Proc. Nat. Acad. Sci. USA
Vol. 69, No. 11, pp. 3350–3354, November 1972

Covalent Structure of Bovine Neurophysin-II: Localization of the Disulfide Bonds

(binding protein/conformation/molecular evolution/disulfides)

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Communicated by Lyman C. Craig, August 16, 1972

ABSTRACT **The completed amino-acid sequence of bovine neurophysin-II, a major neurohypophyseal hormone-binding protein in the hypothalamo–neurohypophyseal complex of cows, set the stage for the localization of the disulfide bonds of this sulfur-rich molecule. Neurophysin-II was digested with subtilisin or a pepsin–trypsin mixture. The resulting peptides were subjected to first-dimensional electrophoresis at pH 6.5, oxidized with performic acid, and subjected to second-dimensional electrophoresis under identical conditions as the first-dimensional separation, but in a perpendicular direction. Cysteic acid peptides were eluted (several after additional electrophoretic purification at pH 3.5) for amino-acid composition and NH_2- and COOH-terminal analyses. Our assignment of the seven disulfide bridges present in neurophysin-II is as follows: Cys_{10}–Cys_{93}; Cys_{13}–Cys_{95}; Cys_{21}–Cys_{27}; Cys_{28}–Cys_{44}; Cys_{54}–Cys_{61}; Cys_{67}–Cys_{73}; Cys_{74}–Cys_{79}. The assignment of disulfide bridges associated with Cys_{27} and Cys_{28} is tentative as it is derived from evolutionary considerations. The high disulfide content reduces drastically the allowed number of biofunctional conformers of neurophysin-II. It is suggested that neurophysin-II possesses a globular topography with minimal α-helix structure.**

In earlier communications, we reported the complete amino-acid sequence of neurophysin-II of cow (BNP-II) (1) and the partial sequence of neurophysin-I of this species (2). These two proteins are the major bovine neurophysins (3), and it has been suggested earlier that neurophysins function physiologically as carriers of the hormones oxytocin and arginine-vasopressin in the hypothalamo–neurohypophyseal tract (4). In line with this hypothesis are the findings that bovine neurosecretory granules rich in neurophysin-I are principally associated with oxytocin and those rich in neurophysin-II are principally associated with vasopressin (5, 6). Moreover, the binding constants between neurophysins and hormones are so low as to ensure a complete dissociation of the peptide–protein complexes once they are released from the posterior pituitary into the systemic circulation (7).

The ionic bond between the NH_2-terminal amino group of the neurohypophyseal hormones and a carboxyl group of the neurophysin protein is the key to the formation of the peptide–protein complex (8, 9), although π–π and hydrophobic interactions involving residues 2 and 3 of the hormone are among those that contribute to the formation of the complex (7, 9, 10). BNP-II, like its congeners, is unusually rich in sulfur; it contains 14 half-cystine residues, which are involved in the formation of seven disulfide bonds (1, 11). This high content of disulfide bridges in neurophysins must dramatically reduce the number of their possible conformers. The consequent restriction in conformational freedom implies a requirement for a highly specific topography in order for these molecules to function as hormone-carrying proteins. As our next step, therefore, towards a three-dimensional definition of the mechanism responsible for the binding of the neurohypophyseal hormones to neurophysins, we embarked on the assignment of all seven disulfide bridges in BNP-II; a preliminary report has been published (12).

Abbreviation: BNP-II, bovine neurophysin-II.

MATERIALS AND METHODS

Tissue Source. A crude powder preparation of acetone-dried bovine posterior pituitary gland was obtained from Parke–Davis (Lot no. 284346).

Reagents and Equipment. Pepsin and trypsin treated with L-(1-tosylamido-2-phenyl)-ethyl chloromethylketone (13), carboxypeptidase A treated with diisopropylphosphofluoridate, carboxypeptidase B, and subtilisin were purchased from Worthington Biochemicals. A 120-C Beckman amino-acid analyzer equipped with a 4- to 5-mV range recorder for scale expansion and an HV 5000A Savant high-voltage electrophoresis apparatus were used.

