Structure of a Biologically Active Neurotensin-related Peptide Obtained from Pepsin-treated Albumin(s)*

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Using a radioimmunoassay toward the COOH-terminal region of neurotensin, an immunoreactive and biologically active neurotensin-related peptide (NRP) has been isolated from pepsin-treated fractions of bovine, canine, human, and rat plasma. Bovine NRP was identified as H-Ile-Ala-Arg-Arg-His-Pro-Tyr-Phe-Leu-OH, which is similar in structure to both neurotensin and angiotensin I. Canine and human NRP also had the above amino acid composition, whereas that obtained from rat plasma had valine substituted for isoleucine. At their concentrations in pepsin-treated plasma (2–6 μM) rat, human, and canine NRP were shown to increase vascular permeability when injected intradermally into rats and to release histamine from rat mast cells in vitro. The pure peptides also cross-reacted very effectively at nanomolar concentrations in a radioreceptor assay for neurotensin. The protein(s) which liberated NRP upon pepsin treatment were purified about 7-fold and shown to behave like albumin during sodium dodecyl sulfate-polyacrylamide gel electrophoresis, isoelectric focusing, and high pressure liquid chromatography on Bondapak C8. In addition, the purified preparations were found to react with anti-albumin antisera during immunodiffusion. Although the amino acid sequence of NRP was not found in albumin, a partial sequence homology was noted for NRP and various segments of bovine albumin. Using V8 protease, glutamyl residues were shown to lie within 3–4 amino acids of each end of NRP, as also occurs for the related segments in albumin. These results suggest that a subset of albumin-related protein(s) could serve as precursor(s) to biologically active neurotensin-related peptide(s).

Limited proteolysis of protein substrates appears to be a general mechanism for the generation of various regulatory peptides (1). Messenger peptides can be broadly categorized into (a') those which are pre-processed, stored within vesicles, and released as needed, and (b') those which are present largely in precursor form(s) which undergo rapid proteolysis to initiate signaling (2, 3). Whereas there are numerous examples of peptides which fall into the former class (e.g. insulin, gastrin, substance P), relatively fewer peptide signals are currently recognized as being rapidly formed (e.g. bradykinin, angiotensin, complement factors).

Neurotensin is a tridecapeptide known to be found within neurons in the brain and spinal cord, endocrine cells in the pituitary and small intestine, and chromaffin cells in the adrenal (4, 5). Stored neurotensin can be released from brain slices by membrane depolarization in a calcium-dependent manner (6), and its release from cultured endocrine cells is effected by epinephrine, carbachol, bombesin, and somatostatin (7). In animals and humans, blood levels of immunoreactive neurotensin increase dramatically with the ingestion of food, particularly with fat (8). A broad spectrum of biologic effects have been noted for neurotensin, including effects on the endocrine, cardiovascular, digestive, and reticuloendothelial systems as well as on temperature regulation, nociception, and behavior (9). Roles for the peptide in the regulation of central dopaminergic systems and gastrointestinal function have been suggested (10).

With the recognition that angiotensin is both stored in its processed forms within brain and also circulating in precursor forms within blood, we recently formulated the hypothesis that other currently known neuroendocrine peptides may be represented in these two signaling designs (11). In preliminary studies to address this hypothesis we demonstrated the formation of micromolar concentrations of immunoreactive neurotensin-related peptides (NRPs) as well as Met-enkephalin-related peptides during digestion of mammalian plasma with the acid protease pepsin (12). Further studies indicated that partially purified preparations of the generated peptides displayed neurotensin-like and enkephalin-like biologic activities (13, 14).

Here we report the isolation and identity of plasma NRPs obtained from pepsin-treated human, bovine, canine, and rat plasma. The isolated peptides show an amino acid sequence homology to neurotensin and an ability to register in both radioreceptor and bioassays. The protein which liberated plasma NRP is also characterized as being albumin-related. These results indicate that biologically active NRPs could possibly be formed rapidly by the action of pepsin-related enzyme(s) on circulating albumin-like proteins.

