Oxidation of Tryptophan-21 Alters the Biological Activity and Receptor Binding Characteristics of Mouse Nerve Growth Factor*

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The tryptophan residues of β nerve growth factor (βNGF) were modified by reaction with varying concentrations of N-bromosuccinimide. Analysis of the oxidized derivatives by electrophoresis under denaturing conditions, before and after reduction or oxidation of disulfide bonds, revealed partial hydrolysis of specific peptide bonds. Analysis of the newly created NH₂ termini by the 5-dimethylaminonaphthalene-1-sulfonyl procedure showed that the peptide bonds contained the carbonyl group of the reacted tryptophan residues and that the oxidation reaction proceeded in the order Trp-21, Trp-99, and Trp-76. To obtain βNGF modified only at Trp-21, 0.9 tryptophan residues/NGF chain were oxidized. The dose-response curves for the βNGF-induced neurite outgrowth on sensory and PC12 cells showed that 20- to 30-fold higher concentrations for the 0.9 Trp-OX-NGF (βNGF oxidized with N-bromosuccinimide) derivative and about 1000-fold higher concentrations for a 2.0 Trp-OX-NGF derivative were required to elicit half-maximum biological response when compared with native βNGF. This decrease in biological activity was paralleled by a decreased binding to NGF receptors on both sensory and PC12 cells. On sensory cells, the affinity of 0.9 Trp-OX-NGF was 2.5% and the affinity of 2.0 Trp-OX-NGF was 0.05% of that of native βNGF. Tested on PC12 cells, 0.9 Trp-OX-NGF bound with 1.2% of the affinity of native βNGF. At the highest concentration tested, 2.0 Trp-OX-NGF did not compete with the binding of βNGF to PC12 cells. That the residual binding affinity and biological activity of 0.9 Trp-OX-NGF were not due to unmodified βNGF in the preparation was ascertained by the βNGF-dependent cytotoxicity assay, which showed that less than 0.2% of unmodified βNGF remained. In contrast to the significant decrease in biological and binding activity, the derivative modified at Trp-21 retained 25% of the antigenicity of the native protein.

Nerve growth factor (NGF) is a polypeptide hormone which has several different functions with respect to its target cells, sympathetic and some embryonic sensory neurons. It is required for the survival (1) and for the growth and differentiation of these cells (2). In the mature nerve cells, NGF is sequestered at the synaptic terminals and carried by retrograde flow to the interior of their cell bodies (3) where it appears to regulate, at least in sympathetic cells, the synthesis of key enzymes involved in neurotransmitter synthesis. The mechanisms by which NGF elicits its several different effects are not yet understood but are under active investigation. It is known, for example, that, like other trophic factors, NGF initiates some of its actions by interacting with specific receptors on the cell surface (4-6). Depending on the cell type studied, the analysis of steady state binding data reveals either homogeneous or heterogeneous binding affinities for NGF. A clonal cell line of rat pheochromocytoma (PC12) binds NGF with homogeneous binding characteristics according to the analysis of steady state binding data (7) but shows heterogeneity of binding when kinetic data are analyzed.2 Sensory neurons from chick embryos, on the other hand, bind NGF specifically with two affinities which differ 50-fold irrespective of whether steady state or kinetic data are analyzed (8). On sensory neurons, the interaction of NGF with the binding sites with the higher affinity (Site I) is sufficient to initiate neurite outgrowth (9). So far, the role of the lower affinity binding site (Site II) in the various functions of NGF on those cells and its relationship to the high affinity binding sites are unknown. Derivatives of NGF with altered biological and binding properties should be valuable tools in investigating the correlation of NGF binding characteristics with its biological activity as well as questions raised by the differences seen in the steady state binding characteristics of various NGF-responsive cell types.

