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Part I

Guest Editor

S.M. LeVine

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METAL-CATALYZED OXIDATION OF BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF): SELECTIVITY AND CONFORMATIONAL CONSEQUENCES OF HISTIDINE MODIFICATION

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Abstract - We have studied the metal-catalyzed oxidation (MCO) of brain-derived neurotrophic factor (BDNF) with regard to target sites and potential conformational changes of the protein. The exposure of BDNF to three different levels of ascorbate/Cu(II)/O_2 [20 μM Cu(II), 2 mM ascorbate (level 1); 20 μM Cu(II), 4 mM ascorbate (level 2); 40 μM Cu(II), 4 mM ascorbate (level 3)], chosen based on the extent of chemical modification of Met and His, respectively, resulted in the exclusive oxidation of a buried Met residue, Met^{92}, at level 1 but in the predominant oxidation of His at level 3. His modification had a significant impact on the structure of BDNF, as quantified by CD and ANSA fluorescence measurements, while Met oxidation had not, also assessed through complementary oxidation of BDNF through hydrogen peroxide. Our ultimate objective was the correlation of the surface exposure of an oxidized His residue in a protein with potential effects on the conformational integrity of the oxidized protein. In a series of three proteins, human growth hormone (hGH), human relaxin (hRlx), and BDNF, we have now observed that His oxidation is paralleled by significant conformational changes when the target His residue is more surface exposed (hRlx, BDNF) while conformational consequences of His modification are less significant when the target His residues are more buried in the interior of the protein (hGH).

Key words: Brain-derived neurotrophic factor (BDNF), metal-catalyzed oxidation (MCO), histidine and methionine oxidation, protein conformation

Abbreviations: **a.m.u.**: atomic mass units; **ANSA**: 8-anilinonaphthalene-1-sulfonic acid; **BDNF**: brain-derived neurotrophic factor; **CD**: circular dichroism; **EDTA**: ethylenediaminetetraacetic acid; **Endo Lys-C**: endoproteinase Lys-C; **ESI-MS**: electrospray ionization mass spectrometry; **HPLC/ESI-MS**: reversed phase HPLC coupled on-line to ESI-MS; **MALDI-TOF MS**: matrix-assisted laser desorption ionization - time of flight mass spectrometry; **MCO**: metal-catalyzed oxidation; **SASA**: solvent accessible surface area; **SDS**: sodium dodecyl sulfate; **SDS-PAGE**: sodium dodecyl sulfate polyacrylamide gel electrophoresis

INTRODUCTION

Redox active transition metals can catalyze the oxidation of proteins in the presence of electron donors (prooxidants), oxygen and/or peroxides (Levine, 1983a, 1983b; Farber and Levine, 1986; Rivett and Levine, 1990; Stadtman, 1990; Li *et al.*, 1995; Schöneich *et al.*, 1997; Zhao *et al.*, 1997). Such metal-catalyzed oxidation (MCO) affects specifically amino acids involved in metal binding or located in

close vicinity to metal binding sites. Often, the MCO of proteins leads to the loss of function and/or conformational changes. Biologically important prooxidants include ascorbate, thiols and hydroquinones (Miller *et al.*, 1990). Their prooxidant activity results from the facile reduction of redox-active transition metals. The reduced transition metals then react with molecular oxygen to generate a series of reactive oxygen species such as superoxide, hydrogen peroxide, and, potentially, hydroxyl radicals (or their metal-bound equivalents) (Miller *et al.*, 1990; Yamazaki and Piette, 1991).

Under normal physiological conditions, cellular levels of "free" redox-active transition metals such as Cu and Fe are very low (Lippard, 1999; Rae *et al.*, 1999). However, certain pathological conditions are associated with elevated concentrations of redox-active transition metals. For example, brain tissue of Alzheimer's disease patients was shown to contain higher levels of redox-active Fe (Smith *et al.*, 1997). Increasing evidence is also mounting that the progression of amyotrophic lateral sclerosis (ALS) is caused by conformationally unstable SOD1 mutants containing a Cu center which not only reacts with superoxide but also generates superoxide through the reaction with prooxidants such as ascorbate (Estévez *et al.*, 1999). Moreover, these SOD1 mutants may ultimately lose Cu to the environment. Hence, pathological conditions may promote the formation of a pool of redox-active transition metals available for binding to and reaction with biomolecules.

The extent to which MCO can affect proteins will depend on the affinity for specific transition metals and the impact of oxidation on the integrity of the proteins. In our laboratory, we specifically focus on structure-reactivity relationships of protein oxidation and the question of how the oxidation of individual amino acids affects protein conformation. Recently, we observed that the Cu-catalyzed oxidation of His in two different proteins, human relaxin (hRlx) and human growth hormone (hGH), had a significantly different impact on these proteins. While hRlx experienced extensive structural changes, ultimately leading to non-covalent aggregation and a pH-dependent precipitation (Li *et al.*, 1995), such characteristics were not observed for hGH (Zhao *et al.*, 1997). A possible rationale for this contrasting behavior is the position of the oxidation-sensitive His residue with regard to the tertiary structure of the protein, buried in hGH but more solvent exposed in Rlx. These observations suggest that the level of solvent exposure may be one parameter controlling the propensity for His oxidation to cause structural changes in the target protein. In order to fully rationalize the biological consequences of protein oxidation *in vivo* it is important that we understand not only the underlying mechanisms but also the parameters which determine the selectivity and conformational impacts of oxidative modifications.

