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Purification and characterization of a neurite extension factor from bovine brain

(neuronal development/cell culture/protein S100 β/disulfide bonds)

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ABSTRACT **The extension of neurites by chicken embryo cerebral cortical neurons can be measured quantitatively at low cell density in serum-free, defined medium. An acidic, heat-stable protein fraction from bovine brain has been shown to have neurite extension activity in this assay. We report the use of reversed-phase HPLC to purify a neurite extension factor from this fraction to apparent homogeneity. The protein was characterized by NaDodSO$_4$/PAGE. In the presence of reducing agents, the protein migrated as a single band, with an apparent molecular weight of 6500. In the absence of reducing agents, the protein showed bands at apparent molecular weights of 6500, 21,000–22,000, 30,000, and 40,000. Reduction and S-carboxymethylation of the protein abolished all biological activity and resulted in a shift of the apparent molecular weight to 11,000. The amino acid composition of the purified neurite-extension factor was nearly identical to that of bovine brain S100 β. The amino acid sequences of peptides derived from trypsin or cyanogen bromide digests of the protein were identical to those found in S100 β and accounted for 71 of 91 amino acids in the protein. However, three peptides obtained from cyanogen bromide digestion of the nonreduced protein appeared to be disulfide-linked dimers. Our results indicate that a biological activity, neurite extension, which is critical for the development of the nervous system, is associated with a disulfide form of S100 β.**

The purification and characterization of proteins that influence neuronal development are essential steps in understanding the development of the nervous system. Such purifications rely on *in vitro* bioassays to identify molecules that have biological activities (1). These molecules can be divided into two broad classes: (*i*) substrate-attached materials, such as laminin (2), that stimulate neurite outgrowth, presumably by promoting adhesion to the substratum, and (*ii*) soluble molecules, such as nerve growth factor, that promote neuronal survival and/or neuronal differentiation (3). Tissue extracts or conditioned media have been used as starting materials for the purification of neurotrophic molecules, but these starting materials may contain multiple growth-promoting activities (1). Thus, the success of purifying any one of these substances depends on the specificity of the bioassay in addition to its reproducibility and sensitivity.

We have studied molecules that influence the development of neurons in the central nervous system. One of us (D.K.) has described a well-defined bioassay for neurotrophic substances active on central neurons (4). This assay quantitatively measures neurite extension from chicken embryo cerebral cortical neurons grown at low cell density in a serum-free, defined medium. By using this assay, an acidic, heat-stable protein fraction was isolated from bovine brain (4).

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In this paper, we report the purification of this neurite extension factor (NEF) to apparent homogeneity by use of reversed-phase HPLC. The amino acid sequences of peptides derived from this protein were identical to those of bovine brain S100 β (5). However, purified NEF appeared to form disulfide-linked oligomers. We suggest that neurite-extension activity is associated with a disulfide-bonded form of S100 β.

EXPERIMENTAL PROCEDURES

Isolation of Neurite Extension Factor. Bovine brain NEF was purified as described (4), with the following modifications. The first ion-exchange step was accomplished by batch adsorption in 0.2 M NaCl/0.02 M Tris Cl, pH 7.4, followed by elution with 0.4 M NaCl in the same buffer, to facilitate handling large volumes. The eluate was diluted to 0.1 M NaCl, and applied to the second ion-exchange column (4), which had been equilibrated in the same buffer. The sample was eluted with a linear gradient from 0.1 to 0.6 M NaCl, and most of the activity was eluted between 0.2 and 0.35 M NaCl. Active fractions from the second ion-exchange step were subjected to reversed-phase HPLC on a 4.6 × 150 mm column of propylsilanyl silica (Beckman RPSC) equilibrated in 0.1% (wt/vol) trifluoroacetic acid in 20% (vol/vol) acetonitrile; elution was achieved with a series of gradients and isocratic steps of acetonitrile as shown in Fig. 1. For preparative HPLC, samples were applied to the column in 0.4 ml. The overall yield of NEF protein was ≈3 mg/kg (wet weight) of bovine brain. Fractions of the HPLC effluent were prepared for bioassay by rotary evaporation in the presence of a carrier protein (bovine serum albumin or gelatin, 0.05 mg/ml) and reconstitution in water.

