

Metal-Catalyzed Oxidation of Brain-Derived Neurotrophic Factor (BDNF): Analytical Challenges for the Identification of Modified Sites

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Purpose. We examined the metal-catalyzed oxidation of brain-derived neurotrophic factor (BDNF) using the Cu(II)/ascorbate/O₂ model oxidative system.

Methods. Electrospray ionization mass spectrometry, peptide mapping and amino acid analysis were utilized to determine the nature of the covalent modification induced by the metal-catalyzed oxidative system. Additionally, analytical ultracentrifugation, the Bradford assay, circular dichroism and ANSA dye-binding were used to determine the nature of any conformational changes induced by the oxidation.

Results. Exposure of BDNF to the Cu(II)/ascorbate/O₂ system led to the modification of ca. 35% of Met⁹² to its sulfoxide, and to subsequent conformational changes. The proteolytic digestion procedure was sensitive to this conformational change, and was unable to detect the modification. Chemical digestion with CNBr, however, was not sensitive to this change, and allowed for the identification of the site of modification.

Conclusions. The modification of Met⁹² to its sulfoxide rendered the oxidized BDNF inaccessible to proteolytic digestion, due to conformational changes associated with the oxidation.

KEY WORDS: brain-derived neurotrophic factor (BDNF); metal-catalyzed oxidation (MCO); cyanogen bromide (CNBr); methionine sulfoxide; quality control; protein conformation.

INTRODUCTION

Recent advances in biotechnology have led to the emergence of recombinant proteins as a significant class of therapeutics. The high degree of complexity of these molecules provides a myriad of possibilities for degradation during production,

storage and delivery. One major pathway for covalent post-translational modification of proteins is oxidation; in particular, metal-catalyzed oxidation constitutes a significant problem for protein pharmaceuticals (1–4). Metals are present as contaminants in water and buffers, and are used in various purification and refolding procedures during the production of recombinant proteins, e.g. metal-affinity chromatography (5–7). Transition metals can promote protein oxidation in the presence of prooxidants, oxygen or peroxides (2–4,8,9), which may lead to undesired loss of drug and/or enrichment of degradation products with unknown properties.

Detection and characterization of oxidative modifications are necessary to ensure quality control of the biotechnology product. Additionally, characterization of the modification is critical for achieving an understanding of the oxidation mechanism, so that rational strategies for avoiding such reactions can be developed. While a variety of analytical techniques are used for these purposes, the predominant technique for monitoring the integrity of protein pharmaceuticals is peptide mapping (10).

In this work, we examine the metal-catalyzed oxidation of a recombinant protein, brain-derived neurotrophic factor (BDNF), by the Cu(II)/ascorbate/O₂ system (general mechanisms of metal-catalyzed protein oxidation are described in reference 8). We will demonstrate the inability of peptide mapping to detect an oxidative modification of BDNF which occurs at significant levels (ca. 35% relative to total protein), and at lower prooxidant concentrations, specifically targets Met⁹².

MATERIALS AND METHODS

Materials

Recombinant BDNF was provided by Amgen, Inc., (Thousand Oaks, CA). The protein was exchanged into doubly distilled deionized water (dd. H₂O) via ultrafiltration using Microcon-3 microconcentrators (3 kD cutoff) from Amicon, Inc. (Beverly, MA). Typically, 500 µl of a 1 mg/ml BDNF solution in phosphate buffered saline (PBS), pH 7.4, was loaded onto a microconcentrator and centrifuged at 12,000 g for 50 minutes, followed by three washes with 300 µl dd. H₂O. The retentate was collected and the BDNF concentration determined by UV spectroscopy (Shimadzu UV-160 spectrophotometer; Kyoto, Japan) using an extinction coefficient of 1.76 cm²/mg at 280 nm. The BDNF solution was aliquotted in amounts of 270 µg/vial which were dried in a vacuum centrifuge (Labconco Centrivap Concentrator; Kansas City, Mo) and stored at –70°C for subsequent experiments. Sequencing grade endoproteinase Lys-C (Endo Lys-C) was obtained from Promega (Madison, WI). 8-Anilino-naphthalen-1-sulfonic acid (ANSA) was obtained from Sigma (St. Louis, MO). For amino acid analysis, 4N methanesulfonic acid, phenyl isothiocyanate and Amino Acid Standard H were from Pierce (Rockford, IL) and ACS grade NaOH (filtered prior to use) was from Fisher (Pittsburgh, PA). Formic acid (99%) was from Fluka (Ronkonkoma, NY), and cyanogen bromide (CNBr), 99.995%, was from Aldrich (Milwaukee, WI). Experiments with CNBr were performed in a fume hood with proper safety attire, including gloves, goggles and a filtered mask. Waste from these experiments was disposed of in a manner appropriate for hazardous chemicals. All other reagents were of the highest grade commercially available.