In the present paper, we have extended our studies to the Cu-catalyzed oxidation of an additional model protein, brain-derived neurotrophic factor (BDNF), in an effort to further elucidate the relationship between the location of target His residues and His oxidation on the integrity of higher-order protein structure. BDNF belongs to the family of neurotrophins which are important for neuronal survival and extracellular control of development and maintainance of neurons (Hofer and Barde, 1988; Robinson *et al.*, 1995; Schuman, 1997; Estévez *et al.*, 1999). Although BDNF does not contain a specific metal-binding site, it contains two highly surface-exposed His residues, located at positions 1 and 75, displayed in fig. 1 (Leibrock *et al.*, 1989; Rosenthal *et al.*, 1991; Acklin *et al.*, 1993). In order to separate the effects of His oxidation from Met oxidation, we compared BDNF exposed to MCO and hydrogen peroxide at three different levels, selected such that the overall yield of oxidized BDNF by both systems was comparable. The following concentrations of ascorbate and Cu(II) were adjusted for MCO: 20 μM Cu(II), 2 mM ascorbate (level 1); 20 μM Cu(II), 4 mM ascorbate (level 2); 40 μM Cu(II), 4 mM ascorbate (level 3). In all cases, these levels of reactants are significantly higher compared to what would be found *in vivo* (typical physiological levels of ascorbate are on the order of a few μM; Rose and Bode, 1993). However, these higher concentrations were selected in order to perform the experiments in a reasonable time.

MATERIALS AND METHODS

Materials

Recombinant BDNF was provided by Amgen (Thousand Oaks, CA). The protein was exchanged into doubly distilled deionized water (dd. H_2O) via ultrafiltration using Microcon-3 microconcentrators (3 kDa cutoff) from Amicon (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 min., followed by three washes with 300 µl dd. H_2O. 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 cm^2/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-Anilinonaphthalene-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), ACS grade NaOH (filtered prior to use) was from Fisher (Pittsburgh, PA) and formic acid (99%) was from Fluka (Ronkonkoma, NY).

Calculations of solvent exposure

Solvent exposure was determined by calculating the solvent accessible surface area (SASA) for each His side chain. The SASA was defined by the center of a spherical probe of a given radius as it rolled over the surface defined by atomic van der Waals radii of the molecule. We used the program NACCESS 2.1.1 (Department of Chemistry and Molecular Biology, University College, London, UK) to calculate atomic accessible surface areas corresponding to a probe of radius 1.4 Å using the Lee and Richards' algorithm (Lee and Richards, 1971). Calculations were performed on an SGI INDIGO2 workstation (SGI, Mountain View, CA). Molecular structures were taken directly from Protein Data Bank files: 1HGU for human growth hormone, 6RLX for relaxin and 1BND for BDNF. The relative SASAs for His were calculated based on an extended Ala-His-Ala peptide as the reference point for 100% solvent exposure.

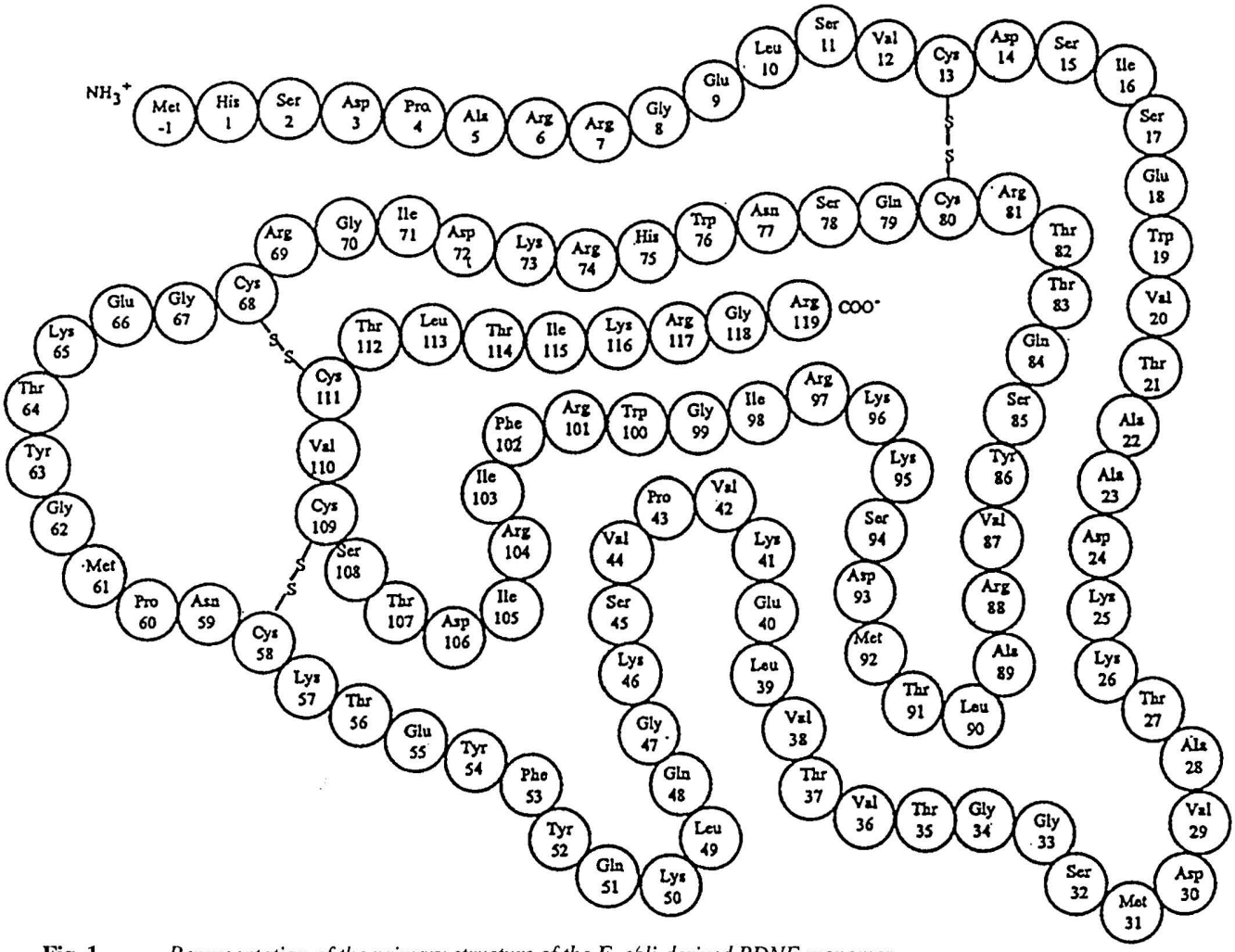

Fig. 1 *Representation of the primary structure of the E. coli-derived BDNF monomer*