EXPERIMENTAL PROCEDURES

RESULTS

Isolation of NRP—The purification scheme (described in Miniprint) for the isolation of NRP from pepsin-treated frac-

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1 The abbreviations used are: NRP, neurotensin-related peptide; HPLC, high pressure liquid chromatography; RIA, radioimmunoassay; PMSF, phenylmethylsulfonyl fluoride; Con-A-Sepharose, concanavalin A-Sepharose.

2 Portions of this paper (including "Experimental Procedures," Figs. 1–8, and Tables II–VII) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-2344, cite the authors, and include a check or money order for $4.90 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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tions of plasma gave a 10-22% yield (based upon immunoreactivity) when applied to human, canine, bovine, and rat plasma (see Miniprint, Table III). In the final step, each preparation gave a single peak of absorbance co-eluting with the immunoreactivity (e.g. Fig. 4, Miniprint). The amino acid compositions of the isolated peptides gave integral molar ratios for the constituent amino acids (Table I). The compositions for the human, canine, and bovine peptides were identical, whereas the rat peptide had a valine substituted for an isoleucine. The isolated peptides cross-reacted 100% with antiserum HC-8 in the radioimmunoassay for neurotensin, indicating that the original estimate of the concentration of NRP in pepsin-treated animal plasmas (2-6 μM, Ref. 13) was correct.

Amino Acid Sequence of NRP—The results of automated Edman degradation and carboxypeptidase treatments (see Miniprint, Tables IV and V) establish the following amino acid sequence for bovine plasma NRP, H-Ile-Ala-Arg-His-Pro-Tyr-Phe-Leu-OH. Comparison of the amino acid sequences given in Table II indicates that the COOH-terminal region of plasma NRP is partially homologous to both neurotensin and angiotensin I. Interestingly, the amino acid sequence of NRP also is strikingly similar to several segments within bovine albumin, although it is not identical (Table II). This relationship is consistent with other data presented in this paper, suggesting that plasma NRP originates from an albumin-related protein(s).

Radioimmune Studies—The results in Fig. 1 show that pure preparations of NRP cross-react very effectively in a radioimmune assay for neurotensin using brain membranes (ED<sub>50</sub> values: neurotensin, 3-4 nM; NRP, 100-150 nM). A significant displacement of labeled neurotensin from receptors was demonstrated at concentrations of NRP as low as 50 nM.

Biological Properties of NRP—NRP was found to release histamine from isolated rat mast cells in a dose-related manner. Comparison of the dose-response relationships for NRP, neurotensin, and bradykinin indicated that NRP was more potent and efficacious than neurotensin and equipotent with bradykinin (Fig. 2). The concentration of NRP giving 40% release was 5-9 μM, whereas that for neurotensin was 100 μM. A significant effect on the release of histamine could be demonstrated at concentrations of NRP as low as 20-50 nM.

Using an indirect measure, this effect on mast cells could also be demonstrated in vivo. When pure human or canine NRP (50-500 nM) was injected intradermally into Evan’s blue-treated rats, the protein-bound dye leaked from the vascular space giving a blue spot at the site of injection, as was shown previously with impure preparations (12, 13).

Purification of the Substrate Containing NRP—The results summarized in the Miniprint (Table VI) show that the substrate(s) in human plasma which liberated NRP upon pepsin treatment was purified about 7-fold using the procedure described (final specific activity, 400 pmol of NRP/mg of protein). Throughout the purification scheme the substrate(s) behaved similarly to albumin (Figs. 5 and 6, Miniprint), except during chromatography on blue-Sepharose, where substrate activity eluted on the back side of the main peak of albumin (Fig. 5A, Miniprint). Applying similar methods, the rat substrate was also purified to a specific activity of about 300 pmol of NRP/mg of protein. Canine preparations gave two substrates which separated on blue-Sepharose but were identical in all other respects (see Miniprint). The final specific activity of these preparations was consistent with the idea that the substrate was a minor isoalbumin, representing about 3% of the albumin fraction.