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ABBREVIATIONS: BDNF, brain-derived neurotrophic factor; Endo Lys-C, endoproteinase Lys-C; ANSA, 8-anilino-naphthalene-1-sulfonic acid; CNBr, cyanogen bromide; EDTA, ethylenediaminetetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption ionization—time of flight mass spectrometry; HPLC/ESI-MS, reversed phase HPLC coupled on-line to ESI-MS; SEC, size exclusion chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CD, circular dichroism; DSC, differential scanning calorimetry; a.m.u., atomic mass units; SDS, sodium dodecyl sulfate.

Reaction Conditions

Oxidation reactions were conducted on samples containing 20 μM BDNF, a concentration at which BDNF exists entirely as a dimer (11). Unless noted otherwise, the oxidation of BDNF by the Cu(II)/ascorbate/O₂ system was conducted under the following conditions: 20 μM BDNF/20 μM CuCl₂/2 mM ascorbate. Reactions were run in a volume of one milliliter in 20 mM phosphate buffer, pH 7.4, and were incubated for one hour at 25°C. The reagents were added in the following order: buffer, BDNF, CuCl₂ and ascorbate. After addition of CuCl₂, the mixture was incubated for five minutes at 25°C to promote metal binding to the protein. The reaction was then started by the addition of ascorbate. For control samples, dd. H₂O was added in place of ascorbate. In some cases, oxidation was stopped by the addition of a final concentration of 20 μM EDTA. All solutions were made with dd. H₂O. The phosphate buffer stock solution was Chelex-treated (5 g Chelex to 100 ml buffer solution; stirred for 1 hour, then filtered) to minimize metal contamination. Stock solutions of CuCl₂ and ascorbate were freshly prepared prior to the reactions.

Electrospray Ionization Mass Spectrometry (ESI-MS)

Samples were prepared for ESI-MS analysis by acetone-HCl precipitation (12), followed by dissolution in dd. H₂O prior to analysis. ESI-MS experiments were performed on an Autospec-Q tandem hybrid mass spectrometer (VG Analytical Ltd., Manchester, UK), equipped with a Mark III ESI source and an OPUS data system. Samples were trapped and desalted prior to ESI by loading onto a trapping column (1.5 cm \times 1 mm of polymeric beads with 400 Å pores, Michrom BioResources, Auburn, CA) with 0.1% acetic acid at 250 $\mu\text{l}/\text{min}$ and eluting the retained sample into the ESI source with 70% methanol/30% H₂O with 0.1% acetic acid at 10 $\mu\text{l}/\text{min}$.

Amino Acid Analysis

Hydrolyses were performed on a Waters PICO•TAG Work Station (Waters, Milford, MA). Samples were prepared for amino acid analysis by acetone-HCl precipitation (12), and were dissolved in formic acid prior to hydrolysis. Vapor-phase HCl hydrolysis was performed with 6 N HCl containing 0.1% (w/v) phenol at 110°C for 24 hours. This method causes loss of Trp residues (12) and can cause conversion of methionine sulfoxide, Met(O), back to methionine (Met) (13–15). To quantitate Trp and Met (O), hydrolysis was also performed with 4 N methanesulfonic acid, which can readily determine Trp, Met and Met(O) residues (16). Hydrolysis and neutralization were conducted following the method of Simpson et al. (16). Briefly, protein samples were hydrolyzed with 4 N methanesulfonic acid at 115°C for 22 hours, then neutralized with 4 N NaOH prior to derivatization. Hydrolyzed amino acids from both procedures were derivatized with phenyl isothiocyanate and separated on a Waters Spherisorb S50DS2 column (4.6 \times 250 mm) (Waters, Milford, MA) at 45°C and 0.8 ml/min, using UV detection at 254 nm. For complementary analysis, samples were also sent to Commonwealth Biotechnologies, Inc. (Richmond, VA) for analysis by alkaline hydrolysis with NaOH.