Reaction conditions
Oxidation reactions were conducted in aqueous phosphate buffer, pH 7.4, containing 20 μM BDNF which, at this concentration exists entirely as dimer (Radziejewski *et al.*, 1992). The oxidation of 20 μM BDNF by Cu(II)/ascorbate/O_2 and hydrogen peroxide (H_2O_2) was performed at three different levels of reactant concentrations, as listed in table 1. These reaction conditions were chosen to obtain different extents of conversion of His and Met by MCO, and comparable extents of Met oxidation alone by H_2O_2. For MCO, 1-ml solutions were incubated for one hr. at 25°C. The reagents were added in the following order: buffer, BDNF, $CuCl_2$ and ascorbate. After addition of $CuCl_2$, the mixture was incubated for five min. at 25°C to promote metal binding to the protein before the reaction was started by the addition of ascorbate. For control samples, dd. H_2O was added in place of ascorbate. In some cases, the oxidation was stopped by the addition of a final concentration of 40 μM EDTA. For the oxidation of BDNF by H_2O_2, 1-ml solutions were incubated for 3 hrs. at 25°C. Control samples did not contain H_2O_2. In this system, the oxidation was stopped by the addition of catalase to a final content of 10 units/ml. All solutions were made with dd. H_2O. Stock solutions of phosphate were treated with 5 g/100 ml Chelex (BIORAD, Hercules, CA) to minimize metal contamination. Stock solutions of $CuCl_2$, ascorbate and H_2O_2 were freshly prepared prior to the reactions.

Electrospray ionization mass spectrometry (ESI-MS)
Samples were prepared for ESI-MS experiments by acetone-HCl precipitation (Ozols, 1990), followed by dissolution in dd. H_2O prior to analysis. ESI-MS experiments were performed on an Autospec-Q tandem hybrid mass spectrometer (VG Analytical, Manchester, UK) equipped with a Mark III ESI source and an OPUS data system. Samples were trapped and desalted prior to ESI by washing the sample onto a trapping column (15 mm x 1 mm of polymeric beads with 400 Å pores, Michrom BioResources, Auburn, CA) with 0.1% acetic acid at 250 μl/min. and eluting retained sample into the ESI source with 70/30 methanol/0.1% acetic acid at 10 μl/min. The relative amount of each BDNF species present after oxidation was determined by measuring the peak heights, using similar baselines for each spectrum.

Table 1 *Reactant concentrations for different levels of oxidation*

	MCO		H_2O_2
Oxidation Level	$CuCl_2$ (μM)	Ascorbate (mM)	H_2O_2 (mM)
1	20	2	2
2	20	4	3
3	40	4	5

Proteolytic digestion
Samples were prepared for digestion by ultrafiltration (three dd. H_2O rinses at 12,000 g for 50 min. 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 min. 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 hrs. at 37°C. At this point, an additional 0.75% (w/w) Endo Lys-C was added and the mixture incubated for 4 additional hrs. at 37°C. The theoretically expected Endo Lys-C fragments are given in table 2.

Peptide mapping by RP-HPLC
Analysis of peptide fragments from the proteolytic digestion of BDNF was performed on a Vydac C18 column (4.6 x 250 mm) (Vydac, Hesperia, CA) at 60°C and 0.7 ml/min., using UV detection at 215 nm. A gradient from 0% to 50% acetonitrile with 0.1% trifluoroacetic acid over 70 min. 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.

MALDI-TOF MS
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

Table 2 *Theoretical endo Lys-C fragments of BDNF*

Fragment	Sequence
K1 (-1-25)	MHSDPARRGELSVCDSISEWVTAADK
K2 (26-26)	K
K3 (27-41)	TAVDMSGGTVTVLEK
K4 (42-46)	VPVSK
K5 (47-50)	GQLK
K6 (51-57)	QYFYETK
K7 (58-65)	CNPMGYTK
K8 (66-73)	EGCRGIDK
K9 (74-95)	RHWNSQCRTTQSYVRALTMDSK
K10 (96-96)	K
K11 (97-116)	RIGWRFIRIDTSCVCTLTIK
K12 (117-119)	RGR

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/H_2O (1: 1 (v/v)).

HPLC/ESI-MS

RP-HPLC coupled on-line to ESI mass spectrometry was used to analyze the peptide maps. The reversed-phase assay for peptide mapping was optimized on a 1 x 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 split post UV detector to 8 µl/min. into the ESI source. Mass spectral data were collected and analyzed on the OPUS data system.

Amino acid analysis

HCl hydrolysis is the most widely used technique for amino acid analysis; however, this method has significant inadequacies. Specifically, HCl hydrolysis causes loss of Trp residues (Ozols, 1990) and can cause conversion of methionine sulfoxide [Met(O)] back to methionine (Met) (Ray and Koshland, 1962; Floyd *et al.*, 1963; Keutmann and Potts, 1969). As both Trp and Met are oxidation sensitive, it was necessary to obtain an accurate quantitation of these residues. Methanesulfonic acid hydrolysis was employed, as this method readily determines Trp and Met/Met(O) residues (Simpson *et al.*, 1976). Samples were prepared for amino acid analysis by performing an acetone-HCl precipitation. Precipitated samples were dissolved in formic acid prior to hydrolysis. Hydrolysis and neutralization were conducted as described (Simpson *et al.*, 1976). Briefly, protein samples were hydrolyzed with 4 N methanesulfonic acid at 115°C for 22 hrs., then neutralized with 4 N NaOH prior to derivatization. Hydrolyzed amino acids were derivatized with phenyl isothiocyanate and separated on a Waters Spherisorb S5ODS2 column (4.6 x 250 mm) (Waters Chromatography, Milford, MA) at 45°C and 0.8 ml/min., using UV detection at 254 nm.

