

**PARTIAL ASSIGNMENT OF DISULFIDE PAIRS IN NEUROPHYSINS<sup>†</sup>**Sudhir Burman\*, Esther Breslow\*, Brian T. Chait<sup>‡</sup> and Tanuja Chaudhary <sup>‡</sup>\*Department of Biochemistry, Cornell University Medical College and  
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Received September 23, 1987

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The original report assigning the pairing of neurophysin's 14 half-cystine residues (Schlesinger *et al.* (1972), Proc. Natl. Acad. Sci., U.S.A., 69,3350-3353) was based on an incorrect amino acid sequence. In the present study, re-investigation of the results of proteolytic fragmentation of bovine neurophysins indicates that the majority of the original assignments were incorrect. Three disulfide pairs are now assigned as Cys<sub>21</sub>-Cys<sub>44</sub>, Cys<sub>67</sub>-Cys<sub>85</sub> and Cys<sub>74</sub>-Cys<sub>79</sub>. The pairing pattern indicates that neurophysin's variable carboxyl terminal region, separately encoded by the third gene exon, does not form a self-contained domain. © 1987 Academic Press, Inc.

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The hormone-binding protein neurophysin (NP)<sup>1</sup> contains 14 half-cystine residues per chain of ~ 95 residues (1). The only reported study of NP disulfide pairing (2) utilized an incorrect sequence and must at least partially be in error (3). Neurophysins are encoded by three exons (4, and Fig. 1). Exon B encodes a highly conserved region containing 12 half-cystines. Exon C encodes a segment which, with the exception of the two remaining half-cystines, Cys<sub>79</sub> and Cys<sub>85</sub>, is relatively variable. Only residues encoded by exons A and B have thus far been assigned a role in hormone-binding (5). Accordingly, it is of interest to determine whether Cys<sub>79</sub> and Cys<sub>85</sub> are paired with each other to form a separate domain, completely encoded by exon C.

We have initiated a study of NP disulfide pairing. Bovine NP-I and -II were digested with a series of proteolytic enzymes. Analysis of enzyme cleavage positions and of released peptides has now allowed identification of 3 of the 7 disulfide pairs, including those containing Cys<sub>79</sub> and Cys<sub>85</sub>.

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<sup>†</sup>Supported by Grant GM-17528 from NIH.

<sup>1</sup>Abbreviations: NP, neurophysin; CPA, carboxypeptidase A; S. protease, *Staphylococcus aureus* protease V8.

### MATERIALS AND METHODS

Bovine neurophysins -I and -II were purified as previously described (6). Carboxypeptidases A and B, trypsin (Diphenylcarbonyl chloride treated) type XI, *Staphylococcus aureus* protease V8 (*S. protease*), thermolysin type X and subtilisin BPN' type VII were Sigma preparations.

Digestion of neurophysins with trypsin: NP-II (8.0 mg/ml) was reacted with trypsin (0.32 mg/ml) in 0.1M, pH 8.0 borate buffer for 5 hr at 37°C and 16 hr at room temperature. The reaction was stopped by lowering the pH to 3 and the mixture applied to a Sephadex G-50 column (0.2M acetic acid eluant) to separate liberated peptides from the protein core. NP-I was reacted with carboxypeptidase A (CPA) prior to trypsin treatment to remove the last three residues as previously described (7).

Digestion of trypsin-treated neurophysins with *S. protease* V8: Trypsin treated protein (7.0 mg/ml) was treated with *S. protease* (0.35 mg/ml) in 0.1M Tris-phosphate buffer, pH 7.0 for 5 hr at 37°C. The mixture was cooled and applied to a G-50 column, as above.

Digestion of trypsin- and *S. protease*-digested neurophysin with thermolysin: The protein (4.0 mg/ml) was reacted with thermolysin (0.2 mg/ml) in pH 7.6, 0.1 M Tris buffer at 40°C for 5 hr and the mixture fractionated on G-50, as above.