Enzymic Digestion of BNP-II. (*A*) Pepsin–trypsin digestion: BNP-II (24.8 mg) was incubated with 0.62 mg of pepsin in 2.23 ml of 0.05 N HCl (pH 1.8) for 14 hr at 37°. The mixture was lyophilized and incubated with 0.62 mg of trypsin in 2.23 ml of 0.2 M NH_4HCO_3 (pH 8.2) for 12 hr at 37°. After acidification of the mixture to pH 3 with 50% acetic acid, the pepsin–trypsin digest was lyophilized. (*B*) Subtilisin digestion: BNP-II(35.15 mg)was incubated with 0.88 mg of subtilisin in 3.13 ml of 0.2 M NH_4HCO_3 (pH 8.2) for 12 hr at 37°. The digest was adjusted to pH 3 and lyophilized.

Electrophoretic Separation and Identification of Cysteic-Acid Peptides. The disulfide bonds were localized according to the general method of Brown and Hartley (14). Preparative digests of BNP-II were applied on Whatman 3MM paper at a peptide concentration of 1.5 mg/cm of paper and subjected to electrophoresis at 3 kV for 75 min in 10% pyridine–0.3% acetate acid buffer (pH 6.5). The peptide material was oxidized by performic acid and then subjected to electrophoresis at pH 6.5 perpendicular to the first dimension. Further separation of selected peptides was achieved by electrophoresis at pH 3.5 before analysis. Cysteic-acid peptides were eluted with

water, lyophilized, and an aliquot of each was hydrolyzed in 6 N HCl for 22 hr under reduced pressure at 110°, dried, and analyzed (15). Selected peptides, which appeared homogeneous by amino-acid analysis, were subjected to (*a*) one cycle of the manual Edman degradation procedure (16); the resulting PTH amino acids were identified by thin-layer (17) and gas (18) chromatography, while base-hydrolyzed samples of PTH amino acids were subjected to amino-acid analysis (19); (*b*) partial digestion with carboxypeptidases A and B (20); the liberated amino acids were identified by amino-acid analysis. In addition, each peptide isolated was tested for the presence of carboxamide groups by the procedure of Offord (21).

RESULTS

Each peptide isolated is characterized in Table 1 by its amino-acid composition, NH_2- and COOH-terminal residues, electrophoretic mobility with respect to aspartic acid at pH 6.5, and the position assigned to the cysteinyl moiety within native BNP-II.

Assignment of disulfide bridge Cys_{74}–Cys_{79}

Three cysteic acid peptides are present in zone A_2 of the two-dimensional electrophoretogram (Fig. 1). Two of these peptides, *A-2-3* and *A-2-1* (the latter peptide streaking in the first dimension), appeared yellow upon staining with ninhydrin; this finding suggested the presence of an NH_2-terminal cysteic acid, which was confirmed by NH_2-terminal analyses (Table 1). The amino-acid composition of *A-2-1* reveals this peptide to be a fragment of *A-2-3*. By the method of Offord (21), the electrophoretic mobility of both *A-2-1* and *A-2-3* (in relation to aspartic acid), with respect to their molecular weight, indicates the presence of one primary carboxamide group in each peptide; the amidated moiety is asparagine. Originally peptide *A-2-3* must have been linked by a disulfide bridge to *A-2-2*.

Peptides *A-2-2* and *A-2-3* are identical in composition (Table 1), except that *A-2-2* contains one valine and one proline residue not found in *A-2-3* and its cysteine residue is not at the NH_2-terminal. From these data it can be concluded that *A-2-3* occupies positions 74–78, *A-2-2* positions 75–81, and that the cysteic-acid peptides *A-2-2* and *A-2-3* arose from the single cystine peptide:

H-Cys_{74}-Ser-Asn-Glu-Glu-OH
|
H-Ser-Asn-Glu-Glu-Cys_{79}-Val-Pro-OH

Partial assignment of the double cystine bridge Cys_{21}–Cys_{27}, Cys_{28}–Cys_{44}