Although the complete covalent structure of mouse βNGF has been determined (10) and crystals of this protein have been obtained (11), little is known about the structural features of the βNGF dimer which determine its biological activities. Neither the COOH-terminal arginine residues nor the NH₂-terminal octapeptide sequences are essential for the neurite-inducing ability of βNGF (12, 13). The relative reactivity of the tryptophan (Trp-21, Trp-76, and Trp-99) and tyrosine (Tyr-52 and Tyr-79) residues in NGF and their relationship to activity have been investigated. Both tyrosine residues are freely available to solvent and are not essential for inducing neurite outgrowth in the sensory ganglion bioassay (14). The Trp-21 residue is the most reactive tryptophan residue and its oxidation by N-bromosuccinimide is reported not to affect the biological activity of NGF in the sensory ganglion bioassay (14). Oxidation of Trp-99 in addition to Trp-21 markedly...

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1 The abbreviations used are: NGF, nerve growth factor; Trp-OX-NGF, βNGF oxidized to different degrees with SucNBr; DHG, dorsal root ganglion; SucNBr, N-bromosuccinimide; NaDodSO₄, sodium dodecyl sulfate; Dns and dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

2 C. E. Landreth, unpublished data.

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reduced the biological activity in the ganglion bioassay (14), although not the binding to specific NGF receptors (15), while oxidation of all three tryptophan residues renders NGF essentially inactive (14).

In this report, we describe the effects of selective oxidation of the Trp-21 residue on the binding characteristics of NGF and on its biological properties and conclude that Trp-21 is a critical residue both for receptor binding and biological activity.

**Materials and Methods**

**Results**

**Analysis of the Products of N-Bromosuccinimide Oxidation of βNGF—** The reaction with SucNBr was performed in order to obtain derivatives of βNGF in which the tryptophan residues were oxidized to various extents. Analysis of the derivatives by acrylamide gel electrophoresis under denaturing conditions showed that, besides a major band co-migrating with βNGF, an increasingly proportion of slower moving proteins (Fig. 1A) appeared with increasing extent of tryptophan oxidation. Since the shift of mobility cannot be due to an increase in the molecular weight of the NGF chains, it appears that the oxidation of the tryptophan residues results either in reduced binding of NaDodSO₄, or in conformational changes, with increasing levels of oxidation (Fig. 1B). Smaller amounts of partially inactive (14).

**The Binding Characteristics of the Oxidized Derivatives of βNGF—** Competition binding assays were carried out with the oxidized derivatives to determine in which way their binding affinities correlated with their lower biological activity. The competition experiments were carried out at two different concentrations of 125I-βNGF. In the first experiment with DRG cells, the concentration of 125I-βNGF (13 pm) was chosen such that, in the absence of competitor, >80% of the 125I-βNGF was bound to the higher affinity Site 1. In the second experiment, a higher concentration of 125I-βNGF (1.5 nm) insured that approximately 86% of the 125I-βNGF was bound to the lower affinity Site 2. The displacement curves in Fig. 4A show that 40 times more 0.9 Trp-OX-NGF and 2500 times more 2.0 Trp-OX-NGF than unmodified βNGF were needed to compete for the same amount of 125I-βNGF bound to Site I of sensory cells. Forty-fold higher concentrations of the 0.9 Trp-OX-NGF derivative than of βNGF were necessary to displace equal amounts of 125I-βNGF bound to Site II (Fig. 5A). At the highest concentrations of the 2.0 Trp-OX-NGF tested (0.35 μM), no displacement of 125I-βNGF bound to Site II occurred (Fig. 5A). The conditions for the competition assays with PCC12 cells were essentially the same as those described for DRG cells. At low 125I-βNGF concentrations (8 pm), the competition curve was shifted to 80-fold higher concentrations for 0.9 Trp-OX-NGF while 2.0 Trp-OX-NGF did not compete at the highest concentration tested (0.38 μM) (Fig. 4B).