Digestion of trypsin treated neurophysins with subtilisin: Trypsin-treated NP-II (or CPA and trypsin treated NP-I) (6.0 mg/ml) was treated with subtilisin (0.15 mg/ml) in 0.2M, pH 8.0 ammonium bicarbonate buffer for 4 hr at 37°C and the mixture fractionated on a G-50 column, as above.

Amino acid analysis: Amino acid analysis was performed as described elsewhere (8). Prior to hydrolysis, samples were oxidized with performic acid following the procedure of Hirs (9), unless otherwise indicated.

HPLC separation of proteins and peptides: Proteins and peptides were routinely purified on an IBM 9533 ternary liquid chromatograph equipped with an IBM 9523 variable UV detector. Cation and anion exchange columns (7.5cm x 7.5mm) were from Waters Associates while the CN column (25cm x 4.6mm) was from Dupont. Ion exchange HPLC employed ammonium acetate buffer 0.02M → 0.50M, pH 4.3 → pH 7.5 (for cation exchange) and pH 7.5 → pH 4.7 (anion exchange). CN HPLC was performed using 0.05% TFA in an H<sub>2</sub>O/CH<sub>3</sub>CN solvent system.

Mass Spectrometry: Mass spectrometric measurements were performed on the 252C<sub>f</sub> fission fragment ionization time-of-flight mass spectrometer described previously (10). Samples were prepared for measurement by adsorbing ~1nmol of peptide from solution (~0.1nmol/μl in 0.1% TFA) on to a thin nitrocellulose film as previously described (11).

### RESULTS

Cleavage of bovine NP-II by trypsin and *S. protease*: Under our conditions, bovine NP-II is clipped by trypsin at positions 8, 18, 20, 43, 66, 86, and 93 (e.g., see reference 13) as shown in Fig. 1. No disulfide-containing peptides are released; the only peptides excised represent residues 1-8, 19-20, and 87-95. The protein core resulting from trypsin treatment was reacted with *S. protease*. Analysis of released peptides indicated quantitative loss of the peptide Ala<sub>41</sub>-Arg<sub>43</sub> (representing cleavage at Glu<sub>40</sub>) and a small amount