Proteolytic Digestion

Samples were prepared for proteolytic digestion by ultrafiltration (three dd. H₂O rinses at 12,000 g for 50 minutes using

Microcon-3 microconcentrators) and vacuum centrifugation. Approximately 270 μg BDNF was dissolved in 0.2 M Tris buffer, pH 8.5, containing 6 M guanidine-HCl and 1 mM EDTA. 20 μl of 0.1 M dithiothreitol was added and the sample was incubated for one hour at 45°C. After cooling to room temperature, 40 μl of 0.1 M iodoacetic acid was added and the sample was placed in the dark for 30 minutes to allow for carboxymethylation of the cysteine residues. The samples were then desalted by extensive ultrafiltration using Microcon-3 microconcentrators and dried by vacuum centrifugation. The reduced and alkylated BDNF was reconstituted in 0.15 M ammonium bicarbonate buffer containing 2.2 M urea. 1.5% (w/w) Endo Lys-C was added and the mixture was incubated for 12 hours at 37°C. At this point, an additional 0.75% (w/w) Endo Lys-C was added and the mixture incubated for an additional 4 hours at 37°C. The primary structure of BDNF and the Endo Lys-C cleavage sites are shown in Fig. 1. This protease was found to give the best cleavage pattern for BDNF, and the most reproducible digestion.

Chemical Digestion

Samples were prepared for chemical digestion by acetone-HCl precipitation. Approximately 270 μg BDNF was dissolved in 900 μl of 70% formic acid. Cyanogen bromide was prepared as a 1 M solution in 70% formic acid, and 100 μl added to the BDNF in formic acid. The sample (in a glass vial covered with aluminum foil) was incubated overnight at room temperature, then vacuum dried. After digestion, samples were denatured, reduced and alkylated as in the proteolytic digestion procedure: samples were dissolved in 0.2 M Tris buffer, pH 8.5, containing 6 M guanidine-HCl and 1 mM EDTA. 20 μl of 0.1 M dithiothreitol was added and the samples were incubated for one hour at 45°C. After cooling to room temperature, 40 μl of 0.1 M iodoacetic acid was added and the samples were placed in the dark for 30 minutes.

Peptide Mapping by RP-HPLC

Analysis of peptide fragments from the proteolytic digestions of BDNF was performed on a Vydac C18 column (4.6 \times 250 mm) (Vydac, Hesperia, CA) at 60°C and 0.7 ml/min, using UV detection at 215 nm. Typically, 1.5 nmol of protein was injected onto the column. A gradient from 0% to 50% acetonitrile with 0.1% trifluoroacetic acid over 70 minutes was employed for the separation and elution of the peptide fragments. Satisfactory signal-to-noise ratios for the separated fragments were achieved for injections of 0.5 nmol protein and above. Samples which had been digested with CNBr and subsequently denatured, reduced and alkylated were desalted on the Vydac C18 column. The resulting fragments were collected together and submitted as a mixture for analysis by ESI-MS.

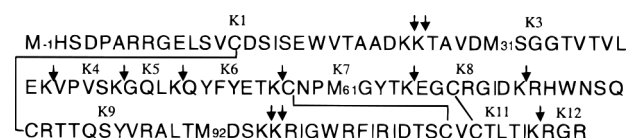


Fig. 1. Primary structure of BDNF. Three disulfide bonds exist: Cys¹³ (S-S-)Cys⁸⁰, Cys⁵⁸(-S-S-)Cys¹⁰⁹ and Cys⁶⁸(-S-S-)Cys¹¹¹. Endo Lys-C cleavage sites are indicated with arrows, and the positions of the four Met residues are indicated.

MALDI-TOF Mass Spectrometry

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra were obtained on a Hewlett Packard model G2025A (Hewlett Packard, Palo Alto, CA), which typically measures molecular masses below 3 kDa with an accuracy of at least 0.1%. Peaks from the peptide map were collected and vacuum centrifuged to dryness. Samples were reconstituted in acetonitrile/isopropanol/0.1% trifluoroacetic acid (1:3:2 (v/v/v)). The matrix used was a standard solution of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) in acetonitrile/H₂O (1:1 (v/v)).