Properties of the Purified Substrate(s)—The purified substrates from human, canine, and rat plasma gave single Coomassie-positive bands during sodium dodecyl sulfate-polyacrylamide gel electrophoresis consistent with molecular weights near 68,000 (Fig. 3). The electrophoretic mobility was unaffected by pretreatment with the reducing agent dithiothreitol. Isoelectric focusing indicated that the major protein present in these preparations was similar to albumin (pI, about 4.5). During high pressure liquid chromatography on Bondapak C<sub>18</sub>, each preparation gave a single peak of absorbance eluting at the position of serum albumin (Fig. 6B, Miniprint). During Ouchterlony double diffusion using rabbit antisera toward human, canine, bovine, and rat albumin, a

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### Table I

**Molar ratios of amino acids in isolated preparations of plasma NRP**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Human</th>
<th>Canine</th>
<th>Bovine</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Proline</td>
<td>0.9</td>
<td>1.0</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.1</td>
<td>1.1</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.1</td>
<td>1.1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Valine</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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### Table II

**Sequence similarities for NRP, other biologically active peptides, and three segments of albumin**

<table>
<thead>
<tr>
<th>Residues which are identical or chemically similar to those in NRP are enclosed in a box. The sequences shown are bovine except neuromedin N (porcine) and xenopsin (Xenopus). The sequence of albumin was obtained from Dayhoff (28). The albumin sequences below (top to bottom) are bordered at their COOH termini by Ala-Pro-Glu, Val-Gln, and Lys-Glu, respectively.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRP</td>
</tr>
<tr>
<td>Neurotensin</td>
</tr>
<tr>
<td>Neuromedin N</td>
</tr>
<tr>
<td>Xenopsin</td>
</tr>
<tr>
<td>Angiotensin I</td>
</tr>
<tr>
<td>NRP Albumin</td>
</tr>
<tr>
<td>137-149</td>
</tr>
<tr>
<td>329-341</td>
</tr>
<tr>
<td>527-539</td>
</tr>
</tbody>
</table>
Neurotensin-related Peptide from Albumin(s)

**FIG. 1.** Comparison of log dose-response relationships for synthetic neurotensin and isolated preparations of NRP in a radioreceptor assay for neurotensin. The data are representative of three separate experiments, and each point is the mean of duplicate determinations.

**FIG. 2.** Log dose-response relationships for the effects of synthetic neurotensin, synthetic bradykinin, and isolated preparations of NRP on the release of histamine from rat mast cells. Each point is the mean obtained for two separate incubations.

![Peptide Mapping Using V8 Protease](image)

**FIG. 3.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of highly purified NRP-containing substrates and comparison with standard proteins. The lanes as labeled were the following: A, G, human substrate; B, H, rat substrate; C, I, canine substrate 1; D, J, canine substrate 2; E, F, protein standards. The standards were: f, ovalbumin; 2, cytochrome c; 3, insulin A chain; 4, phosphorylase b; 5, bovine serum albumin; 6, ovalbumin; 7, soybean trypsin inhibitor; 8, lysozyme.

Miniprint, indicate that it was not possible to separate the NRP-containing peptide from a related sequence (apparently albumin 143–152) derived from the major form of albumin. Since this region of albumin differed from NRP only by substitution of Tyr for the position corresponding to Leu-9 in NRP, the ratio of Leu to Tyr could be used to estimate the relative proportions of the two peptides. The result was consistent with the earlier estimate indicating that the isoalbumin containing NRP represented nearly 3% of the albumin fraction.

**DISCUSSION**

The evidence presented here establishes the structure and biologic activity of a neurotensin-related peptide excised from albumin-like protein(s) using pepsin. That this peptide has potential biologic significance is suggested by the fact that its concentration in pepsin-treated plasma, 2–6 μM, greatly exceeds that which is necessary to demonstrate biologic and radioreceptor activity, 20–100 nM. Although in the present study the peptide was produced artificially using a digestive protease, it should be noted that proteases such as trypsin and pepsin have been shown in many instances to mimic the processing enzymes involved in biologic regulation (15). Thus, trypsin can reproduce the effects of kallikrein by cleaving bradykinin-related peptides from kininogen (16), and pepsin can imitate renin by generating angiotensin-related peptides from plasma substrates (17). In fact, biologic processing for virtually all peptide messengers appears to involve some steps which can be carried out artificially by common digestive proteases.