Zone A_1 of the electrophoretogram (Fig. 1) contains three cysteic-acid peptides. On the basis of its amino-acid composition (Table 1), peptide *A-1-3* can be placed uniquely in positions 26–30. Its mobility of +1.0 indicates a formal charge of −3 at pH 6.5 that, in conjunction with its amino-acid composition, supports this placement of *A-1-3*. In native BNP-II, the adjacent half-cystine residues in positions 27 and 28 must be bridged to the two peptides *A-1-1* and *A-1-2* (Fig. 1). Because of its amino-acid composition, *A-1-1* can be placed unequivocally in positions 21–25, and therefore Cys_{21} must be linked in BNP-II to either Cys_{27} or Cys_{28}. Peptide *A-1-2* possesses the sequence Cys–Gln as revealed by amino-acid and NH_2-terminal analyses and by its mobility at pH 6.5 (Table 1). The dipeptide with sequence H-Cys-Gln-OH occurs at positions 44–45 and 54–55 in the protein. However, we isolated a tetrapeptide with the sequence H-Cys-Gln-Glu-Glu-OH from a pepsin–trypsin digest of BNP-II. The electrophoretogram of this digest gave two well-resolved peptides referred to as *A-3-1* and *A-3-2*. Peptide *A-3-1*, with the sequence H-Cys-Cys-Gly-Asp-Gln-OH, is the dicysteic acid-containing peptide, which corresponds to *A-1-3* of the subtilisin digest (Fig. 1). *A-3-2* is the tetrapeptide with the sequence H-Cys-Gln-Glu-Glu-OH that can be singularly placed in positions 44–47 in the protein; this last finding proves that *A-1-2*, which is the NH_2-terminal dipeptide of *A-3-2*, is located in positions 44 and 45. From the above results, it can be concluded that Cys_{44} is linked to Cys_{27} or Cys_{28} in BNP-II.

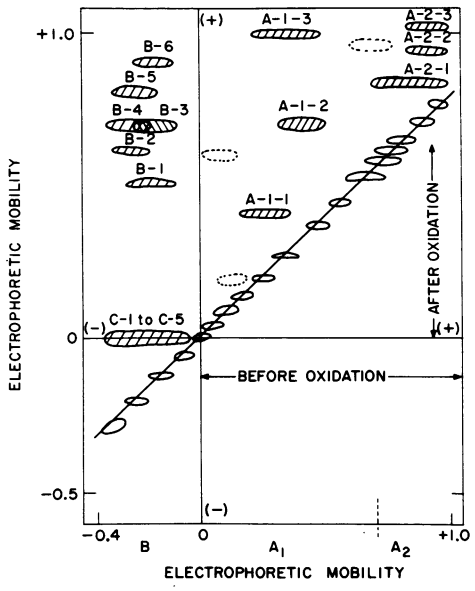

FIG. 1. Diagonal peptide map of the first subtilisin digest of native BNP-II at pH 6.5. Conditions of protein digestion and development of the electrophoretogram are detailed in the text. The first-dimensional electrophoresis (nonoxidized peptides) was performed horizontally, the cathode being on the left side of the electrophoretogram. The second-dimensional electrophoresis (performic acid-oxidized) was performed vertically with the negative pole at the bottom. The electrophoretic mobility of the peptides in the two-dimensional map is expressed relative to that of aspartic acid (+1.0). Those cysteic-acid peptides that have been characterized are the *hatched* areas; those present in trace amounts are indicated by *dashes*; the spots along the diagonal represent peptides that do not contain cysteine.

Preliminary analysis of basic peptides containing cystine

The subtilisin digestion yielded not only three acidic *cystine*-containing peptides but also a number of basic *cystine*-containing peptides (first dimension, cathodal peptides, Fig. 1). Six peptide fragments containing *cysteic acid* were resolved after oxidation (second dimension, anodal peptides, Fig. 1), and are listed in Table 1 (*B-1* through *B-6*). However, several cysteic acid-containing peptides stayed at the point of origin after oxidation (second dimension, neutral peptides). These peptides were completely resolved electrophoretically at pH 3.5 into five cysteic-acid peptides (Table 1, *C-1* through *C-5*). Thus, region *B* of the electrophoretogram of the subtilisin digest of BNP-II (Fig. 1) yielded 11 cysteic acid-containing peptides. Eight of these peptides must have been originally disulfide-bridged, while three must be fragments of those eight. Since varying electrophoretic conditions (pH 6.5 and

TABLE 1. *Characterization of cysteic acid-containing peptides obtained after subtilisin digestion of native bovine neurophysin-II and performic acid oxidation of the resulting peptides*