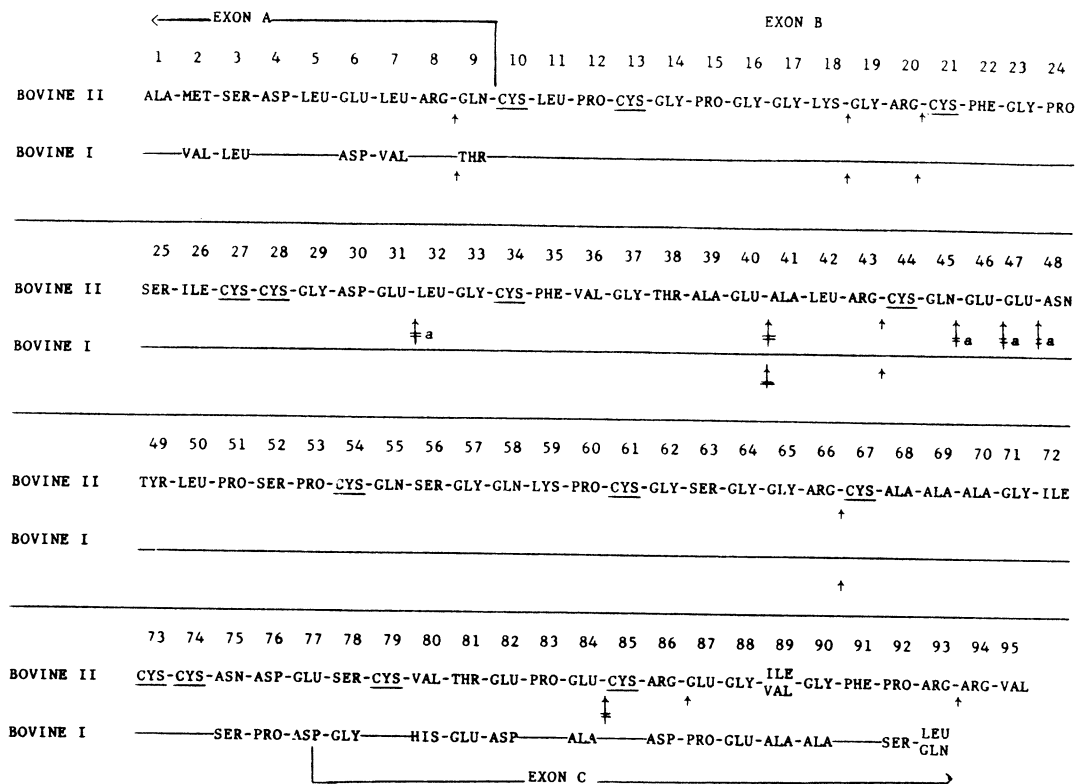


Figure 1. Amino acid sequences of bovine neurophysins -I and -II in relation to gene exon structure and positions of cleavage by trypsin and S. protease. The complete sequence of neurophysin-II is shown, while for neurophysin-I only the substitutions are listed (12).

† Positions of cleavage by trypsin as described in the text. ‡ Positions of cleavage of trypsin treated neurophysins by S. protease V8. The points of cleavage of neurophysin-II were established as described in the text. For neurophysin-I, only cleavage at position 40 has been established.

<sup>a</sup>Cleavage in this region did not go to completion at each position. For example, cleavage did not occur at positions 46 and 47 in the same molecule; i.e., no free Glu was released. Cleavage at position 45 is anomalous.

of Glu in peptide form; again, no half-cystine was lost (data not shown). To identify other points of protease cleavage, the protein core from the sequential enzyme treatment was performic acid oxidized and the resulting peptide fragments analyzed by anion exchange HPLC. The amino acid composition of the oxidized peptides (data not shown) demonstrated cleavage at the positions indicated in Fig. 1; e.g., cleavage at positions 31, 46, and 84 was indicated by isolation of peptides Leu<sub>32</sub>-Glu<sub>40</sub>, Glu<sub>47</sub>-Arg<sub>66</sub> and Cys<sub>85</sub>-Arg<sub>86</sub>. Evidence for cleavage at Glu<sub>77</sub> and Glu<sub>82</sub> was ambiguous. The fact that no disulfide-containing peptides are released by sequential treatment with trypsin and S. protease, given the firmly identified cleavage positions, precludes the following cystine pairs: Cys<sub>10</sub>-Cys<sub>13</sub>, Cys<sub>34</sub>-Cys<sub>44</sub>, Cys<sub>34</sub>-Cys<sub>85</sub>,

Table I. Comparison of amino acid compositions of peptide fractions obtained after thermolysin digestion of trypsin- and S. protease-treated NP-I and NP-II<sup>a</sup>

	NP-I Peptides	NP-II Peptides	NP-II-NP-I <sup>b</sup>
Asp	1.9	2.1	0.2
Thr	0.9	1.4	<u>0.5</u>
Ser	1.1	1.7	<u>0.6</u>
Glu	4.0	5.0	<u>1.0</u>
Pro	1.1	1.6	<u>0.5</u>
Gly	3.3	4.8	<u>1.5</u>
Ala	1.4	3.9	<u>2.5</u>
Half-Cys <sup>c</sup>	1.4 (2)	3.0 (4)	<u>(2)</u>
Val	0.9	1.4	<u>0.5</u>
Met	Absent	Absent	
Ile	0.1	0.4	0.3
Leu	0.2	0.6	0.4
Tyr	0.4	0.4	0.0
Phe	2.0	2.0	0.0
His	Absent	Absent	
Lys	0.1	0.3	0.2
Arg	Absent	1.1	<u>1.1</u>

<sup>a</sup>Results are given as moles amino acid released into peptide fraction per mole of protein.

<sup>b</sup>Difference between peptides released from NP-II and NP-I. Significant differences are underlined.

<sup>c</sup>Cystine values are corrected for up to 20% loss due to decomposition during hydrolysis and then rounded off to nearest integer. These samples were not oxidized with performic acid prior to hydrolysis.

Cys<sub>44</sub>-Cys<sub>85</sub>, and Cys<sub>54</sub>-Cys<sub>61</sub>. The pairing Cys<sub>54</sub>-Cys<sub>61</sub> had been suggested earlier (2).