Assay of Neurite-Extension Activity. Neurite-extension activity was measured as described (4), using 7-day chicken embryo cerebral cortical neurons at low cell density in a serum-free, defined medium. Fractions to be assayed were added to the cultures, and after 20 hr the cells were fixed and scored for neurite extension. One unit of activity is that amount in 1 ml of culture medium that gives a half-maximal response.

Amino Acid Analysis. Amino acid analysis was performed on samples hydrolyzed *in vacuo* for 24 hr in 6 N HCl containing 1% (vol/vol) phenol. The samples were dried, derivatized with phenylisothiocyanate, and analyzed by HPLC, essentially as described (6, 7). The elution buffer was adjusted to pH 6.50 with acetic acid and the column was operated at 39°C. Cysteine was determined as *S*-carboxymethylcysteine after alkylation with iodoacetic acid. The procedure for S-carboxymethylation of the protein was that described for spinach calmodulin (8).

Protein Sequence Analysis. Automated, repetitive Edman degradations of peptide samples were performed with an Applied Biosystems 470A protein sequencer. All procedures, including identification of phenylthiohydantoin derivatives,

Abbreviation: NEF, neurite extension factor.

were done as described (9), using a Hewlett-Packard 1090A liquid chromatograph and 3392 integrator.

Purification of Peptides. Purified NEF (6 nmol) was digested with cyanogen bromide (10) or trypsin (11). The resulting peptides were separated by HPLC on a 2.1 × 150 mm column of octadecylsilanyl silica (Hewlett-Packard) equilibrated in 0.01 M HCl/10% (vol/vol) acetonitrile. The peptides were eluted with increasing concentrations of acetonitrile in the mobile phase on a Hewlett-Packard 1090A liquid chromatograph, according to the following programmed steps: 5 min, 10% acetonitrile; 10 min, 25%; 15 min, 25%; 20 min, 50%; 25 min, 50%; 30 min, 75%. The absorbance of the effluent was monitored at 210 nm, and regions of peak absorbance were collected. Absorption spectra from 200 to 350 nm were recorded for each peak, using a Hewlett-Packard diode array detector.

Polyacrylamide Gel Electrophoresis. Samples were subjected to $NaDodSO_4$/17.5% (wt/vol) PAGE using the buffer system described by Laemmli (12). Proteins were visualized by use of a photochemical silver-staining method (13). Protein standards were obtained from Bethesda Research Laboratories.

Gel Permeation Chromatography. Chromatography was performed with a Hewlett-Packard 1084B liquid chromatograph using a column (7.5 × 600 mm) of diol-silica (TSK G3000 SW, Bio-Rad) in buffer containing 0.05 M ammonium bicarbonate (pH 7.8). Elution was at 1 ml/min, and the absorbance of the eluate was monitored at 210 nm.

Materials. Adult bovine brains were obtained from Pel Freez and stored in liquid nitrogen. Solvents for HPLC were obtained from Fisher or from Burdick and Jackson (Muskegon, MI).

RESULTS

NEF was purified from bovine brain as described above, using as a final step reversed-phase HPLC on propylsilanyl silica. An example of this chromatographic separation is shown in Fig. 1. Under these conditions, the neurite-extension activity was eluted as a single, symmetrical peak at a higher acetonitrile concentration (45%, vol/vol) than did the major contaminating proteins. This procedure separated the neurite-extension activity from a protein with apparent M_r 37,000 that was not separable by gel filtration under nondenaturing conditions (4).

Purified NEF was half-maximally active at about 125 ng/ml and had a specific activity of 8000 units/mg. These values represent minimal estimates of the specific activity because the protein concentration was determined by amino acid analysis of the chromatographic effluent, whereas the activity was determined after concentration and reconstitution of the sample. The recovery of activity at this step was ≈50%. Thus, some loss of activity occurred during the concentration and reconstitution of the purified NEF in the presence of carrier protein.