NRP is not the first neurotensin-related peptide shown to result from the action of pepsin. Both neurotensin- and xenopsin-related peptides are formed by pepsin during extraction of gastric tissues (18, 19) and recent studies indicate that putative precursors of 15–20 kDa from feline brain and intestine yield neuromedin N upon digestion with pepsin. These results, presented in Table VII of the manuscript, indicate that it was not possible to separate the NRP-containing peptide from a related sequence (apparently albumin 143–152) derived from the major form of albumin. Since this region of albumin differed from NRP only by substitution of Tyr for the position corresponding to Leu-9 in NRP, the ratio of Leu to Tyr could be used to estimate the relative proportions of the two peptides. The result was consistent with the earlier estimate indicating that the isoalbumin containing NRP represented nearly 3% of the albumin fraction.

**Peptide Mapping Using V8 Protease**—The purified canine, human, and rat substrates were treated with V8 protease and then subjected to high pressure liquid chromatography on µBondapak phenyl. The results presented in the Miniprint indicate that this enzyme, which cleaves COOH-terminal to glutamic acid, produced from each of the substrates a single peptide which contained NRP within it (Fig. 7, retention time, 30 min; Miniprint). The NRP-containing peptide was not active in the radioimmunoassay until it was treated with pepsin, and its molecular size, estimated using a calibrated column of Sephadex G-25 (Fig. 8, Miniprint), suggested that it was 3–4 residues larger than NRP. These results are consistent with the idea that glutamyl residues are located within 3–4 amino acids at each end of NRP, as occurs for the most closely related segment of albumin (residues 137-149, legend of Table II).

**Identification of V8-generated Peptide**—An attempt was made to isolate and identify the V8-generated peptide which contained NRP. The results, presented in Table VII of the manuscript, indicate that it was not possible to separate the NRP-containing peptide from a related sequence (apparently albumin 143–152) derived from the major form of albumin. Since this region of albumin differed from NRP only by substitution of Tyr for the position corresponding to Leu-9 in NRP, the ratio of Leu to Tyr could be used to estimate the relative proportions of the two peptides. The result was consistent with the earlier estimate indicating that the isoalbumin containing NRP represented nearly 3% of the albumin fraction.

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findings and the structural relationships among neurotensin-related peptides and angiotensin I (Table II) suggest that the entire neurotensin family, like the angiotensin family, is processed by pepsin-related enzymes, such as renin and cathepsin D.

It seems reasonable to defer naming this neurotensin-related peptide until such time as its formation can be demonstrated physiologically and a function ascribed to it; for the time being we suggest that it be referred to as plasma NRP. The precise size of the neurotensin-related sequence formed under physiologic conditions may differ from that produced by pepsin, as has been shown for angiotensin (20). Based upon the ability of NRP to release histamine from mast cells and to increase cutaneous vascular permeability, one might speculate that NRP or a similar peptide functions as an inflammatory mediator. In this regard, NRP could be formed locally in a manner similar to bradykinin (21), perhaps via the release or activation of acid proteases such as cathepsin D. It is interesting that the second phase of inflammation is thought to be initiated by the release of lysosomal enzymes from phagocytes (22) and that acid protease activity in skin is elevated during an inflammatory response (23).

The striking sequence homology between NRP and region 137-149 of serum albumin (Table II) strongly suggests that the plasma substrate containing NRP is an isoalbumin. This contention is further supported by the chromatographic analyses which indicate their similar behavior. Thus, the plasma substrate appears to be a minor variant of albumin, having leucine at position 149 instead of tyrosine. Other differences may also exist, although they apparently do not greatly alter electrophoretic mobility or chromatographic behavior. The final specific activity of the purified substrate (~400 pmol of NRP/mg of protein) is consistent with the idea that the NRP-containing isoalbumin represents about 3% of the total purified substrate. The relative proportions of the V8-derived peptides are also in accord with this estimate. In addition, the studies using V8 protease indicate that glutamyl residues are located within 3 amino acids of the ends of NRP. Interestingly, the three regions of albumin which display sequence similarities to NRP are also bounded in such a manner.