Peptide	Electrophoretic Mobility	NH_2-Terminal Amino Acid	COOH-Terminal Amino Acid	Amino Acid Composition	Probable Sequence	Position of half-cystine Residue in BNP-II
A-1-1	0.40	--	--	$Cys_{1.1}$, $Ser_{0.9}$, $Pro_{1.0}$, $Gly_{1.1}$, $Phe_{1.0}$	Cys-Phe-Gly-Pro-Ser	21
A-1-2	0.70	--	--	$Cys_{1.0}$, $Gln_{0.9}$	Cys-Gln	44
A-1-3	1.10	--	--	$Cys_{2.0}$, $Asp_{1.0}$, $Gly_{0.8}$, $Ile_{0.8}$	Ile-Cys-Cys-Gly-Asp	27,28
A-2-1	0.72	Cys	--	$Cys_{1.0}$, $Ser_{0.8}$, $Asn_{1.0}$	Cys-Ser-Asn	74
A-2-2	0.90	Ser	--	$Cys_{1.0}$, $Ser_{1.0}$, $Asn_{1.0}$, $Glu_{2.0}$, $Pro_{0.9}$, $Val_{1.0}$	Ser-Asn-Glu-Glu-Cys-Val-Pro	79
A-2-3	1.10	Cys	Glu	$Cys_{1.0}$, $Ser_{0.8}$, $Asn_{1.0}$, $Glu_{1.8}$	Cys-Ser-Asn-Glu-Glu	74
B-1	0.50	--	--	$Cys_{1.0}$, $Thr_{0.8}$, $Ala_{1.0}$, $Ile_{0.7}$	Ala-Thr-Ile-Cys	73
B-2*	0.64	Cys	--	$Cys_{1.0}$, $Gly_{1.0}$	Cys-Gly	13 or 61
B-3	0.70	Gln	--	$Cys_{1.0}$, $Gln_{1.0}$	Gln-Cys	10
B-4	0.70	Cys	--	$Cys_{0.9}$, $Gln_{1.0}$	Cys-Gln	54
B-5	0.80	--	--	$Cys_{1.0}$, $Ile_{0.9}$	Ile-Cys	73
B-6	0.83	--	--	$Cys_{1.0}$, $Ala_{0.9}$	Cys-Ala	67
C-1	0.00	--	--	$Cys_{1.0}$, $Gly_{3.1}$, $Arg_{0.9}$	Gly-Arg-Gly-Gly-Cys	93
C-2	0.00	--	--	$Cys_{1.0}$, $Gly_{2.1}$, $Ala_{1.1}$, $Arg_{1.1}$	Gly-Gly-Arg-Cys-Ala	67
C-3	0.00	--	--	$Cys_{1.0}$, $Gln_{1.2}$, $Pro_{1.1}$, $Gly_{1.1}$, $Arg_{1.1}$	Gln-Arg-Pro-Cys-Gly	61
C-4	0.00	--	Lys	$Cys_{1.0}$, $Pro_{0.9}$, $Gly_{2.8}$, $Lys_{1.0}$	Cys-Gly-Pro-Gly-Gly-Lys	13
C-5	0.00	Cys	--	$Cys_{1.0}$, $Arg_{1.0}$	Cys-Arg	95

* Peptide *B-2*, obtained from the first subtilisin digest (Fig. 1), can be recognized as an acidic fragment of *C-3* and/or *C-4*, and is therefore not discussed further.

3.5) were used for resolution of the peptides, no disulfide assignments could be made as yet. In order to localize the remaining four disulfides, a second subtilisin digest was subjected to electrophoresis for an extended time period (160 min instead of 60 min) at pH 6.5 and 3 kV. Many of the peptides isolated from the second digestion had the same composition as those from the first and are therefore labeled identically.

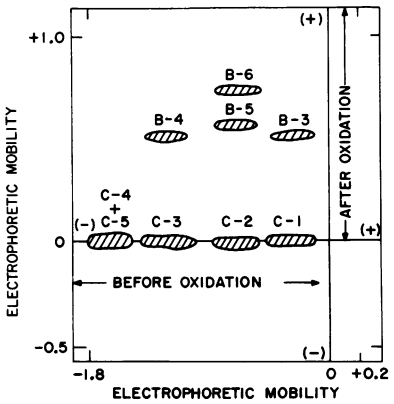

FIG. 2. Diagonal map (pH 6.5) of the basic cystine-containing peptides derived from the second digestion of BNP-II with subtilisin. Protein digestion is described in the text. Electrophoretic conditions are the same as in the legend to Fig. 1, except that the first-dimensional electrophoresis was performed for 160 min.

