Characteristics of a precursor to vasopressin-associated bovine neurophysin

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1. INTRODUCTION

The neurophysin produced by vasopressinergic neurons of the ox has been called vasopressin-associated bovine neurophysin (VP-BNP) [1] and it has long been suspected that such neurophysins are initially translated as part of the prohormone structure of vasopressin [2,3,4; for reviews see 5,6]. Some elegant work was recently performed to obtain the sequence of a cDNA encoding both vasopressin and its neurophysin from bovine hypothalamus [7]. This provided proof that both molecules arise as part of the same precursor. The primary structure of this precursor derived from a knowledge of the nucleotide sequence of an mRNA, places vasopressin on the N-terminal side of VP-BNP and joined to it by the tripeptide sequence, -Gly-Lys-Arg-. A 39-residue glycopeptide is bridged to the C-terminus of VP-BNP by an Arg. On the N-terminal side of vasopressin there is a 19-residue peptide believed to represent the leader sequence of the translated prohormone. The prohormone (possibly minus the leader sequence) is believed to be processed within neurosecretory granules during transport from the perikarya of neurons to axonal storage sites in the neural lobe from where the products of intragranular proteolysis, vasopressin and VP-BNP, are released [5,6]. We have earlier demonstrated the presence in neurosecretory granules of an enzyme which might be involved in such a transformation [8]. Here we describe the characteristics of precursors to VP-BNP which were isolated from bovine neural lobes. These proteins provide us with a partial confirmation of the structure derived for the prohormone from nucleotide sequencing, and also provide information concerning the manner in which processing from prohormone to products might occur within the neurosecretory granules.

2. MATERIALS AND METHODS

Fresh frozen bovine neural lobes were extracted as in [9], in 0.1 M HCl at pH 1.5 in order to destroy proteolytic activity [10]. Extracts were processed through 2.0 × 60 cm columns of Sephadex G-25 equilibrated with 1.0 M formic acid and the protein fraction was lyophilised. Protein was reconstituted in 10 mM ammonium acetate – 0.15 M NaCl (pH 5.7) and passed through a 1.0 × 10 cm column of Sepharose-bound Concanavalin A (ConA-Sepharose 4B, Sigma). The resin was washed with 5 column volumes of acetate, and bound material then eluted with concentrations of 0.05 M and 0.5 M α-methyl-D-mannoside in the 10 mM acetate buffer. Material removed from the ConA-Sepharose by 0.05 M α-methyl-D-mannoside (~10 mg protein) was dialysed against 4 changes of 100 volumes of 0.1 M acetic acid, lyophilised, reconstituted in 5 ml of 0.1 M acetic acid, and passed through a 1.2 × 60 cm column of Sephadex G-75 superfine. The eluant from this gel filtration was combined on the basis of absorbance at 280 nm to give 5 protein fractions. These fractions were represented by absorbance peaks having app. M_r of 65000, 30000, 17000, 10000 and 1000