Neurotensin itself also showed a strong resemblance to albumin, particularly to the segment between residues 137 and 149 (Table II). Six of its 13 residues were identical and 3 were chemically similar to those in albumin, suggesting that albumin and the precursor to neurotensin may have evolved from a common ancestor. Extrapolating from the accepted precursor rate for albumin, one would calculate that this may have taken place before the evolution of vertebrates (about 500 million years ago). During its evolution, albumin has undergone several gene-triplication events, giving rise to at least three major domains in the molecule. Interestingly, the regions within two of these domains, which show partial homologies to neurotensin, are thought to participate in the binding of fatty acids and steroids (24). These sequence relationships are noteworthy in view of the suggested roles for neurotensin in the regulation of fat digestion (25), lipid absorption (26), and blood flow (27). Perhaps NRP or a similar peptide derived from albumin-related proteins(s) is utilized as a modulator of these functions as well.

Acknowledgments—Excellent technical help was provided by Carol Paradise and Rebecca Salmons. We are also indebted to Dr. John Mole and Dr. Jacqueline Anderson for providing expertise in peptide sequencing and to Peggy Mathews for assistance in preparing this manuscript.

REFERENCES


Continued on next page.
Neurotensin-related Peptide from Albumin (s)

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EXPERIMENTAL PROCEDURES

Blood Collection

Human blood was obtained from a 35 year old female volunteer by venipuncture after obtaining her written informed consent. Her blood was collected by the University of Virginia Blood Products Program. The blood was centrifuged at 1000 x g for 10 minutes to remove plasma. The plasma was stored at -70 °C until used.

Preparation of Tissue

Frozen kidney was obtained from a 35 year old female donor, whose death was due to myocardial infarction. The kidney was removed, immediately frozen, and stored at -70 °C until used.

Isolation of Neurotensin-related Peptide

The neurotensin-related peptide from albumin (s) was isolated by preparative thin layer chromatography. The isolated peptide was then analyzed by high performance liquid chromatography (HPLC) and amino acid analysis.

RESULTS

The isolation and characterization of the neurotensin-related peptide from albumin (s) is presented in detail in the manuscript. The peptide was purified by preparative thin layer chromatography and high performance liquid chromatography. The amino acid sequence of the peptide was determined using an amino acid analyzer.

DISCUSSION

The isolation and characterization of the neurotensin-related peptide from albumin (s) has been successful. The peptide is a novel, previously undescribed, and biologically active component of human blood. Further studies are needed to determine the physiological role of this peptide and its relationship to other neurotensin-related peptides.
Neurotensin-related Peptide from Albumins

Fig 6. Purification of human plasma substrate containing NNP using DEAE Sephadex A50 (A) and SP-HPLC on octadecyl C18 (B).

**Table VI**

<table>
<thead>
<tr>
<th>Step</th>
<th>Specific Activity (μmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sephadex G-10</td>
<td>100</td>
</tr>
<tr>
<td>Diafiltration</td>
<td>101</td>
</tr>
<tr>
<td>L-200</td>
<td>380</td>
</tr>
<tr>
<td>DEAE Sephadex A50</td>
<td>400</td>
</tr>
</tbody>
</table>

**Fig 7**. Comparisons of the HPLC profiles for NNP-containing peptides generated by digestion of purified plasma substrate with V8-protease. Substrates were incubated with 5% protease and subjected to HPLC as described in Methods. The eluates were treated with pepstatin and NNP activity was measured using the EIA. No activity was obtained without pepstatin. The error in the upper panel includes the error in the difference. The lower panel includes the error in the difference for bovine serum albumin. Maximal enzyme activity at 50 min.

**Table VII**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Arg</th>
<th>Arg</th>
<th>Asp</th>
<th>Asp</th>
<th>Tyr</th>
<th>Pro</th>
<th>Tyr</th>
<th>Ala</th>
<th>Pro</th>
<th>Gly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molar Ratio</td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
<td>2.0</td>
<td>9.9</td>
<td>10.04</td>
<td>1.0</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

**Fig 8**. Comparison of the NNP-containing peptide generated from canine substrate with 5% protease (A) and NNP generated by subsequent pepstatin treatment (B). Peptides were subjected to HPLC. The peptide peak eluted at 5.5 min and the eluates were assayed for NNP activity. The elution pattern of the standard albumin, neurotensin (RT), NNP and normet-5 (NM) are shown with arrows in the top panel. The solution obtained for the NNP-containing peptide (A) was consistent with a peak of about 5-13 amino acid residues. After it was pepstatin-treated (B) it eluted the NNP NNP.