Assignment of disulfide bridge Cys_{13}–Cys_{95}

The basic material possessing the greatest electrophoretic mobility towards the cathode, in the first dimension, became electrophoretically neutral at pH 6.5 after oxidation (Fig. 2). Electrophoresis of this material at pH 3.5 yielded two cysteic acid-containing peptides, designated *C-4* and *C-5* (Table 1). Peptide *C-4* is uniquely placed in positions 13–18 and *C-5*, a dipeptide possessing an NH_2-terminal cysteic acid, in positions 95–96. Hence, Cys_{13} and Cys_{95} are bound in BNP-II in the following manner:

H-Cys_{13}-Gly-Pro-Gly-Gly-Lys-OH
|
H-Cys_{95}-Arg-OH.

Assignment of disulfide bridge Cys_{54}–Cys_{61}

The ninhydrin-active material adjacent to *C-4* and *C-5* in the first dimensional electrophoretogram (Fig. 2) was separated to give *B-4* and *C-3* during the second-dimensional electrophoresis. Peptide *B-4* possesses the amino-acid com-

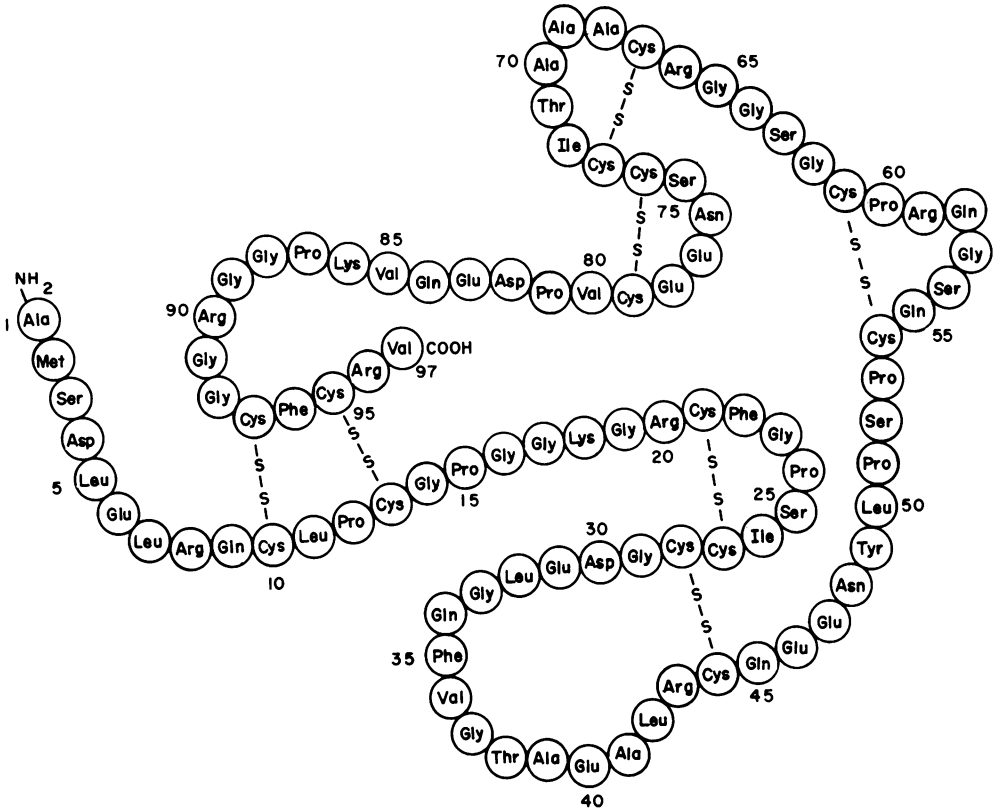

FIG. 3. Complete covalent structure of bovine neurophysin-II. *Numbers* indicate positions of amino-acid residues starting from the NH_2-terminus. Upon reexamination of the published amino-acid sequence of the protein (1), we found that BNP-II possesses an Asp instead of an Asn in position 30, an Asn instead of an Asp in position 76, and a Glu instead of a Gln in position 84 (Schlesinger, Capra, and Walter, unpublished data).