Purified NEF yielded a single, broad band at M_r 6500 when subjected to electrophoresis in 17.5% polyacrylamide gels in the presence of $NaDodSO_4$ under reducing conditions (Fig. 2, lane a). In the absence of reducing agents, the preparation showed a doublet at apparent M_r 21,000–22,000 and additional bands at apparent M_r 6500, 30,000, and 40,000 (Fig. 2, lane b). In addition, the reduced NEF stained differently than the nonreduced sample. The reduced form was visible only after reexposure to the silver stain, whereas the nonreduced form was visible after the first development cycle (13). Although equal amounts of protein were analyzed, the reduced form was lighter in color than the nonreduced form after the second development cycle.

Purified NEF was also subjected to gel-permeation chromatography without added reducing or denaturing agents. Under these conditions, the protein was eluted in several peaks, with the major peak at apparent M_r 20,000, and additional peaks at higher apparent M_r (data not shown). These results, together with the results from electrophoresis, suggested that the protein might form disulfide-bonded oligomers with a subunit $M_r \approx 10{,}000$.

To characterize the biological activity of the reduced NEF monomer, the protein was reduced and alkylated with iodoacetic acid under denaturing conditions. Upon analysis by PAGE, the protein migrated as a broad band at apparent M_r 11,000. The S-carboxymethylated protein was tested for biological activity in the neurite-extension assay and was found to have no activity at levels 40 times those required for half-maximal stimulation by unmodified NEF.

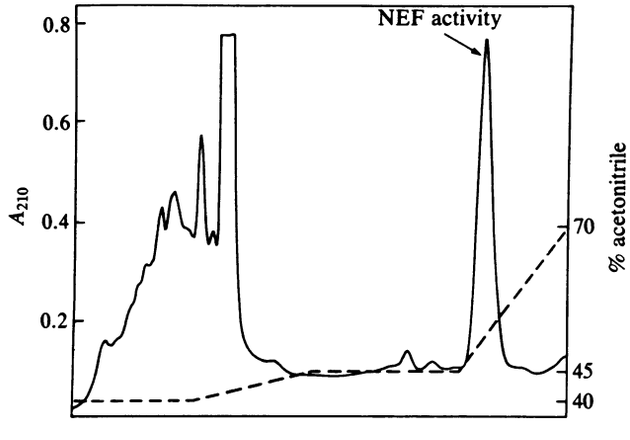

FIG. 1. Purification of NEF by reversed-phase HPLC. A 25-μl aliquot of a biologically active fraction of the eluate from the second ion-exchange step was applied to a 4.6 × 150 mm column of propylsilanyl silica equilibrated in 0.1% (wt/vol) trifluoroacetic acid/20% (vol/vol) acetonitrile and eluted with a series of gradient and isocratic steps of increasing acetonitrile concentrations: 5 min, 20%; 10 min, 20–40%; 15 min, 40%; 20 min, 40–45%; 25 min, 45%; 30 min, 45–70%. The absorbance of the effluent was monitored at 210 nm. The peak containing biologically active NEF was eluted at 45% (vol/vol) acetonitrile (arrow).