Protein assay

Soluble protein concentration was determined using the Coomassie® plus protein assay reagent from Pierce (Rockford, Il), which is a modification of the Bradford method (Bradford, 1976). Sample analysis involved the addition of the Coomassie dye to the protein solution, with the subsequent measurement of absorbance at 595 nm on a Shimadzu UV-160 spectrophotometer (Shimadzu, Kyoto, Japan).

SDS-PAGE

Samples were electrophoresed on a PhastSystem (Pharmacia Biotech, Piscataway, NJ) using precast PhastGel Gradient 8-25% acrylamide gels at 250 volts for ca. 1 hr. Approximately 0.8 µg protein was loaded into each lane. Gels were stained using the PhastGel Silver Kit (Pharmacia Biotech).

Analytical ultracentrifugation

Sedimentation equilibrium and velocity experiments were performed on a Beckman XL-I analytical ultracentrifuge (Beckman Coulter, 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 µM. Samples were centrifuged at three different speeds (15,000, 20,000 and 27,000 rpm) for 24 hrs. at 20°C. Equilibrium was confirmed by monitoring the UV absorbance at 229 nm, measured every 5 hrs. Raw data were imported into the software program Kdalton (developed by John Philo, Amgen) for analysis via non-linear curve fitting. For sedimentation velocity experiments, samples of 20 µM BDNF were equilibrated to 25°C and centrifuged at 40,000 rpm. Sedimentation was monitored using UV absorbance at 229 nm. The data generated were 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).

8-Anilino-1-naphthalenesulfonic acid (ANSA) fluorescence

Fluorescence spectra were obtained using a Photon Technology International (Monmouth Junction, NJ) spectrofluorometer (Quanta Master Luminescence Spectrometer, QM1). ANSA (100 µM) containing 20 µM EDTA, was incubated with 10 µM BDNF (from either an oxidized or control sample) in 20 mM sodium phosphate buffer, pH 7.4, at 37°C for 30 min. 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.

CD spectroscopy

Samples were prepared for CD experiments by ultrafiltration and solvent exchange into dd. H_2O (three dd. H_2O rinses at 12,000 g for 50 min. 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 100 µg/ml. Measurements were taken at intervals of 0.5 nm with a speed of 20 nm/min. and were averaged over 3 accumulated scans.

RESULTS

Solvent exposure of His

The relative SASAs for the His residues of hGH are as follows: His^{18} - 37%, His^{21} - 26% and His^{151} - 89%. His^{18} and His^{21} were modified at levels of 63.5 and 49.1%, respectively, by MCO (10 µM Cu(II), 100 µM ascorbate) while His^{151} was not (Zhao *et al.*, 1997). The His residue in hRlx (position 12) has an SASA of 54%, and was modified 40% by MCO (200 µM Cu(II), 4 mM ascorbate) (Li *et al.*, 1995). BDNF contains two His residues, one at the N-terminus and one at position 75. No X-ray coordinates are available for His^1 (Robinson *et al.*, 1995) indicating high flexibility and most likely high surface exposue.

His^{75} is characterized by an SASA of 73%.

Mass spectrometry

ESI-MS experiments were performed to determine the extent of covalent modification of BDNF exposed to three different levels of the Cu(II)/ascorbate/O_2 and H_2O_2 systems, respectively. Fig. 2 shows representative mass spectra indicating the respective extents of BDNF oxidation at each oxidation level. The reactant concentrations and reaction times were selected such that they led to approximately similar extents of modification at each oxidation level, quantified in terms of oxygen incorporation into the protein. As revealed in fig. 2e, BDNF exposed to the harshest metal-catalyzed conditions (oxidation level 3) was extensively modified. Very little native BDNF (M.W. = 13637) remained, while significant amounts of species with molecular weights of 13653, 13669 and 13685 (all ± 1) appeared. These molecular weights correspond to M+16, M+32 and M+48, respectively, indicating the incorporation of 1-3 equivalents of oxygen into the BDNF monomer (M = molecular weight of native BDNF). The oxidized species were not present in a control sample (data not shown). Table 3 summarizes the relative amounts of BDNF species present at each