HPLC/ESI-MS

RP-HPLC coupled on-line to ESI mass spectrometry was used to analyze the peptide maps. The reverse-phase assay for peptide mapping was optimized at a flow rate of 50 μ l/min on a 1 \times 250 mm Vydac C18 column (Vydac, Hesperia, CA), which was coupled to the Autospec-Q tandem hybrid mass spectrometer. The column effluent was monitored with UV detection at 215 nm, and post UV detector, 8 μ l/min were split off into the ESI source. Mass spectral data were collected and analyzed on the OPUS data system.

Analytical Ultracentrifugation

Sedimentation equilibrium and velocity experiments were performed on a Beckman XL-1 analytical ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA), equipped with an eight cell rotor. Samples were dialyzed extensively against 10 mM sodium phosphate buffer, pH 7.4, at 4°C prior to centrifugation. For the sedimentation equilibrium experiments, samples were diluted with 10 mM sodium phosphate buffer, pH 7.4 to concentrations of 5, 10 and 20 μ M. Samples were centrifuged at three different speeds (15,000, 20,000 and 27,000 rpm) for 24 hours at 20°C. Equilibrium was confirmed by monitoring the UV absorbance at 229 nm, measured every 5 hours. Raw data were imported into the software program Kdalton (developed by John Philo, Amgen) for analysis via nonlinear curve fitting. For sedimentation velocity experiments, samples of 20 μ M BDNF were equilibrated to 25°C and centrifuged at 40,000 rpm. Sedimentation was monitored using UV absorbance at 229 nm. The data generated was fit to a single species model using the modified Fujita-MacCosham function in the software program SVEDBERG, version 6.10 (developed by John Philo, Amgen).

Protein Concentration

Soluble protein concentration was determined using the Coomassie® Plus Protein Assay Reagent from Pierce (Rockford, IL), which is a modification of the Bradford method (17). Sample analysis involved the addition of the Coomassie dye to the protein solution, with subsequent measurement of absorbance at 595 nm on a Shimadzu UV-160 spectrophotometer (Shimadzu, Kyoto, Japan).

Circular Dichroism Spectroscopy

Samples were prepared for CD experiments by ultrafiltration (three dd. H₂O rinses at 12,000 g for 50 minutes using

Microcon-3 microconcentrators). Far-UV CD spectra (185–260 nm) were obtained on a Jasco J-720 spectropolarimeter (Jasco, Easton, MD), using a 0.1 cm path length quartz cell. The spectra were recorded at 25°C at a concentration of approximately 100 μ g/ml. Measurements were taken at intervals of 0.5 nm with a speed of 20 nm/min, and were averaged over 3 accumulated scans.

Fluorescence Spectroscopy of 8-Anilino-naphthalene-1-sulfonic Acid (ANSA)

Fluorescence spectra were obtained using a Photon Technology International (Monmouth Junction, NJ) spectrofluorometer (Quanta Master Luminescence Spectrometer, QM1). ANSA (100 μ M) containing 20 μ M EDTA, was incubated with 10 μ M BDNF (from either an oxidized or control sample) in 20 mM sodium phosphate buffer, pH 7.4, at 37°C for 30 minutes. The fluorescence emission spectra (excitation, 370 nm) were monitored between 400 and 600 nm. Binding of ANSA to protein was determined by subtracting the emission spectrum of ANSA from that of ANSA in the presence of BDNF.