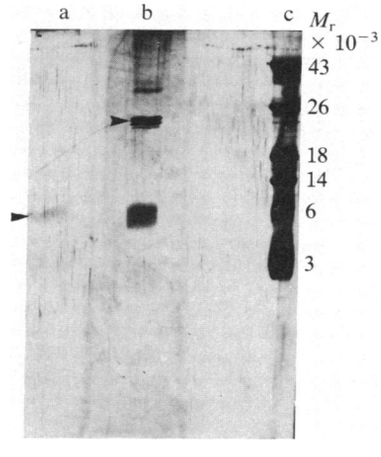

FIG. 2. $NaDodSO_4$/PAGE of NEF. Purified NEF (6 μg) was treated with (lane a) or without (lane b) 5% (vol/vol) 2-mercaptoethanol in sample buffer (12) containing 1% (wt/vol) $NaDodSO_4$ for 5 min at 90°C and subjected to electrophoresis in a 17.5% (wt/vol) polyacrylamide gel in the presence of $NaDodSO_4$. Arrowheads indicate the monomer (lane a) and the proposed dimer (lane b) of NEF. The molecular weight standards (lane c; 5 μg each) were ovalbumin (M_r 43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), lysozyme (13,300), bovine trypsin inhibitor (6200), and insulin (3000). The gel was silver-stained by a photochemical procedure (13).

To further characterize the structure of NEF, we subjected the purified protein to amino acid analysis and partial amino acid sequence determination. As shown in Table 1, the amino acid composition of NEF was similar to that of bovine brain S100 β (5), with no detectable tryptophan, an amino acid found in S100 α but not in S100 β (5, 14). Sequential Edman degradation of the intact protein released no detectable phenylthiohydantoin amino acid derivatives, indicating that the protein has a blocked amino terminus. Therefore, the protein was digested with trypsin or cyanogen bromide, and the resulting peptides were isolated by reversed-phase HPLC. Fig. 3 gives a summary of the peptides isolated and their amino acid sequences. All of the peptides isolated had amino acid sequences identical to those found in bovine brain S100 β (5). These structural data provide evidence for the homogeneity of the preparation but do not exclude the possibility that there is biological activity in a minor contaminant present at levels below the limits of detection of our techniques.

The cyanogen bromide digest of NEF yielded three peptides that were not identified in previous studies of S100 β (5). One of the additional peptides had an amino acid sequence identical to that of peptide CB 58–74, a second had a sequence identical to that of CB 80–91, and the third showed two sequences in equimolar amounts identical to both residues 58–74 and residues 80–91. These peptides were eluted at 35–50% (vol/vol) acetonitrile, a concentration significantly higher than that (10–25%) required to elute CB 58–74, CB 75–79, and CB 80–91. These new peptides were not observed when the S-carboxymethylated protein was subjected to cyanogen bromide digestion.

DISCUSSION

In the present paper, we have described the purification to apparent chemical homogeneity of a soluble protein, NEF, that is active in a well-defined assay (4). This assay measures factors that stimulate neurite extension from individual neurons in cell culture. Neurite extension could be either a primary response or a phenomenon secondary to an overall metabolic effect on these cells. Differentiation of these possibilities depends on understanding the molecular structure and mechanism of action of NEF.

Table 1. Amino acid composition of bovine brain NEF

	Residues, mol per 10,507 g of NEF	Residues in S100 β*
Aspartic acid	8.3	9
Glutamic acid	20.7	19
Serine	2.5	5
Glycine	6.6	4
Histidine	5.0	5
Arginine	1.2	1
Threonine	2.2	3
Alanine	5.5	5
Proline	<0.3	0
Tyrosine	0.8	1
Valine	6.7	7
Methionine†	2.9	3
Isoleucine	4.0	4
Leucine	8.7	8
Phenylalanine	6.7	7
Lysine	7.8	8
Tryptophan	0.0‡	0
Cysteine§	2.0	2

*Calculated from the amino acid sequence of bovine brain S100 β (5).
†Calculated as the sum of methionine and methionine sulfoxide.
‡Excluded spectrophotometrically.
§Determined as *S*-carboxymethylcysteine after modification with iodoacetic acid.