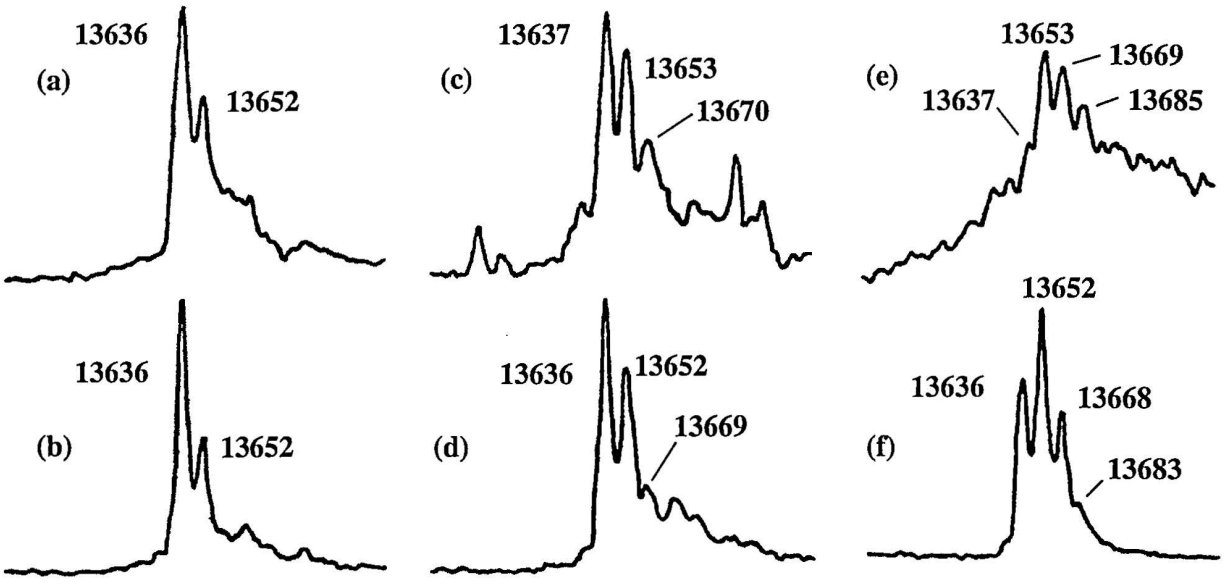

Fig. 2 *ESI-MS spectra for BDNF oxidized at various levels.* Oxidation level 1: **a)** MCO, **b)** H_2O_2; Oxidation level 2: **c)** MCO, **d)** H_2O_2; Oxidation level 3: **e)** MCO, **f)** H_2O_2. For all graphs: x-axis: molecular weight, y-axis: abundance of ions

Table 3 *Relative amounts of BDNF products for different levels of oxidation*

	MCO				H_2O_2			
Oxidation Level	Unoxidized (%)	Mono-Oxidized (%)	Di-Oxidized (%)	Tri-Oxidized (%)	Unoxidized (%)	Mono-Oxidized (%)	Di-Oxidized (%)	Tri-Oxidized (%)
1	62	38	—	—	63	31	6	—
2	45	37	18	—	46	33	12	9
3	13	36	31	20	28	41	24	7

oxidation level for both oxidizing systems.

Proteolytic digest

In order to localize covalent modifications induced by MCO, BDNF, oxidized at levels 1 and 3, was subjected to Endo Lys-C digestion followed by RP-HPLC separation of the resulting peptide fragments. All fragments, except K12 (Arg^{117}-Arg^{119}), were easily identified via LC-MS or peak collection followed by MALDI-TOF MS and/or ESI-MS. The peak assignments are given in fig. 3. Several combination fragments were seen, resulting from incomplete cleavage at various Lys residues. The peptide map of BDNF oxidized at level 1 (data not shown) gave no evidence for any additional fragment being 16 a.m.u. heavier than the identified native sequences, consistent with our earlier data (Jensen *et al.*, 2000). For BDNF oxidized at level 3, however, major differences were seen between the peptide maps of the oxidized and control samples (Fig. 3). A number of peaks were greatly decreased in the oxidized sample relative to the control; however, only one new product peak was observed in the map of the oxidized sample (indicated by an arrow). The identities of the Endo Lys-C fragments which experienced changes upon exposure to MCO are given in table 4. The only observed oxidation product was K7 + 16, i.e. the K7 fragment modified by a single oxygen addition.

Several further attempts were made to obtain a peptide map which not only showed the loss of modified fragments, but also detected all product peaks. The first approach involved increasing the urea level to 4 M during the digestion process. As expected, Endo Lys-C was inactivated under these conditions leading to incomplete digestion of BDNF. The second approach was to use sodium dodecyl