In a second experiment (Fig. 5B) at higher tracer concentrations (0.7 nm), competition with 0.9 Trp-OX-NGF resulted in an 85-fold shift in the curve relative to βNGF. As at the lower tracer concentration, no displacement of 125I-βNGF was seen up to the highest concentrations of 2.0 Trp-OX-NGF tested. These data indicate that the affinity of 0.9 Trp-OX-NGF is reduced to 2.5% of that of βNGF when assayed on DRG cells and to 1.2% of that of βNGF when assayed on PCC12 cells. These values correlate well with the residual neurite-inducing abilities of this derivative on DRG cells (5% of that of βNGF) and PCC12 cells (3% of that of βNGF), respectively.

In order to determine to what degree the tryptophan oxidation of the 0.9 Trp-OX-NGF changed the antigenic properties of the protein, native βNGF and 0.9 Trp-OX-NGF were compared in coated tube radioimmunoassays (31). In contrast...
to the drastic decrease in the ability of 0.9 Trp-OX-NGF to compete with native βNGF for the binding sites on DRG and PC12 cells, its ability to compete for the antibody binding sites was only reduced by a factor of 4 (data not shown).

Estimation of Unmodified βNGF by Cytotoxicity Assay—

It could be argued that the residual activity exhibited by the 0.9 Trp-OX-NGF preparation arises from the presence of a corresponding amount of unmodified βNGF in the reaction mixture. The theoretical amount of unmodified βNGF dimers in 0.9 Trp-OX-NGF can be calculated as follows. In this derivative, 81% (0.9 × 100%) of the protein has oxidized Trp-21 residues in both chains. The remaining 0.09 oxidized Trp-21 residues are in dimers where only one chain carries the modified residue. Since these dimers must also contain a chain with an unmodified Trp-21 residue, the fraction of the total protein present in this form is 18%. In other words, the 0.9 Trp-OX-NGF contains only 1% of unmodified βNGF chains, too little to account for the residual activity of this derivative. Since this calculation depends entirely on the accuracy of the determination of the number of oxidized tryptophans in the derivative, an alternative method of determining the fraction of unmodified βNGF, based on the cytotoxicity assay of Zinnbauer et al. (23), was used. In this assay, cells carrying NGF binding Site I are lysed in the presence of βNGF, anti-βNGF antisem, and complement. The assay can also be used to detect minute quantities of βNGF. As shown in Fig. 5, the proportion of DRG cells which are lysed increases with increasing βNGF concentration until it reaches a maximum at 3.8 × 10^{-11} \text{M} \betaNGF. Under the same conditions, amounts of 0.9 Trp-OX-NGF 500-fold greater than the βNGF concentration necessary to produce a detectable proportion of lysed cells did not give any cell lysis. The amount of unmodified βNGF in the 0.9 Trp-OX-NGF preparation is therefore less than 0.2%. When the amount of 0.9 Trp-OX-NGF in the assay was increased to 1000-fold greater than the βNGF concentration producing detectable cell death, then cell lysis was observed (Fig. 5). Finally, a derivative in which 1.1 residues of tryptophan/βNGF chain were modified was prepared. Theoretically, this should contain no unmodified βNGF dimers. It required 25 times more of this derivative than unmodified βNGF to compete for the same amount of 125I-βNGF bound to Sites I or II. The binding characteristics of this derivative are, therefore, very similar to those of 0.9 Trp-OX-NGF and reflect the residual activity of the derivative.