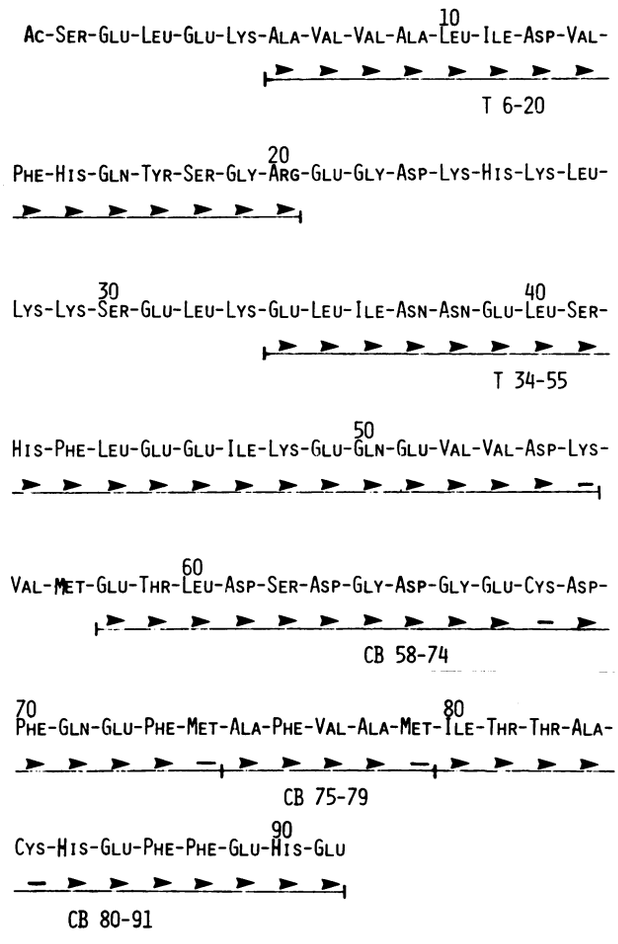

FIG. 3. Amino acid sequence analysis of NEF. The amino acid sequence of bovine brain S100 β (5, 14) is shown, and peptides obtained from trypsin (T) or cyanogen bromide (CB) treatment of NEF are aligned with that sequence. Arrowheads indicate identification of the amino acid residue by automated, repetitive Edman degradation. Dashes indicate the inference of the identity of the residue from amino acid composition.

Characterization of NEF indicated that the primary structure of the protein is similar to that of bovine brain S100 β (5). NEF is an acidic protein that exhibits an anomalous mobility on PAGE in the presence of $NaDodSO_4$ and reducing agents, as does S100 β (15). After reduction and S-carboxymethylation, NEF protein had an apparent molecular weight of 11,000 by electrophoresis, close to the formula molecular weight of S100 β, 10,507 (5). The amino acid composition of NEF was indistinguishable from that of S100 β. All of the peptides obtained from digestion of the protein had amino acid sequences that were identical to those of bovine brain S100 β. These sequences accounted for 71 of the 91 amino acids in S100 β. These data indicate that the primary structure of NEF is identical to or nearly identical to that of bovine brain S100 β.

Several lines of evidence indicate that the biologically active species of NEF contains disulfide bonds. First, all biological activity was lost after reduction and alkylation of the cysteine residues in the protein. Second, electrophoresis of NEF in the absence of reducing agents resulted in several bands at apparent molecular weights higher than that of the reduced form of the protein. Third, the elution profile showed several peaks when NEF was subjected to gel-permeation chromatography in the absence of reducing and denaturing

agents. Thus, NEF may form monomers containing one disulfide bond or form disulfide-linked dimers, trimers, or higher oligomers. Disulfide-linked oligomers have been observed in partially purified S100 protein fractions (16, 17). However, it is possible that some oligomer formation may be due to noncovalent interactions between S100 subunits (14). A commercial preparation (Calbiochem) of S100 protein had no detectable activity in the neurite-extension assay at concentrations of 0.1–10 μg/ml.