Cleavage of the trypsin, S. protease products by thermolysin: Protein cores from the above enzyme treatment were digested with thermolysin. The total amino acid compositions of the peptides released from neurophysins-I and -II are shown in Table I. Approximately two cystine-containing peptide sequences are quantitatively released from NP-II and one from NP-I. Differences between the two proteins suggested that the additional Cys-containing peptide released from NP-II represented the sequence Cys<sub>67</sub>-Gly<sub>71</sub> bonded to Cys<sub>85</sub>-Arg<sub>86</sub>, the differences accountable for by the presence of a S. protease-sensitive Glu in position 84 of NP-II. The other differences appeared to represent partial release of the peptide Val<sub>80</sub>-Glu<sub>84</sub> in NP-II. The common amino acids released from the two proteins indicated that the sequences Phe<sub>22</sub>-Ser<sub>25</sub>, Phe<sub>35</sub>-Glu<sub>40</sub>, Gln<sub>45</sub>-Asn<sub>48</sub>, and perhaps also Gly<sub>29</sub>-Glu<sub>31</sub>, were completely released by thermolysin. This suggested that the released half/Cys residues were two from the set 21, 28, 34 and 44, excluding (see above) the possibility of both 34 and 44. Attempts to directly analyze the released peptides were frustrated by the fact that thermolysin tended to cleave the released half-cystines from adjacent amino acids, leaving the Cys residues without an identifying tag.

Table II. Purification and analysis of peptides obtained from subtilisin digestion of trypsin-treated NP-II

Peptide #1: Obtained from cation exchange HPLC; elution time 14.23 min

<u>Observed amino acid composition:</u>	<u>Assigned sequence</u>
Cys 2.0; Ala 2.1; Gly 0.5; Arg 0.9	Cys <sub>67</sub> -Ala-Ala Cys <sub>85</sub> -Arg

Peptide #2: A peak eluting at 2.35 min from cation exchange HPLC was collected and refractionated on a reverse phase CN column where a sharp peak eluting at 17.09 min was collected and analysed.

<u>Observed amino acid composition:</u>	<u>Assigned sequence</u>
Cys 1.9; Phe 1.0; Gly 1.2; Pro 1.2; Ser 1.0; Glu 3.0; Asp 1.0	Cys <sub>21</sub> -Phe-Gly-Pro-Ser Cys <sub>44</sub> -Gln-Glu-Glu-Asn

Peptide #3: The peak eluting at 2.35 min on cation exchange HPLC was collected and subsequently purified on anion exchange HPLC where a broad peak eluting at 21.11 min was collected and analyzed.

<u>Observed amino acid composition:</u>	<u>Assigned sequence</u>
Cys 1.9; Asp 2.2; Ser 0.9; Thr 1.0; Glu 3.7; Gly 1.0; Val 0.9; Pro 1.2	Cys <sub>74</sub> -Asn-Asp-Glu-Ser-Cys <sub>79</sub> - Val-Thr-Glu-Pro-Glu

Treatment of trypsin-digested neurophysin with subtilisin: Subtilisin treatment of trypsin-digested NP-II led to the release of a number of Cys-containing peptides. Peptides were fractionated by HPLC. Purification schemes, amino acid compositions and assigned structures (based on composition) for three peptides are shown in Table II.

Peptide #1 is analogous to that tentatively identified above as representing the pairing Cys<sub>67</sub>-Cys<sub>85</sub>. Its identity was confirmed by mass spectrometry, which indicated the presence of a peptide with mass 538.3 amu (lowest isotopic component). This measured mass corresponds closely with that calculated (538.2 amu) for peptide #1. In addition, a mass of 467.3 amu was observed, representing loss of Ala<sub>69</sub> from peptide #1. The presence of Gly in the peptide (Table II) suggests the presence of an extended component containing Ala<sub>70</sub> and Gly<sub>71</sub>, but this was not observed in the mass spectrum. Note that peptide #1 was purified only by ion-exchange HPLC, so that the presence of more than a single component of like charge is not unexpected.