position Cys(0.9), Gln(1.0) with an NH_2-terminal cysteic acid. The sequence of *B-4* is encountered twice in BNP-II, placing the Cys residue at positions 44 or 54; since we have already established that Cys_{44} is linked to Cys_{27} or Cys_{28} and since no *basic* fragments that could contain solely the cysteinyl residues Cys_{27} and/or Cys_{28} can be derived from the subtilisin digest of BNP-II, we conclude that *B-4* has to be placed at positions 54–55. Peptide *C-3* (Table 1) is uniquely placed in positions 58–62. Since no other ninhydrin-positive peptide was detected in this section of the electrophoretogram (*B-4* and *C-3* also cannot be fragments of each other), we conclude that *B-4* and *C-3* arose from the single cystine peptide:

H-Cys_{54}-Gln-OH
|
H-Gln-Arg-Pro-Cys_{61}-Gly-OH.

Assignment of disulfide bridge Cys_{67}–Cys_{73}

The next zone of ninhydrin-active material in the first dimensional electrophoretogram gave three bands after oxidation and electrophoretic separation: *C-2*, *B-5*, and *B-6* (Fig. 2, Table 1). On the basis of its amino-acid composition, *C-2* (Table 1) can be unequivocally placed at positions 64–68 of BNP-II. Dipeptide *B-6* is recognized by virtue of its amino-acid composition as a fragment of *C-2*. Similarly, the amino-acid composition of *B-5* suggests it to be a fragment of *B-1* (first subtilisin digest, Fig. 1). Peptide *B-1* in turn can be uniquely localized in positions 70–73, and it is the only half-cystine peptide isolated that contains the sequence Ile-Cys, and that is capable of migrating towards the cathode in the first dimensional electrophoretogram; hence, *B-5* must be a fragment of *B-1* in order to be a "basic cysteinyl peptide." Thus, *C-2* and *B-1* originate from the cystine peptide:

H-Ala-Thr-Ile-Cys_{73}-OH
|
H-Gly-Gly-Arg-Cys_{67}-Ala-OH.

Assignment of disulfide bridge Cys_{10}–Cys_{93}

The material with the slowest electrophoretic mobility in the first dimension yielded two peptides after oxidation, *C-1* and *B-3*, which separated completely in the second dimension. The amino-acid composition of *C-1* places this peptide singularly at positions 89–93 in BNP-II; the composition of *B-3*, together with the finding that Gln is NH_2-terminal, localizes *B-3* at positions 9–10. Thus, *C-1* and *B-3* are originally joined in the following manner:

H-Gln-Cys_{10}-OH
|
H-Gly-Arg-Gly-Gly-Cys_{93}-OH.

DISCUSSION

This study deals with the localization of the disulfide bridges in BNP-II. This protein presents a challenge not only because of its high cystine content but also because of the presence of two Cys–Cys sequences in the molecule.

Cleavage of BNP-II by treatment with subtilisin gave rise to several cystine-containing peptides. Electrophoretic separation of a first enzymatic digest gave an excellent resolution of the *acidic* cystine-containing peptides (Fig. 1). Selec-

tive oxidation and second-dimensional electrophoresis resulted in well-resolved acidic cysteic-acid peptides. Isolation and characterization of these peptides allowed unequivocal assignment of the disulfide bridge linking Cys_{74} to Cys_{79} in native BNP-II. This assignment was made possible by the fortuitous cleavage by subtilisin of the peptide bond between the vicinal Cys_{73} and Cys_{74} residues. However, under identical digestion conditions the peptide bond of the vicinal Cys_{27}–Cys_{28} remained intact. Experiments involving dilute acid hydrolysis, successful in cleaving the Cys_6–Cys_7 amide bond in insulin (22) and prolonged acidolysis, which cleaved the peptide bond between neighboring half-cystine residues in cobrotoxin (23), were not attempted in this investigation. Therefore, on the basis of chemical analysis alone Cys_{27}–Cys_{28} could be present in either of the two covalent structures:

H-Cys_{21}-Phe-Gly-Pro-Ser-OH
|
H-Ile-Cys_{27}-Cys_{28}-Gly-Asp-OH
|
H-Cys_{44}-Gln-OH

H-Cys_{21}-Phe-Gly-Pro-Ser-OH
|
H-Ile-Cys_{27}-Cys_{28}-Gly-Asp-OH
|
H-Cys_{44}-Gln-OH

Although a definitive localization of the two disulfide bridges depends upon the hydrolysis of the Cys_{27}–Cys_{28} peptide bond, a tentative assignment based on other considerations is presented below.