**DISCUSSION**

The relationship of the three-dimensional structure of βNGF to its biological activities has not yet been explored in detail. The present data show, in agreement with earlier studies (14), that the chemical modification of the tryptophan residues of βNGF reduces both its capacity to induce neurite outgrowth and its ability to bind to the specific NGF receptor. The identification of the tryptophan residues oxidized by increasing concentrations of SucNBr was based on the fact that the oxidation of the indole ring of the tryptophan side chain is known to cause, besides the indole-oxindole conversion, cleavage of the peptide bond on the carbonyl side of this residue (32, 33). In the present study, it was found that the cleavage occurred at about 13% of the susceptible peptide bonds during the oxidation of the first tryptophan residue, Trp-21. The shorter peptide chains resulting from this cleavage were only released after the intrachain disulfide bonds were reduced. The appearance of the new NH-terminal residues indicated that the tryptophan residues are attacked by SucNBr in the order Trp-21, Trp-99, and Trp-76. This agrees with the conclusions of Frazier et al. (14) based on kinetic measurements. They found that the relative rate constants for the reaction were 2040, 34, and 1 for Trp-21, Trp-99, and Trp-76, respectively. The relatively high reaction rate for Trp-21 suggests that 0.9 Trp-OX-NGF is oxidized exclusively (>98%) at Trp-21. The absence of detectable amounts of Dns-Asp or Dns-Arg in the acid hydrolysis of danylated 0.9 Trp-OX-NGF is consistent with this assessment. Because about 87% of the βNGF chains in 0.9 Trp-OX-NGF remain intact, its lower biological activity (<5% of that of βNGF) and binding capacity can be attributed to the oxidation of the indole ring. That the residual activity of 0.9 Trp-OX-NGF is not due to contamination by unmodified βNGF is apparent from the cytotoxicity assay for this derivative. The 0.9 Trp-OX-NGF derivative is therefore an NGF agonist with decreased but still detectable activity.

The parallel displacement of the dose-response curves in the bioassay and the competition binding curves for the high affinity Site I noted for 0.9 Trp-OX-NGF with sensory cells emphasizes the crucial role of these receptors in mediating neurite outgrowth. The same parallel loss of biological activity and binding capacity to Site I on sensory cells also occurs with 2.0 Trp-OX-NGF. It is clear from the data obtained with 0.9 Trp-OX-NGF that its binding to the low affinity Site II of DRG cells is decreased to the same extent as its binding to the high affinity Site I. One possible explanation for this finding is that Sites I and II share common structural features which are equally sensitive to changes in the βNGF structure. Whereas the changes in 0.9 Trp-OX-NGF lead to a considerable reduction in its affinities for both binding sites, they have much less effect on its antigenicity.

With PC12 cells, as with chick DRG cells, a parallel shift in both the dose-response curves in the bioassay and the competition curves is observed with 0.9 Trp-OX-NGF. The two concentrations of tracer NGF used for the competition assays with PC12 cells were chosen in analogy to those for sensory cells. However, PC12 cells differ from chick DRG cells in that the high affinity component of the binding of NGF observed in competition assays on DRG cells (Fig. 4A) is not seen on PC12 cells (Fig. 4B). The affinity for the low affinity component of NGF observed in competition assays on DRG cells (Fig. 4A) is not seen on PC12 cells (Fig. 4B). This difference is also reflected in the binding behavior of the NGF derivatives. Approximately 100-fold higher concentrations of 0.9 Trp-OX-NGF are required for the same degree of displacement of low tracer NGF concentrations on PC12 cells than on DRG cells (Fig. 4, A and B). The significance of the difference of chick DRG cells and of PC12 cells in respect to their steady state binding characteristics for NGF and its Trp-OX derivatives, as is apparent from the competition experiments, is not yet clear.

The data presented here are in general agreement with the data of Frazier et al. (14) in showing that the oxidation of the tryptophan residues of βNGF alters the biological activity of the protein. The use of the single cell bioassay and an improved method for measuring receptor binding, however, allow a more quantitative assessment of the biological activity and binding characteristics of the Trp-OX-NGF derivatives. The results from the single cell bioassays show that oxidation of even the first of the 3 tryptophan residues in the βNGF monomer drastically impairs one of the biological activities of NGF, namely its ability to induce neurite outgrowth.