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by reference to the elution of bovine serum albumin, pepsin (pig), chymotrypsinogen (bovine), cytochrome *c* (emu) and vasopressin from such columns. Aliquots of each fraction were reduced and S-alkylated using *N*-ethylmaleimide [10] and subjected to sodium dodecyl sulphate (SDS)-electrophoresis on 12.5% polyacrylamide [12] with ^{14}C-labelled molecular markers purchased from Amersham (CFA 626, CFA 645). The fraction from Sephadex which corresponded to the M_r range from 20000–12000 (app. M_r of V_{max}, 17000) was found on SDS-electrophoresis to comprise a single major protein component of M_r 16000 with about 10% contamination by proteins of M_r 20000 and 12000. One-half of the protein in this fraction (0.5 mg) was reduced with 2-mercaptoethanol (0.7 mmol) for 4 h and S-alkylated with ^{14}C-labelled iodoacetamide (1.44 mmol, 0.17 mCi/ml) in the presence of 8 M urea at pH 8.6 as in [1]. The S-alkylated mixture was passed through a 1.0 × 60 cm column of Sephadex G-50 superfine wrapped in aluminum foil to eliminate light and the eluant monitored for ^{14}C. This chromatography clearly resolved 3 peaks of radioactivity in the protein region, 2 minor peaks on either side of a major peak. The major peak represented > 80% of the ^{14}C counts and the protein in this peak which displayed a M_r on SDS-electrophoresis of 16000 was used for further analysis. Some of this material (100 μg, ~7 nmol) was hydrolysed in constant boiling HCl for 40 h at 110°C and subjected to amino acid analysis on a Beckman 121MB amino acid analyzer. Another sample (300 μg, ~20 nmol) was subjected to automated amino acid sequence analysis on a Beckman 890C Sequencer using a 0.1 M Quadrol buffer system [13]. All PTH-amino acid were identified by using high-pressure liquid chromatography (HPLC) and ^{14}C was counted in a Packard scintillation spectrometer, model 3255 (1). PTH-amino acid reference standards used were obtained from Sigma. HPLC was performed on a Beckman unit, model 332 plus model 100A pump, an Hitachi model 155-40 variable wavelength spectrophotometer with flow cell, and a model C-RIA Shimadzu Integrator-recorder. Automation in sample handling was possible through use of a WISP 710A automatic sample injector from Waters Associates. The remaining S-alkylated protein (100 μg, ~7 nmol) was subjected to digestion with Carboxypeptidase Y (Pierce, 60 units/mg) at 40°C for 15 min and 30 min using a substrate concentration of 0.1 mM and an E/S molar ratio of 1:400 at pH 5.5. In each case the reaction was stopped by adding sulphosalicylic acid to a final concentration of 2.5%. Each sample was centrifuged at 10 000 × *g* for 2 min, the supernatants filtered through 0.22 micron filters, and then subjected to amino acid analysis using a 200 min lithium citrate physiological gradient on the Beckman 121MB analyzer. Control studies were performed with enzyme alone, and with VP-BNP as substrate. A small amount of non-modified protein (~50 μg, ~3 nmol) from the Sephadex G-75 fraction was reacted with 3 × recrystalled α-chymotrypsin (Worthington Biochemicals) at a substrate concentration of 0.1 mM and E/S molar ratio of 1:100 for 4 h at 40°C and pH 7.6. The product was passed through a 1.5 × 2 cm column of ConA-Sepharose at pH 5.7 and bound material eluted as earlier described. This material was divided into two fractions: one was hydrolysed in constant boiling HCl for 40 h at 110°C and subjected to amino acid analysis, the other underwent partial sequencing on the 890C Beckman sequencer.

3. RESULTS

As judged by the criterion of SDS-electrophoresis 13 proteins ranging from M_r > 65000–> 10000 were bound to ConA-Sepharose and displaced by α-methyl-D-mannoside (fig.1). They were therefore, likely, glycoproteins. Sephadex G-75 filtration resolved these glycoproteins into 5 fractions, one of which chiefly comprised (> 85%) a single species of M_r 16000. Following *S*-carboxamidomethylation and filtration on Sephadex G-50, the major glycoprotein of this fraction appeared to be free of contaminants on SDS-electrophoresis. The amino acid analysis of the modified protein obtained from a single 40 h hydrolysis is shown in table 1 together with the amino acid composition of the proposed bovine precursor structure to vasopressin from residues 1–147 [7]. Because of the compositional similarities, the calculations for residue number were based on a M_r of 14000 after removal of 2000 for contribution of the carbohydrate moiety. The differences in amino acid composition might be explained if the pituitary glycoprotein represented a