RESULTS

Mass Spectrometry

ESI-MS experiments were performed to determine if exposure to the Cu(II)/ascorbate/O₂ system caused any covalent modification of BDNF. The spectrum in Fig. 2a reveals a significant amount of product (ca. 35% of total protein) with a molecular weight of 13652 ± 1 , i.e. 16 a.m.u. higher than that of the

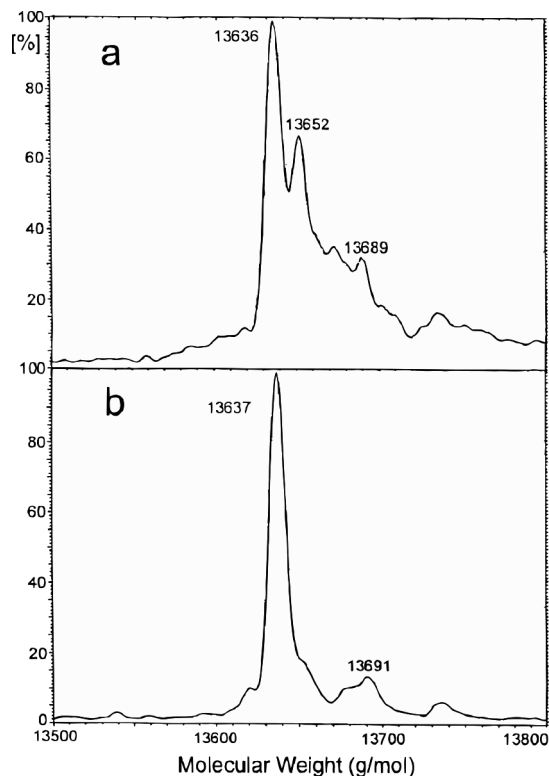


Fig. 2. ESI-MS spectra for oxidized and control BDNF. (a) Oxidized; (b) control.

native protein (M.W. = 13636), for the sample exposed to 20 μM CuCl_2 and 2 mM ascorbate. This modified species is absent in the control sample (Fig. 2b). The existence of an $M + 16$ peak in the spectrum of the oxidized sample is indicative of BDNF modification via a single oxygen addition.

We investigated the possibility that the oxidation seen in the ESI-MS data was an artifact of the procedure itself, as it has been reported that oxidation of analytes can occur during ionization in the mass spectrometer (18). Although acetone-HCl precipitation of samples was performed prior to ESI-MS analysis, it is possible that minute amounts of ascorbic acid and CuCl_2 remained in the sample and promoted oxidation during ionization. In order to rule out this possibility, a control experiment was performed. A sample containing 20 μM BDNF and 20 μM CuCl_2 in 20 mM phosphate buffer was prepared and incubated at room temperature for one hour, after which an acetone-HCl precipitation was performed. Immediately prior to analysis by ESI-MS, a solution of 2 mM ascorbic acid was added to the sample. No evidence for oxidation of the BDNF was seen under these conditions (data not shown). Clearly the +16 modification seen in the oxidized BDNF sample did not occur during ionization in the mass spectrometer.

Amino Acid Analysis

Results from HCl hydrolysis of oxidized BDNF and its control revealed no change in the content of any of the amino acid residues. One amino acid particularly sensitive to metal-catalyzed oxidation is His (2,3,8). In order to control whether HCl hydrolysis would have indicated the potential loss of His from BDNF, we subjected BDNF to more extensive oxidation by 40 μM Cu(II) and 4 mM ascorbate. Subsequent to HCl hydrolysis, amino acid analysis indicated a loss of up to 63% of His (relative to the initial content). Hence, our experimental method would have indicated the loss of His if it had occurred under our original reaction conditions employing 20 μM Cu(II) and 2 mM ascorbate. After oxidation by 20 μM Cu(II) and 2 mM ascorbate, methanesulfonic acid and NaOH hydrolysis revealed some loss of Met (ca. 5%), and the formation of Met(O) (ca. 16% relative to the original content of Met). These results do not quantitatively agree with those from ESI-MS analysis; however, they do provide qualitative identification of the modified amino acid residues and indicate that Met oxidation is the only detectable chemical modification.

Proteolytic Digest

In order to localize the chemical modification to a particular sequence in the protein, oxidized BDNF was subjected to proteolytic digestion, followed by RP-HPLC separation of the proteolytic fragments. All fragments of the native protein, except fragment K12 (Arg¹¹⁷-Arg¹¹⁹), were easily identified via on-line ESI-MS or peak collection followed by MALDI-TOF MS. The peak assignments are given in Fig. 3. Neither procedure showed evidence for a fragment being 16 a.m.u. heavier than the identified native sequences of BDNF for the oxidized sample.