In the absence of detailed information on the location of Trp-21 in the three-dimensional structure of the βNGF dimer, it is difficult to envision how modification of these side chains can so drastically affect its ability to bind to the specific NGF receptors and thus its biological activity. It should be noted that, in the earlier, detailed investigation by Frazier et al. (14), modification of residues other than tryptophan, e.g. histidine, tyrosine, and methionine, was ruled out. It is clear from studies on the susceptibility of the different tryptophan residues of NGF to chemical modification and from a comparison
of a model structure of βNGF based on its homology with the known structure of insulin (14) that Trp-21 is on the outside of the molecule, freely available to solvent. Also, it should be noted that the region around the conserved cystine residue (B20) in insulin, insulin-like growth Factors I and II, relaxin, and NGF has been implicated in the receptor-binding site of insulin (34). This region includes the Phe-B25 residue in insulin and the corresponding Trp-21 residue in NGF (35). Since the hydrophobic modification of Trp-21 with hydroxynitrobenzyl groups does not alter the biological activity of βNGF (14), it seems likely therefore that the planar, hydrophobic nature of the indole ring of Trp-21 is a key feature in the interaction with the receptor.

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REFERENCES

Activity of Oxidized NGF

Supplementary Material:

Oxidation of Tryptophan-21 Alters the Biological Activity and Receptor Binding Characteristics of Mouse Neurotrophin Growth Factor


MATERIALS AND METHODS

Protein and peptide synthesis

The NGF preparation was lyophilized from the subcutaneous gland of male Swiss Webster mice by the procedure of Heath et al. (18). The radiolabeled or NGF was purified as described elsewhere (19) to give a product with a specific activity of 330 mCi per mg. Soluble oxidized NGF was prepared according to the procedure described by Bregman and Weinreb (17). Intact cells were obtained by homogenizing tissue with commercial or home-made buffer. Aliquots (1 ml of a 10 mM triethanolamine solution) of oxidative (295) were added stepwise to 1 ml of a 10 to 30 mM solution of NGF on 0.1 M sodium acetate buffer, pH 6.0, such that the molar ratio of reagents to tryptophan residues (12,13) was 1:2.5. After each addition, the 235 spectrum was recorded on a Beckman 25 spectrophotometer. The number of tryptophan residues modified per NGF molecule (12,13) was calculated using the value of 0.480 for the molar extinction factor (14,15). The reaction was carried out to the desired extent and the samples were then dialyzed against 0.25 M sodium acetate. Prior to the final concentration of the oxidized oxidized derivatives was determined by measuring the absorbance at the characteristic of the indole-3methylnitrore 32 mg of 25%, 22% of the sample was taken (16). An extinction coefficient of 22 mg of 25% 0.154 was taken for native NGF. Electrophoresis was carried out on 12.5% acrylamide gels containing 0.15 M sodium acetate, and 4 M urea according to Davis and Studier (18). Phenolic acid oxidation of the NGF derivatives was performed as described by Kita (19). Analysis of the biotinylated derivatives by reaction with dodecyl (19) or dodecyl (20) for peptides. The Des peptides were determined by chemical analysis on a 5 × 5 cm polyethylene sheets (Chung Chiu, Taiwan) according to Gray (21) as modified by Lee and Section (22).

Table I

Identification of the N-terminal residues of NGF and oxidized NGF derivatives by reaction with dodecyl chloride.

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<td>2.6 Sep-Ch-NGF</td>
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* Hydrolysis of proteins and desamidation of the hydrolytic products was performed on as described in Materials and Methods. Each determination was repeated at least twice in duplicate.
Activity of Oxidized NGF

Fig. 1. Electrophoretic behavior of the oxidized derivatives of NGF before and after denaturation of the disulfide bridges. a. Polyclonal rabbit anti-NGF antiserum (2.55 ml of 0.1 M Tris-HCl, pH 8.0) was reacted with NGF before and after reduction and alkylation with iodoacetamide in 0.1 M Tris-HCl, pH 8.0 for 24 h at room temperature. The gel was then electrophoresed in the absence and presence of NaDodSO4. The same quantity of protein was loaded per gel. Migration is from top to bottom. b. Same as in a but after periodic acid oxidation of NGF (A), 0.9 Trp-de-NGF (B) and 2.0 Tyr-de-NGF (C). In these gel scans migration is from left to right. Disulfide bridges were reduced and carboxymethylated in 33% acetic acid, 0.1 M Tris-HCl, pH 8.0, bovine trypsin inhibitor (0.5 mg/ml), dithiothreitol (50 mM), neuacin (300 μM). Amino acid analysis (2300).