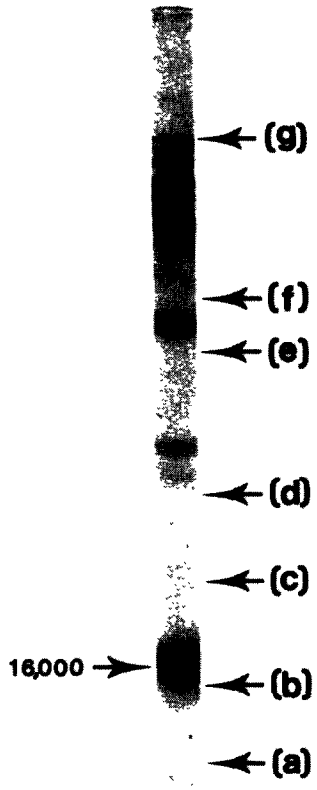

Fig.1. SDS-electrophoresis on 10% polyacrylamide of *N*-ethylmaleated proteins isolated from bovine neural lobes using ConA-affinity chromatography and elution with 50 mM α-methyl-D-mannoside. Staining was performed with Coomassie Brilliant Blue. The positions of ^{14}C-methylated reference proteins employed as internal M_r markers are indicated: (a) cytochrome *c*; (b) lysozyme; (c) trypsin inhibitor; (d) carbonic anhydrase; (e) ovalbumin; (f) bovine serum albumin; (g) phosphorylase *b*. Also indicated is one major protein component of M_r 16000 which was purified and characterised.

Table 1

Amino acid	[a]PLG	[b]Pro-hormone	[c]VP-BNP	[d]GPP
CM-CYs (1/2 Cys)	15.4	16	14	–
Asp	12.5	10	5	1.5
Thr	4.1	3	2	0.8
Ser	8.7	8	6	2.0
Glu	18.9	18	13	1.2
Pro	11.5	14	7	0.7
Gly	16.7	21	14	2.2
Ala	9.5	13	6	1.0
Val	3.4	6	3	–
Met	0.74	1	1	–
Ile	1.8	2	2	–
Leu	10.8	13	6	1.4
Tyr	1.4	3	1	–
Phe	2.8	4	3	–
Lys	2.1	3	2	0.9
His	0.65	0	0	–
Arg	7.9	12	7	1.1

[a] Amino acid composition of a carboxamidomethylated posterior lobe glycoprotein(s) from 40-h acid hydrolysis

[b] Amino acid composition of provasopressin (1–146) from the sequence derived in [7]

[c] Amino acid composition of VP-BNP from the sequence in [16]

[d] Amino acid composition of the glycopeptide isolated following α-chymotryptic digestion of PLG as determined from a 40-h acid hydrolysate

truncated form of the proposed precursor structure with an exception that the former appears to contain a single residue of histidine.

Edman degradation of the modified glycoprotein showed it to be > 90% represented by a single N-terminal sequence. This sequence was unequivocally identified with the exception of one residue through 25 steps and is given in fig.2(a). It is identical to the N-terminal sequence of VP-BNP. Another sequence was present as a 5–10% contamination of the major protein, and is shown in fig.2(b). Some of the residues in this minor sequence could not be identified because of overlap with the major sequence and noise from this structure, but it appeared to commence with the sequence of arginine vasopressin. This finding was

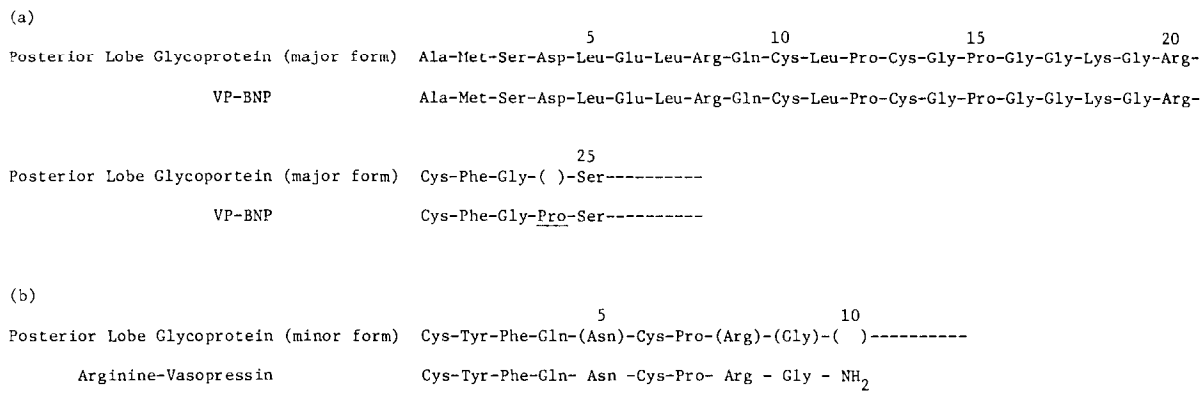

Fig.2. Comparisons of (a) the amino-terminal sequences of a posterior lobe glycoprotein of M_r 16000 and of vasopressin-associated bovine neurophysin, VP-BNP, showing sequence identity through 25 residues and (b) the amino-terminal sequence of a second posterior lobe glycoprotein present as a 5–10% contaminant of the glycoprotein in (a) with the sequence of arginine–vasopressin showing sequence identity. ( ) indicates residues not clearly defined.