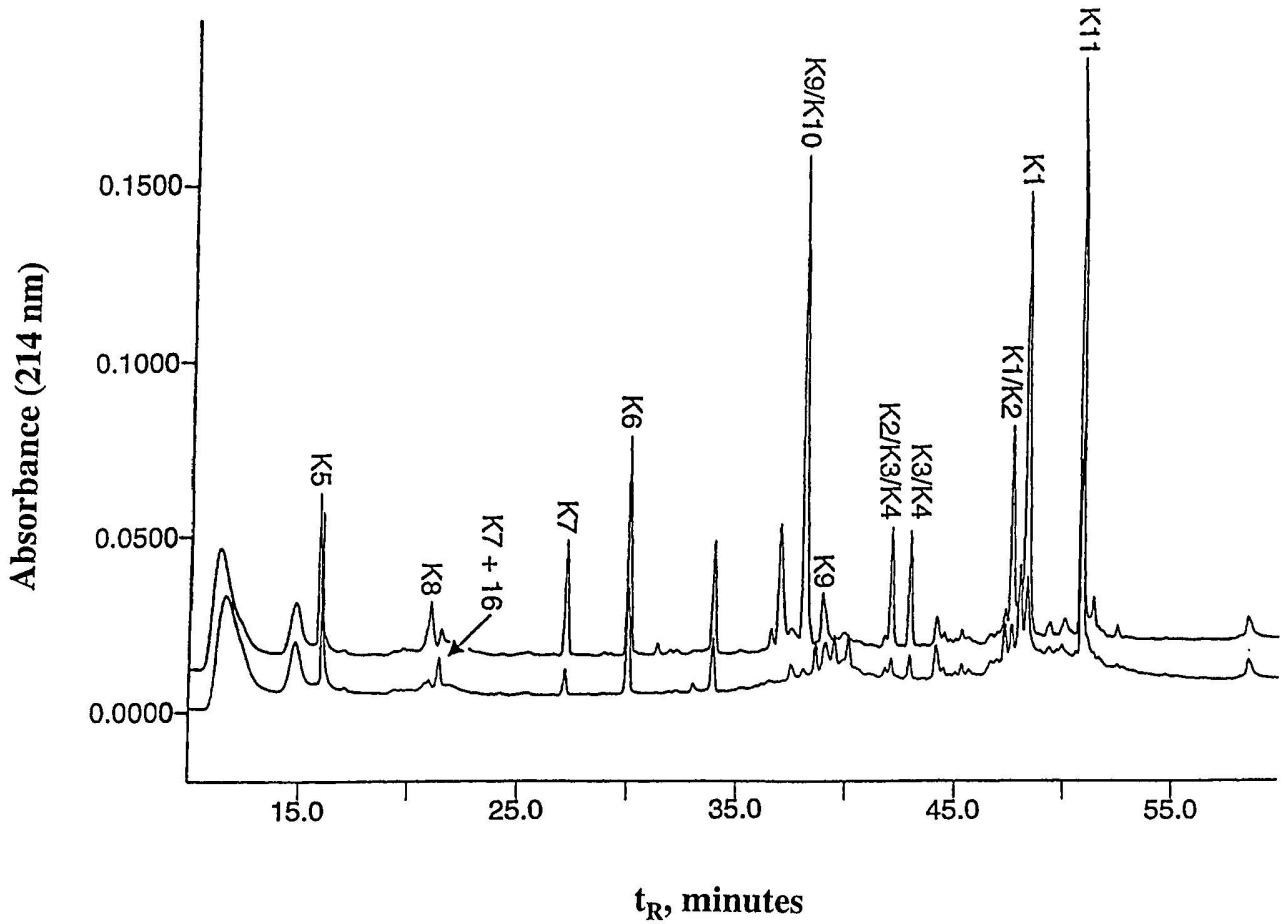

Fig. 3 *Endo Lys-C maps of BDNF, exposed to MCO at level 3* (lower line) *and control* (upper line).

sulfate (SDS) as a solubilizing agent in place of guanidine/HCl and urea during the denaturation, reduction, alkylation and digestion procedure. Although more product peaks were evident here, the SDS interfered with subsequent analysis by ESI-MS, and fragment identification was impossible.

Amino acid analysis

Each of the modified fragments listed in table 3 contains residues which are potentially oxidation sensitive (Met, His, Trp and Tyr). Amino acid analysis was employed to identify the modified residue(s) in BDNF exposed to MCO at oxidation levels 1 and 3. For oxidation level 1, methanesulfonic acid and NaOH hydrolysis of BDNF revealed some loss of Met (ca. 5%) and formation of Met(O) (ca. 16% relative to the original content of Met. For oxidation level 3, methanesulfonic acid hydrolysis of BDNF revealed the loss of 63 ± 1% of the total His residues (2.5 mol/mol BDNF dimer). Additionally, a 12 ± 1% loss of Met residues with a corresponding gain of Met(O) was seen. Notably, no changes in the contents of any other amino acid were detected.

Determination of soluble protein

The possible loss of soluble protein was investigated using a modified Bradford assay. For BDNF reacted with the Cu(II)/ascorbate/O_2 system, increasing levels of oxidation led to decreasing levels of soluble protein. At the harshest conditions, a loss of ca. 15% of soluble protein relative to the control sample occurred. This loss indicated possible aggregation or surface adhesion of BDNF. Although a higher degree of variability was seen in the BDNF samples oxidized by H_2O_2, no trend toward decreasing levels of soluble BDNF was seen with increasing levels of oxidation. There was no visible evidence for precipitation in any of the samples.

SDS-PAGE

The presence or absence of covalent aggregates in BDNF samples exposed to MCO and H_2O_2 (oxidation level 3), and control conditions was examined by SDS-PAGE. None of the samples exhibited bands representing a species which was larger and slower migrating than the native BDNF. However, a protein band of smaller size than native BDNF was evident in the sample exposed to MCO, indicating some fragmentation.

Analytical ultracentrifugation

The possibility of aggregation was further investigated by analytical ultracentrifugation. Results from sedimentation equilibrium experiments confirmed that BDNF existed entirely as a dimer under the conditions used for the oxidation reactions. Additionally, the results showed that BDNF remained in the dimeric form after the exposure to MCO and H_2O_2, even at level 3. Data from sedimentation velocity experiments confirmed the existence of a single species in all samples, with molecular weights corresponding to that of the dimer. It is not surprising that dissociation of the dimer did not occur upon oxidation, as the dimer is known to be extremely stable. In fact, Radziejewski *et al.* (1992) showed that the BDNF dimer is stable even in solutions of 8 M urea. Furthermore, no evidence for the presence of multimers was seen by either technique, indicating that the oxidation of BDNF did not induce aggregation beyond the native dimeric state, confirming our SDS-PAGE results.

ANSA fluorescence

ANSA is a fluorescent probe that is known to bind to exposed hydrophobic sites in proteins, and has been used extensively to study the "molten globule" state of proteins (Stryer, 1965; Turner and Brand, 1968; Slavik, 1982; Semisotnov *et al.*, 1991). The probe is essentially non-fluorescent in water/buffer, but exhibits strong fluorescence upon binding to a protein. Fig. 4 shows the increase in fluorescence for ANSA in the presence of BDNF exposed to the various oxidation conditions. A clear relationship between the increase in ANSA fluorescence intensity and the extent of oxidation was evident for BDNF exposed to MCO, i.e. the fluorescence intensity of ANSA increased concomitantly with the level of BDNF oxidation; however, no such relationship was seen for BDNF modified by H_2O_2. Additionally, derivative analysis of the spectra revealed a blue shift of ca. 10 nm in the peak maximum for the MCO of BDNF. In contrast, no peak shift was evident upon

oxidation of BDNF by H_2O_2. These results suggest that MCO leads to a progressive increase in the exposure of hydrophobic regions in BDNF. Remarkably, the oxidation of BDNF by H_2O_2 did not show a similar trend. The substantial increase in ANSA fluorescence for BDNF exposed to MCO at level 3 occurred despite the loss of 15% of the soluble protein. Assuming that the fraction of insoluble protein is unable to bind ANSA, the increase in ANSA fluorescence is entirely due to the exposed hydrophobic regions on the remaining 85% of soluble protein.

