNF Does Not Result in Increased Thermal Stability

Despite the doubling of rates of degradation between BDNF and PEG-BDNF in identical NaCl-free formulations (see Table 2), this does not translate to an appreciable difference in the observed conformational stability. This can be shown by examining the differences between BDNF and PEG-BDNF onset of melt temperatures for the same formulations. PEGylation did not change the onset of melting in H7 and H7S formulation buffers. Likewise, the onset of melt temperature did not vary between BDNF and PEG-BDNF in all of the salt formulations tested. Thus, attaching PEG to BDNF did not have an impact on the onset of melt in all formulation environments examined when compared to BDNF without PEG attachment. Since the full denaturation of the structure was not observed by temperature, but only the initial melting of tertiary structure, the impact of salt on the overall globular conformation was not determined.

In salt-free formulations, the strength of the dimer interface upon PEGylation may be an important consideration in understanding differences in stability compared to unPEGylated BDNF. Attaching PEG to BDNF could weaken the dimer interface, leading to increased rates of degradation.

The BDNF Monomer Is More Chemically Degraded than the Dimer

To examine if chemical degradative changes accompany dissociation in BDNF, the main peak and peak 2 (corresponding to BDNF monomer on SDS-PAGE) were collected after size-exclusion chromatography from the NaCl-free H7S sample after storage at 52°C (Figure 5A). The main peak and peak 2 fractions were reinjected on reversed-phase HPLC (Figure 5B). A comparison of the reinjected peaks showed that the main peak was reasonably intact, and coeluted with BDNF standard material that had not been degraded. The size-exclusion peak 2 on reversed-phase, however, showed substantial chemical degradative changes, seen as multiple unresolvable broad peaks before and after the main peak.

Thus, it appears that degradation of BDNF to an apparent monomer, induced by elevated temperature storage, has negative implications for the stability of this protein. It is not

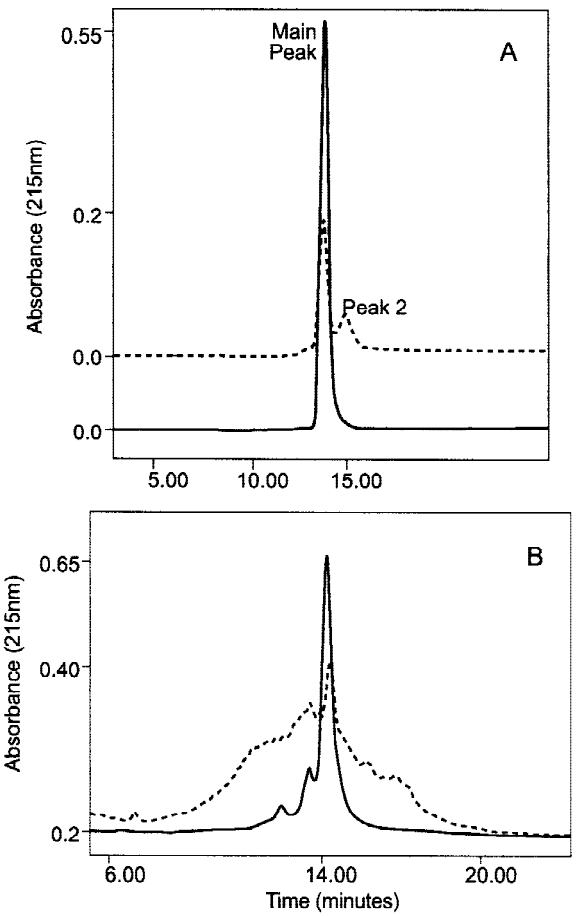

Fig. 5. Comparison of BDNF monomer and dimer SEC peaks upon reversed-phase reinjection. Panel A, Size-exclusion chromatography of BDNF in H7S and H7N buffer after storage at 52°C for 3 weeks. The dashed line represents BDNF in 10 mM histidine, pH 7.0, containing 5% sorbitol. The solid line represents BDNF in 10 mM histidine, pH 7.0, containing 150 mM NaCl showing the absence of the degraded monomer peak 2 seen in the formulation sample with sorbitol. Panel B, Reinjection of collected dimer and monomer peaks from SEC of BDNF in H7S buffer on a reversed- phase HPLC column. The solid line represents the main peak SEC fraction and the dashed line represents the peak 2 fraction from Panel A.

known, however, if dissociation precedes major chemical and physical changes of BDNF.

CONCLUSION

PEGylation of BDNF accelerated the rate of degradation, in all formulations examined, compared to the rate measured in non-PEGylated BDNF. When either protein was exposed to formulation conditions without NaCl, the protein underwent a different route of degradation that is more rapid. A comparison of onset of melts showed that the addition of NaCl to formulation buffers resulted in increased resistance to thermal denaturation; this was also supported by size-exclusion and light scattering results showing that the dimer state was maintained after elevated temperature storage in these conditions. The BDNF monomer degradation product, isolated after storage at elevated temperature, has been shown to be more chemically degraded than the dimer degradation product. Thus, the addition of NaCl in formulations minimized degradation and resulted in improved conformational and thermodynamic stability.

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