We investigated the possibility that the modified Met residue (Met(O)) in oxidized BDNF was reduced during sample preparation procedures prior to the enzymatic digestion. A sample which was exposed to the $\text{Cu(II)/ascorbate/O}_2$ system was

denatured, reduced and alkylated as described in the Experimental section. The sample was then prepared for ESI-MS experiments via ultrafiltration, followed by an acetone-HCl precipitation. ESI-MS spectra clearly showed oxidized carboxymethylated BDNF (M.W. = 14006) at a level of ca. 35% of total protein, similar to that seen for BDNF exposed to metal-catalyzed oxidation without further reduction and alkylation. The observed molecular mass indicated that the oxidized BDNF was completely reduced and alkylated at all six cysteine residues. This control effectively eliminates the possibility that the +16 modification on the oxidized BDNF was reduced during sample preparation for enzymatic digestion.

A discrepancy exists between ESI-MS, which showed clear evidence for oxidized BDNF, and proteolytic mapping, which could not resolve any product peaks. A possible rationale for this discrepancy is that oxidized BDNF is not accessible to Endo Lys-C for proteolytic digestion due to aggregation and/or conformational changes, or likewise, oxidatively modified proteolytic fragments may undergo aggregation or adhesion to surfaces. A range of analytical techniques was employed to determine the nature of any potential structural changes induced by the oxidation of BDNF, as described below.

Analytical Ultracentrifugation

The possibility of aggregation beyond the native dimer was investigated by analytical ultracentrifugation. Results from sedimentation equilibrium experiments revealed that BDNF existed as dimer under the conditions used for the oxidation reactions, and also showed that BDNF remained entirely in the dimer form after oxidative modification. Data from sedimentation velocity experiments confirmed the existence of a single species in all samples, with molecular weights corresponding to that of the dimer. No evidence for the presence of multimers was seen by either ultracentrifugation technique, indicating that metal-catalyzed oxidation of BDNF did not induce further aggregation.

Determination of Soluble Protein

The possible loss of soluble protein was investigated using a modified Bradford assay. No loss of soluble protein occurred upon oxidation of BDNF.

Circular Dichroism

Protein secondary structure can be assessed through the use of far-UV CD spectroscopy. Figure 4 compares the CD spectra of control and oxidized BDNF. Native BDNF has been estimated to consist of ca. 74% β -sheet and 21% β -turn structure, based on deconvolution analysis of the circular dichroism spectrum (11). The broad negative signal between 190 and 220 nm has been attributed to a combination of β -sheet and β -turn structure (11,19,20), while the distinctive peak at 232 nm has been attributed to both tertiary structural elements (20,21) and a combination of secondary β -structure elements (20). Oxidized BDNF did not show a significant change in the peak at 232 nm. However, a slight increase was seen in the magnitude of the negative peak at 190–220 nm for the oxidized sample relative to the control, suggesting an increase in β -structure.

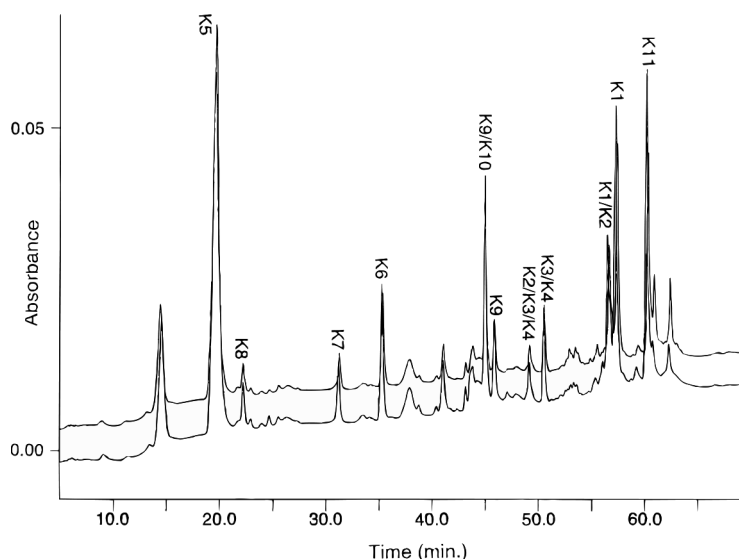


Fig. 3. Endo Lys-C maps of oxidized (upper) and control (lower) BDNF, monitored by RP-HPLC at 215 nm.