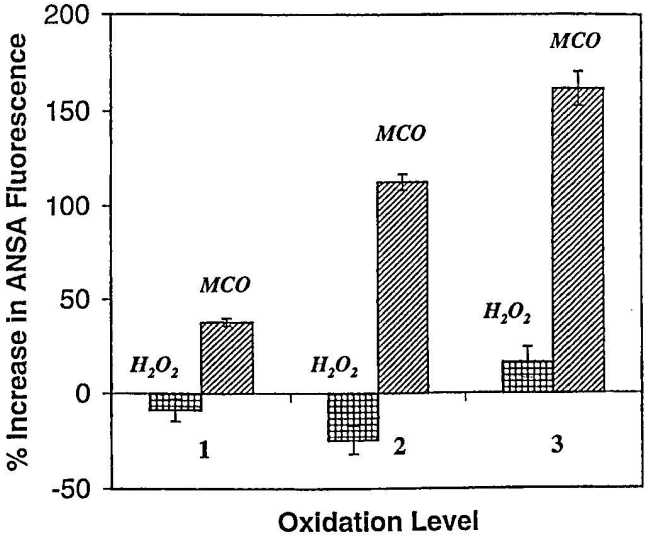

Fig. 4 *Changes of ANSA fluorescence* (relative to control) *as a result of BDNF exposure to MCO and H_2O_2 at the different oxidation levels.*

Circular dichroism

Protein secondary structure can be assessed through the use of far-UV CD spectroscopy. Fig. 5 displays the CD spectra for BDNF exposed to MCO (oxidation levels 1 and 3) and H_2O_2 (oxidation level 3), and their respective controls. Native BDNF is estimated to consist of ca. 74% β-sheet and 21% β-turn structure, based on deconvolution analysis of the circular dichroism spectrum (Radziejewski *et al.*, 1992). The broad negative signal between 190 and 220 nm has been attributed to a combination of β-sheet and β-turn structure (Neumann and Snatzke, 1990; Radziejewski *et al.*, 1992; Narhi *et al.*, 1993), while the distinctive peak at 232 nm has been attributed to both tertiary structural elements (Narhi *et al.*, 1993; Philo *et al.*, 1993) and a combination of secondary β-structure elements (Radziejewski *et al.*, 1992). While BDNF exposed to MCO at level 1 showed a slight increase in the magnitude of the negative peak at 190-220 nm relative to the control (Fig. 5a), BDNF oxidized at level 3 showed a very large decrease in the magnitude of this peak (Fig. 5b). This indicates a considerable MCO-dependent loss of β-structure. For BDNF exposed to H_2O_2, however, no significant change was seen in the negative peak at 190-220 nm relative to the control (Fig. 5c) suggesting that H_2O_2-dependent oxidation does not

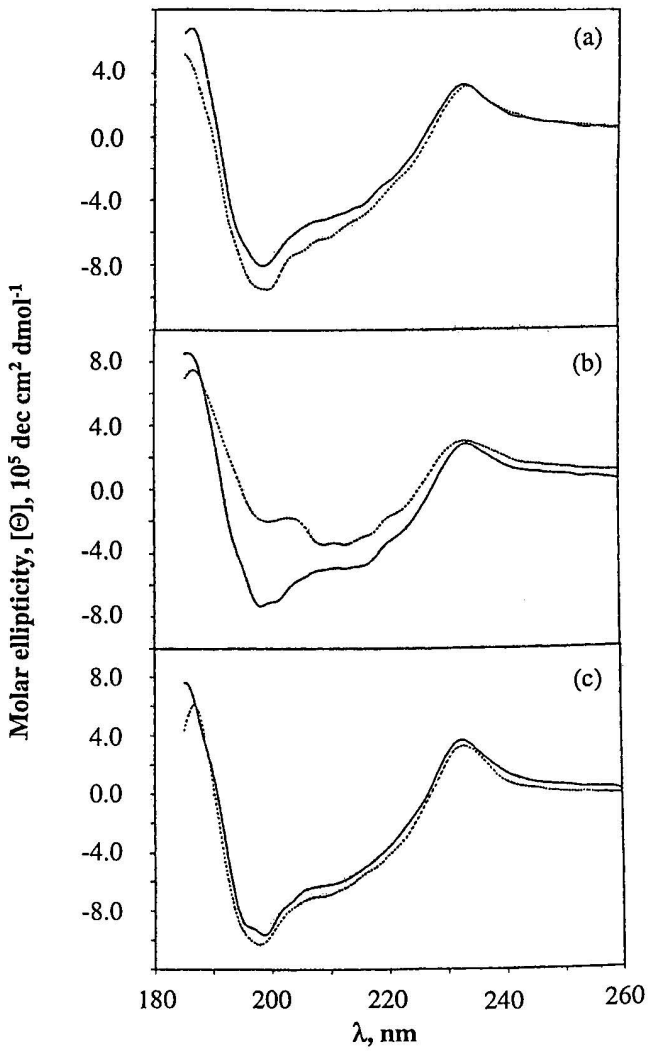

Fig. 5 *Far-UV CD spectra of oxidized and control BDNF.* **a)** MCO, level 1: (---) oxidized; (—) control. **b)** MCO, level 3: (---) oxidized; (—) control. **c)** H_2O_2, level 3 (---) oxidized; (—) control.

affect the β-structure. Neither oxidation system induced a change in the peak at 232 nm.