Fig. 2. Topology of cysteine residues and disulfide bridges in NGF.

Fig. 3. Biological activity of the oxidized derivatives of NGF. a. Stimulation of neurite outgrowth in single cell cultures of NGF neurons. NGF (C), 0.9 Trp-de-NGF (C) and 2.0 Trp-de-NGF (H) were cultured in medium containing either 10 mM NGF, 100 μM NGF, or control medium (A). The data were plotted as percentage of the control values obtained at 5 ng/ml NGF. Each point represents the mean of the response of a minimum of 100 cells in cultures analyzed in duplicate. b. Stimulation of neurite outgrowth from PC12 cells. NGF (C), 0.9 Trp-de-NGF (C) and 2.0 Trp-de-NGF (H). PC12 cells were plated 7 to 10 days in the presence of 2 ng/ml NGF and replaced in 15-mm wells containing complete medium and NGF at its derivatives. The fraction of cell-bearing clones was determined after 24 h. The data are plotted as percentage of the control values obtained at 10 ng/ml NGF. Each point represents the mean of triplicate determinations. The dose-response curve for NGF represents results from three independent samples, and for 0.9 Trp-de-NGF of two independent assays. A minimum of 100 clones were counted per well.

Fig. 4. Competition assay at low 125I-NGF concentrations on NGF and PS3 cells. a. Inhibition of 125I-NGF binding to site I of NGF cells by NGF (C), 0.9 Trp-de-NGF (Δ), and 2.0 Trp-de-NGF (C). 2.0 × 10⁶ cells per ml were incubated at 37°C for 45 min in the presence of 25 ng/ml 125I-NGF and of increasing amounts of the competitors. For each point triplicate aliquots (100 μl) were processed as described in Materials and Methods. Results are expressed as percentage of maximum bound 125I-NGF in absence of competitor. The bars indicate the standard deviation. b. Same as a but on PS3 cells. The cell suspension contains 10⁶ cells per ml and 0.5 μg 125I-NGF. NGF (C), 0.9 Trp-de-NGF (Δ), and 2.0 Trp-de-NGF (C).

Fig. 5. Competition assay at high 125I-NGF concentrations on NGF and PS3 cells. a. Inhibition of 125I-NGF binding to site II (NGF cells) by NGF (C), 0.9 Trp-de-NGF (Δ), and 2.0 Trp-de-NGF (C). 2 × 10⁵ cells per ml were incubated for 1 h at 37°C in the presence of 1 mg/ml 125I-NGF and of increasing amounts of competitor. Triplicate aliquots (100 μl) of each sample were processed as described under Materials and Methods. Results are expressed as percentage of maximum bound 125I-NGF in absence of competitor. The bars indicate the standard deviation. b. Same as a but on PS3 cells. The cell suspension contains 10⁶ cells per ml and 0.7 mg 125I-NGF. NGF (C), 0.9 Trp-de-NGF (Δ), and 2.0 Trp-de-NGF (C).

Fig. 6. anti-NGF antiserum and complement mediated cytolytic assay on PC12 neurons. After 24 h preincubation of 100,000 cells/well with various concentrations of NGF (C) or 0.9 Trp-de-NGF (Δ) anti-NGF antiserum (400 μl) and complement (1:1:4) were added to the cell monolayers and incubated for another 20 min at 37°C. The percentage of dead cells was determined under the microscope using the Trypan Blue exclusion test. The data are given as % of maximum cytolytic index measured as 3.5 × 10⁶ 125I-NGF where the cytolytic index 4 dead cells/ 4 dead cells in normal/complement control.

100 - 8 dead cells in normal/complement control
The maximum cytolytic index under these conditions was 100.
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