supported by the pattern of ^{14}C liberated from the preparation. The major peaks of radioactivity were at positions 10, 13 and 21 representing the neurophysin structure, but there were also present smaller peaks at positions 1, 6, 19 and possibly 22.

α-Chymotryptic digestion of the unmodified protein preparation gave a product which bound to ConA and had the amino acid composition shown in table 1. The composition of this product is compatible with it being portion(s) of the glycopeptide described in [14] which represents the C-terminal portion of the vasopressin/neurophysin precursor molecule described in [7]. Edman degradation of this glycopeptide (from digestion with α-chymotrypsin) indicated that it possessed an N-terminal Ala.

C-terminal analysis of VP-BNP using carboxypeptidase Y gave the result shown in table 2a with the time-dependent liberation of Val, Arg, Phe and Pro, seemingly in that order. A substrate-free control gave only a small peak in the position of Asn. The glycoprotein isolated from posterior pituitaries gave a liberation of amino acids shown in table 2b. Tyr, Leu, Val, Ala, Gly, Glu, Arg and Phe were all liberated. The large amount of Leu indicated that a number of Leu residues are near the C-terminus of the protein, but the patterns of liberation suggest the existence of two (or more) C-terminal structures, one possibly involving Tyr and Val, and also perhaps Phe, and the other involving Leu, Ala, Gly, Glu and Arg.

Table 2

Carboxypeptidase Y digestions (40°C, pH 5.5) of VP-BNP and a posterior lobe glycoprotein (PLG) for 15 min and 30 min at an E/S molar ratio of 1:400 and substrate concentration of 0.1 mM

	Residues/mol	
	15 min	30 min
a. VP-BNP		
Pro	0.26	1.02
Val	0.97	0.99
Phe	0.32	1.03
Arg	0.47	2.04
b. PLG		
Gln	0.27	0.39
Glu	0.82	1.50
Gly	0.54	1.12
Ala	0.60	1.18
Val	0.15	0.24
Leu	1.60	2.57
Tyr	0.50	0.50
Phe	0.12	0.35
Arg	0.55	0.74

4. DISCUSSION

The mRNA sequence encoding a common precursor for vasopressin and VP-BNP has been used to deduce its amino acid sequence [7], but no

sequence information has been obtained directly on the isolated molecule. Also, the manner in which processing of the precursor takes place to liberate hormone and neurophysin within neurosecretory granules is not yet known. The data obtained here from sequencing and compositional analysis shows that the major proteins of a glycosylated preparation from bovine neural lobes are C-terminally elongated forms of VP-BNP. These glycoproteins of about M_r 16000 represent most of the material in the M_r range 20000–14000. There appeared to be two such proteins with the N-terminal structure of VP-BNP. One of these had a C-terminal structure compatible with that of the proposed precursor, while the prevalent form contained a shortened glycopeptide that probably resulted from cleavage in the region of Leu_{129} of the precursor structure. We suggest the name 'proneurophysins' for these proteins to distinguish them from forms of the precursor that contain the structure of vasopressin, the prohormone and preprohormone.

A single contaminant of the isolated proteins was shown by Edman degradation to have the structure of vasopressin at its N-terminus. Since this was present in the M_r range of 20000–14000 and was glycosylated, we believe it to be the vasopressin precursor (1–147) in [7], the prohormone.