DISCUSSION

Chemical characterization of modified BDNF

ESI-MS analysis clearly showed that BDNF was progressively modified by both increasing levels of H_2O_2 and MCO. Earlier results had shown that various levels of H_2O_2 selectively oxidized Met^{-1}, Met^{31}, and Met^{61} (Garcia *et al.*, 1994). In contrast, the Cu(II)/ascorbate/O_2 system at level 1 selectively oxidized the buried Met^{92} to Met(O) (Jensen *et al.*, 2000). Interestingly, an increase of the MCO conditions to level 3 did only marginally increase the conversion of Met but instead led to a pronounced loss of His, 63% relative to the total His residues, corresponding to 2.5 mol His/mol BDNF dimer. His residues are prominent targets of MCO (Levine, 1983b; Farber and Levine, 1986; Amici *et al.*, 1989; Li *et al.*, 1995; Maria *et al.*, 1995; Zhao *et al.*, 1997), due to their strong metal-binding propensities (Levine, 1983a, 1983b; Farber and Levine, 1986; Stadtman, 1993). While Met residues are not primary metal-binding residues, they are vulnerable to a broad range of oxidants (Sysak *et al.*, 1977; Brot and Weissbach, 1991; Hall and Roberts, 1992; Li *et al.*, 1995). The physico-chemical basis for the switch of target from Met to predominantly His on going from MCO level 1 to 3 is not known. Eventually, the oxidation of Met^{92} causes small local conformational changes which modify the specificity of metal-binding in the BDNF dimer.

Earlier, we had reported on the difficulties associated with the proteolytic (Endo Lys-C) mapping of BDNF exposed to Cu(II)/ascorbate/O_2 at level 1, most likely due to an increased resistance of the oxidized protein to digestion (Jensen *et al.*, 2000). Peptide mapping was only possible after chemical, CNBr, digestion which revealed the oxidation of exclusively Met^{92}. After exposure to Cu(II)/ascorbate/O_2 at level 3, the oxidized BDNF was more amenable to digestion by Endo Lys-C, indicated by the loss of several fragments, displayed in fig. 3 and summarized in table 4. Based on the results from amino acid analysis, the loss of K7 and of the combination fragment K2/K3/K4 is likely due to the oxidation of Met^{31} and Met^{61}, respectively. The loss of combination fragment K1/K2 should be caused by the oxidation of Met^{-1} or His^{1}, or a combination thereof. Finally, the loss of fragment K9/K10 would be consistent with the loss of both Met^{92} and His^{75}. Based on these results, the combined loss of Met and His, any correlation of structural changes with the modification of His would be impossible. However, such a correlation can be achieved by comparison of results from MCO to the oxidation of BDNF by H_2O_2, as described below.

Table 4 *Identification of fragments which experienced changes upon exposure to MCO at level 3* (from the peptide map displayed in Fig. 3)

Endo Lys-C Fragment	Sequence Identification
K1/K2	M^{-1}HSDPARRGELSVCDSISEWVTAADKK
K2/K3/K4	KTAVDM^{31}SGGTVTVLEKVPVSK
K7	CNPM^{61}GYTK (K7 + 16; seen in the chromatogram)
K9/K10	RHWNSQCRTTQSYVRALTM^{92}DSKK

Structural consequences of MCO

Results from the Bradford assay indicated that higher levels of MCO cause some loss of soluble protein; however, analytical ultracentrifugation and SDS-PAGE experiments did not reveal any aggregation beyond the native dimeric state of the protein. ANSA fluorescence showed a progressive exposure of hydrophobic sites with increasing exposure to MCO but not to H_2O_2. Similarly, CD spectroscopy attested to a significant loss of β-structure as a result of MCO but not exposure to H_2O_2. As H_2O_2 selectively targets Met^{-1}, Met^{31} and Met^{61} (Garcia *et al.*, 1994), we conclude that the oxidation of these Met residues has little consequence on 2° and 3° structure of BDNF. Amino acid analysis revealed that level 1 MCO exclusively targets Met^{92} (Jensen *et al.*, 2000) while advanced levels of MCO did not lead to an increased modification of Met but, rather, a predominant modification of His. Hence, a quantitative comparison of the differences in the changes of

2°and 3° structure of BDNF between MCO levels 1 and 3 approximately yields the contribution of His oxidation to structural changes. In fact, only small structural modifications are visible at MCO level 1, entirely due to the modification of Met^{92} (Jensen *et al.*, 2000) whereas significant structural changes appear at MCO level 3, predominantly due to His modification with smaller contributions from the oxidation of Met^{92}. In conclusion, the MCO-dependent modification of His causes significant conformational changes of the protein.

Comparison to hGH and hRlx

Earlier we had shown that the MCO of hGH resulted in the efficient oxidation of two relatively buried His residues, His^{18} and His^{21}, with little consequences for the 2° and 3° structure, assessed by CD and fluorescence spectroscopy (Zhao *et al.*, 1997). The relative SASAs of these two His residues amount to 37 and 26%, respectively. In contrast, the exposure of hRlx to MCO resulted in a pH-dependent aggregation and precipitation of the protein due to oxidation of His^{12} (Li *et al.*, 1995) which has an SASA of 54%. The present results of BDNF show that the MCO of highly surface exposed His residues, where His^{75} has an SASA of 73% and His^{1} an SASA of likely >73%, is accompanied by significant structural changes of the protein. While it is certainly too early to generalize from these three proteins, our findings would support our hypothesis that the level of surface exposure of a target His residue controls, in part, the MCO-dependent conformational consequences for a protein. The physico-chemical basis of solvent exposure controlling the MCO-dependent conformational modifications may be: a) the nature of the His oxidation products (Tomita *et al.*, 1969; Uchida and Kawakishi, 1989, 1990); b) breakage of existing hydrogen bonds or salt bridges involving the target His residue(s), or c) effects of His oxidation of the solubility of the protein or hydrophobic/hydrophilic properties of the His-containing domain. Further experiments with a selection of proteins containing His at different positions shall bring an answer to these questions.

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