Peptide #2 represents the pairing Cys<sub>21</sub>-Cys<sub>44</sub>, also compatible with the thermolysin data. The identity of this peptide was confirmed both by mass spectrometry and by sequencing, employing the Applied Biosystems 470A gas phase sequencer(14). The mass spectrum indicated the presence of a peptide

with molecular weight 1129.2 amu, corresponding closely to the isotopically averaged mass (1129.0 amu) calculated for peptide #2.

Peptide #3. The amino acid composition of peptide #3 indicated the possibility of either Cys<sub>28</sub> or Cys<sub>74</sub> paired to Cys<sub>79</sub>. Accordingly, this peptide was subjected to automated Edman degradation. The sequence obtained was Cys-Asn-Asp-Glu-Ser-Cys-Val-Thr, unequivocally assigning it the sequence Cys<sub>74</sub>-Cys<sub>79</sub>. It is relevant that the peptide was sequenced without breaking the cystine disulfide bridge. Cys was released at step 6 of the degradation, but no significant Cys was released at step 1, indicating the internal bridging of Cys<sub>74</sub> to Cys<sub>79</sub> in the peptide. Mass spectrometry confirmed the peptide sequence, demonstrating the presence of a peptide with molecular weight 1223.3 amu. This measured mass corresponds closely with the isotopically averaged mass (1223.1 amu) calculated for peptide #3. Note that the apparent presence of Gly in the peptide (see amino acid composition) is not confirmed by either mass or sequence data.

#### DISCUSSION

Concern over disulfide interchange is warranted in any analysis of disulfide pairing. In the present study, we have assigned 3 of neurophysin's 7 disulfide bonds. These are unlikely to represent products of disulfide rearrangement. First, both the Cys<sub>67</sub>-Cys<sub>85</sub> pair and the Cys<sub>21</sub>-Cys<sub>44</sub> pair are released quantitatively by thermolysin. Thus, any disulfide rearrangement to generate these pairs would have to be quantitative, which seems unlikely. Second, the Cys<sub>74</sub>-Cys<sub>79</sub> peptide obtained from subtilisin-digested trypsin-treated NP, is also seen in peptides derived from subtilisin digestion of native NP (data not shown) and similarly reported by Schlesinger *et al.* (2). Last, no Cys-containing peptides are released by sequential trypsin and S. protease treatment. The multiple clips produced by these two enzymes should lead to the random release of Cys-containing peptides if significant interchange occurred at these steps.

Of the pairs we report, only the Cys<sub>74</sub>-Cys<sub>79</sub> pair is analogous to that reported earlier. By contrast, our studies indicate that the pairings of Cys<sub>21</sub>, Cys<sub>27</sub>, Cys<sub>28</sub>, Cys<sub>44</sub>, Cys<sub>54</sub>, Cys<sub>61</sub>, Cys<sub>67</sub> and Cys<sub>73</sub> are different from those originally assigned; i.e., the original assignments were 21-27, 28-44, 54-61 and 67-73. The pairing assignments for Cys<sub>79</sub> and Cys<sub>85</sub> are of interest, demonstrating that these two half-Cys residues, which lie within the variable carboxyl-terminal region of the protein, are not paired to each other. They therefore indicate that the gene product of Exon C is not an independent domain.

It is also relevant that, of the six half-Cys residues in the last 40% of the protein (Cys-61,67,73,74,79 and 85), the present studies pair four of them to each other. Should Cys-61 and -73 be paired with each other, neurophysin's two duplicated segments (12 through 31 and 60 through 74) would be placed in separate disulfide-linked domains and perhaps allow the significance of this puzzling internal duplication (e.g., 1) to be better understood.

**ACKNOWLEDGEMENTS:** We are grateful to Dr. Daniel Wellner, Cornell University Medical College, Department of Biochemistry for his help in amino acid analysis and gas phase sequencing. The gas phase sequencer was purchased with grant (1S10 RR 02855-01) from NIH. The mass spectrometric measurements were made by the Rockefeller Mass Spectrometry Biotechnology Research Resource supported by the Division of Research Resources, NIH.

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