A resolution of the *basic* cystine-containing peptides, comparable to that achieved for the acidic cystine-containing peptides, required a second enzymatic digestion of BNP-II and a tripling of the time for the first dimensional electrophoresis. The four remaining disulfide bridges were then localized as Cys_{10}–Cys_{93}, Cys_{13}–Cys_{95}, Cys_{54}–Cys_{61}, and Cys_{67}–Cys_{73}.

We pointed out previously that BNP-II exhibits an extraordinarily high degree of intrachain homology and suggested that a primitive gene, which encoded a "mini" neurophysin molecule, had undergone unequal crossing-over, resulting in partial gene duplications with fusion (2). The positions of the five disulfide loops that we have chemically localized in BNP-II reinforce our present theory of the molecular evolution of neurophysin proteins. For example, Cys_{13} is contained in the sequence Pro_{12}-Cys-Gly-Pro-Gly-Gly-Lys-Gly-Arg_{20}, and similar sequences occur twice more in the BNP-II structure, i.e., Pro_{53}-Cys-Gln-Ser-Gly-Gln-[ ]-Arg_{59} and Pro_{60}-Cys-Gly-Ser-Gly-Gly-[ ]-Arg_{66}. The single half-cystine residues contained in each of the last two sequences are covalently linked to form the Cys_{54}–Cys_{61} disulfide loop. As noted above, the successful hydrolysis of the Cys_{73}–Cys_{74} peptide bond during digestion of BNP-II with subtilisin allowed the definitive localization of the two disulfide loops Cys_{67}–Cys_{73} and Cys_{74}–Cys_{79}. In order to more specifically assign the remaining two disulfide bonds in BNP-II, we have drawn on further considerations of the molecular biogenesis of neurophysins. The two partially assigned disulfide bonds, Cys_{21}–Cys_{27} and Cys_{28}–Cys_{44}, occur in a region of the peptide sequence in BNP-II that is highly homologous to the region containing the definitively localized disulfide bridges Cys_{67}–Cys_{73} and Cys_{74}–Cys_{79}. In close analogy to the assignment of the 23-membered disulfide ring Cys_{67}–Cys_{73}, we propose that Cys_{21} forms a 23-membered ring by virtue of a disulfide bond with Cys_{27}. Thus, by elimination, the remaining ring structure is formed by the disulfide bridge Cys_{28}–Cys_{44}.

Disulfide bonds play a critical role in establishing and maintaining the three-dimensional structure of proteins. The large number of disulfide bridges present in BNP-II greatly diminishes the number of allowed conformations for this molecule. Moreover, the fairly regular spacing of rather small-membered disulfide loops throughout the protein sequence (Fig. 3), militates against a substantial α-helical structure. In fact, the only peptide region that could possibly exhibit α-helical structure is the sequence 29–53, because this region contains no disulfide loops. Indeed, a model proposed by Kotelchuck and Scheraga (24) predicts that amino-acid residues 31–35 initiate a helical segment that extends through residue 47, at which point it is terminated by the dipeptide sequence Asn-Tyr (residues 48 and 49).

The four disulfide loops, Cys_{21}–Cys_{27}, Cys_{28}–Cys_{44}, Cys_{67}–Cys_{73}, and Cys_{74}–Cys_{79}, in the region of great internal sequence homologies lend a certain symmetry to the molecular structure of the carrier protein. This symmetry is further reinforced by the two disulfide bonds, Cys_{10}–Cys_{93} and Cys_{13}–Cys_{95}, which result in a juxtaposition of the amino- and carboxy-segments of BNP-II. Thus, the topographical surface of BNP-II should be globular (Fig. 3).

This work was supported by USPHS Grants AM-13567, AM-01431, and AM-02594. B. F. is a Senior Fellow of the Arthritis Foundation.

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