The characterization of the structure of purified NEF supports our suggestion that disulfide bonds exist in the biologically active protein. The purification protocol was designed to optimize the recovery of activity, and the buffers employed contained no added reducing or chelating agents. This protocol resulted in the isolation of a protein that had biological activity in the neurite-extension assay, had a primary structure similar to S100 β, and had multiple disulfide-bonded forms. Following digestion of NEF with cyanogen bromide, three peptides were identified that had not been previously described for S100 β (5) and that were not observed in CNBr digests of S-carboxymethylated NEF. Two of these peptides had amino acid sequences identical to peptide CB 58–74 and peptide CB 80–91, respectively (Fig. 3), and thus may be dimers of peptides with disulfide bonds between cysteine residues at identical positions in the monomers $\begin{pmatrix}\text{-Cys}^{68}\text{-} & \text{-Cys}^{84}\text{-}\\ \text{-Cys}^{68}\text{-} & \text{-Cys}^{84}\text{-}\end{pmatrix}$ or $\begin{pmatrix}\text{-Cys}^{68}\text{-} \\ \text{-Cys}^{84}\text{-}\end{pmatrix}$. Such dimers may be derived from parallel dimers of S100 β (5). In addition, one peptide was isolated that showed the sequence of both peptide CB 58–74 and peptide CB 80-91, suggesting that disulfide bonds may also form between cysteines at two different positions in the molecule $\left(\text{-Cys}^{68}\text{- . . . -Cys}^{84}\text{- or } \begin{matrix}\text{-Cys}^{68}\text{-} \\ \text{-Cys}^{84}\text{-}\end{matrix}\right)$. This peptide may be derived from an intrachain disulfide monomer, an antiparallel dimer, or from higher oligomers of S100 β. Such disulfide bonding may account for the two species with apparent M_r 21,000–22,000 observed on PAGE. This type of parallel and antiparallel disulfide dimerization has been described for human atrial natriuretic polypeptides (18) and for human fibrinogen (19). The relative potencies for neurite-extension activity of the disulfide-bonded species of NEF were not established by our experiments. Nevertheless, a disulfide form of the protein is required for biological activity in the neurite-extension assay.

Our analysis of the structure of NEF indicated that it is a form of S100 β. The name S100 β refers to one species identified (5, 15) as part of the S100 fraction originally isolated from bovine brain (20) on the basis of structural properties. In contrast, our purification was designed to isolate an activity fundamental to the development of the nervous system, the extension of neural processes. Our finding that this biological activity resides in a molecule that is structurally related to S100 β is consistent with earlier studies that suggested that S100 protein had a role in the development of the nervous system. The quantity of S100 protein increased during those periods of chicken embryo brain development when processes were elongating (21). In addition, levels of translatable mRNA for S100 β increased during similar periods of development of the rat brain (22). Thus, the neurite-extension activity that we observe in cell culture may reflect an activity of S100 β protein *in vivo*.

We propose that glial cells in the central nervous system secrete NEF, a form of S100 β, to stimulate the outgrowth of neural processes during development of the nervous system. Conditioned medium from C6 rat glioma cell cultures contains neurite-extension activity (23). C6 cells are known to contain S100 protein (24). Secretion of S100 from another cell line has been described (25). Our proposal that glial cells in the central nervous system secrete NEF during development does not exclude the possibilities that (*i*) neurons may have endogenous S100 protein, (*ii*) other neurotrophic factors exist in the nervous system, (*iii*) S100 proteins have other physiological activities in the nervous system or other tissues, or (*iv*) NEF may be active in the peripheral nervous system.

The primary structure of S100 β is highly conserved among vertebrates (26, 27). This evolutionary conservation implies that the protein has a critical physiological role in the organism. Neurite extension may be one physiological activity of S100 β that is essential to the development of the vertebrate nervous system. Additionally, S100 β has been found in tissues outside the nervous system (28) and may have other physiological roles in those tissues. Furthermore, S100 proteins are members of a class of structurally and functionally related calcium-modulated proteins (29) that bind calcium ions reversibly under physiological conditions. S100 β has been shown to bind zinc ions with higher affinity than it does calcium ions (30). This observation may be relevant to neurite extension in light of recent suggestions that zinc ions are associated with neurotrophic activities (31). Our studies of the structure and function of NEF provide the foundation for continuing studies of the mechanism of action of this protein.

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