ANSA Binding

ANSA is one of a class of fluorescent probes that has been used to study the surface hydrophobicity of proteins (22,23). It was thought that the intense fluorescence exhibited by the dye in the presence of proteins was due to hydrophobic interactions, i.e. binding, between the naphthalene moiety and exposed hydrophobic sites on proteins. Recent work, however, has shown that ANSA binding is actually dominated by electrostatic forces between the sulfonate group of ANSA and exposed cationic groups on the protein (24,25). Furthermore, ANSA fluorescence upon binding to a protein depends on many variables, of which hydrophobicity is only one. Nonetheless, binding and subsequent fluorescence of ANSA can indicate changes in overall structure of the protein molecule. Here, ANSA binding was used to determine if any changes in 3° structure were induced upon oxidation. Figure 5 shows the fluorescence spectra of ANSA in the presence of non-oxidized and oxidized BDNF. The increase in ANSA fluorescence intensity suggests an oxidation-dependent conformational change of BDNF resulting in either exposure of additional sites to which ANSA can bind,

or a change in the local environment of the bound ANSA which enhances its fluorescence. Derivative analysis of the spectra revealed a blue shift of ca. 10 nm in the peak maximum upon metal-catalyzed oxidation, indicating that the ANSA is located in a more apolar or hydrophobic region in the oxidized BDNF than in the control BDNF. Although ANSA may induce further conformational changes upon binding to the protein (induced-fit binding model) (24,26), it is evident that metal-catalyzed oxidation of BDNF results in conformational changes which promote ANSA binding.

Alternate Approaches Using Denaturants

Clearly BDNF experienced structural modifications upon oxidation by the Cu(II)/ascorbate/O₂ oxidative system. Oxidized BDNF may exist in a conformation which is not fully accessible to Endo Lys-C for proteolytic digestion. Two approaches were taken to overcome this problem. One approach involved increasing the urea level to 4 M during the digestion procedure, in an effort to unfold the protein to a greater extent.

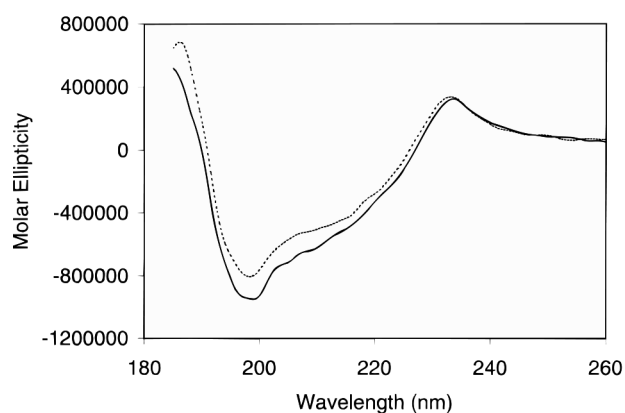


Fig. 4. Far-UV CD spectra of oxidized (---) and control (—) BDNF. (Molar ellipticity: $[\Theta]$, dec cm² dmol⁻¹).

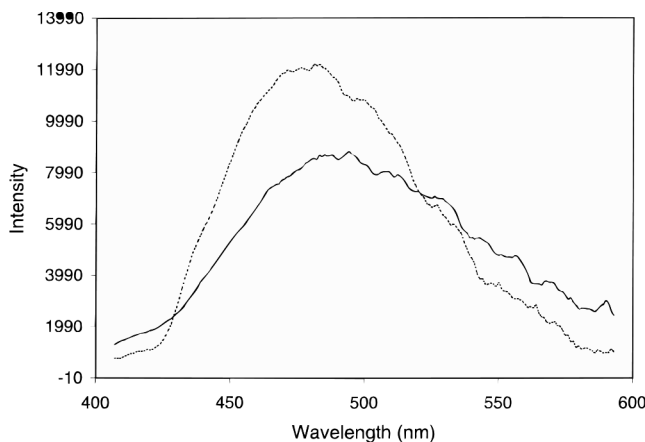


Fig. 5. Fluorescence spectra of ANSA in the presence of oxidized (---) and control (—) BDNF.

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