The presence of these molecules and their relative amounts in neural lobes suggests to us that the processing of prohormone within neurosecretory granules takes place according to the scheme illustrated in fig.3 with intermediate

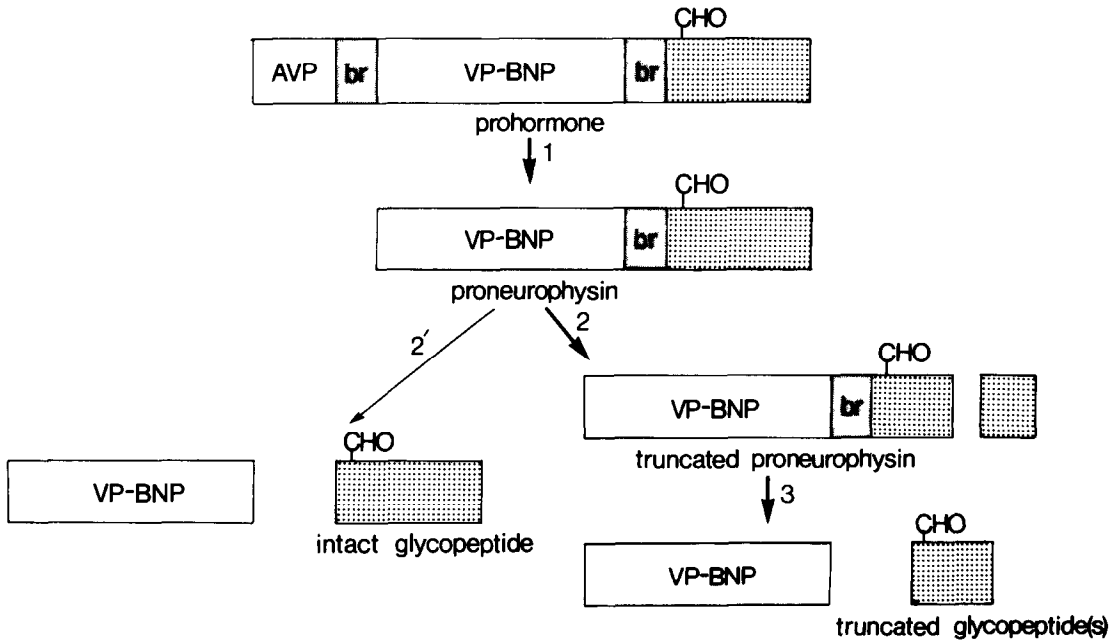

Fig.3. A proposed scheme for the manner in which prohormone is processed to VP-BNP in vasopressinergic neurons of hypothalamic projections. The scheme is based on the presence and relative amounts of truncated 'proneurophysin(s)', 'proneurophysin', and prohormone in bovine neural lobes. The seemingly preferred route is: (1) the liberation of vasopressin and the bridging sequence (br) from the N-terminal structure of the prohormone to form 'proneurophysin'; (2) the cleavage of a C-terminal fragment(s) from 'proneurophysin' to form a truncated 'proneurophysin(s)'; (3) the cleavage of the bridging sequence between VP-BNP and the shortened glycopeptide(s). As intact glycopeptide has also been located in bovine neural lobes [14], the direct conversion of 'proneurophysin' to VP-BNP and glycopeptide (step 2^1) must also occur. Such a scheme may not apply to processing by vasopressinergic neurons in extra-hypothalamic regions.

formation of 'proneurophysins' which lack the hormone sequence. The isolation of C-terminally truncated glycopeptides from bovine neural lobes [14] is further support for such a scheme. α-Chymotrypsin seems to be capable of liberating glycopeptides from 'proneurophysins'.

Unfortunately, it was not clear in this study what sequences joined hormone to neurophysin and neurophysin to glycopeptide. Also, there are some questions raised by the data about the proposed structure of the vasopressin precursor. The presence of Lys and Phe in the composition of the glycopeptide is puzzling, and in this respect it is of interest that the glycopeptide of human neural lobes contains a Phe at position 22 [15]; there appears to be a smaller amount of Leu than expected in the composition of our proneurophysins; and while the ^{14}C peaks at positions 1, 6, 10, 13, 21 and 22 would be expected from N-terminal degradation of the mixture of the provasopressin and 'proneurophysins' obvious from HPLC, the appearance of a ^{14}C peak after 19 steps of Edman degradation is hard to reconcile with the published data on these sequences.

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