

## Complete assignment of neurophysin disulfides indicates pairing in two separate domains

(cystine pairing/gas-phase sequencing/proteolytic enzymes/peptide-binding sites)

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**ABSTRACT** The pairing of the 14 half-cystine residues of bovine neurophysin was established by sequential proteolytic digestion. Purified released peptides and the residual disulfide-linked core were monitored at each step by use of amino acid analysis, gas-phase sequencing, and mass spectrometry. The approach included application of gas-phase sequencing to assign disulfide pairs in peptides containing multiple disulfides. The results demonstrate that neurophysin disulfides are paired in two distinct domains—an NH<sub>2</sub> domain (residues 10–54) containing four disulfides and a COOH domain (residues 61–85) containing three disulfides. The specific disulfide bridges are Cys-10 to Cys-54, Cys-13 to Cys-27, Cys-21 to Cys-44, Cys-28 to Cys-34, Cys-61 to Cys-73, Cys-74 to Cys-79, and Cys-67 to Cys-85. The results place the internally duplicated segments of neurophysin (residues 12–31 and 60–77) in separate domains. Disulfide-pairing patterns within each domain are homologous with the exception of the Cys-10 to Cys-54 bond, which is unique to the NH<sub>2</sub> domain and which links the two ends of this domain together. The potential role of the Cys-10 to Cys-54 bond in organizing the hormone-binding site is discussed.

Neurophysins (NPs) are closely related pituitary proteins that bind oxytocin and vasopressin and share with the hormones common biosynthetic precursors (1, 2). NPs contain 14 conserved half-cystines per ≈95 residues (see Fig. 1) and are one of the richest cystine-containing proteins on a weight basis; disulfide reduction is associated with loss of activity (3). In 1972 Schlesinger *et al.* (4) reported a disulfide-pairing scheme for NP based on the analysis of proteolytically derived peptides; this scheme used an incorrect sequence and hence was incorrect (5, 6). In 1981, Drenth (7) proposed a pairing scheme based solely on comparison of half-cystine positions in NP with established disulfide pairs in other proteins. The essential feature of this pairing, which differed significantly from that of the Schlesinger model, was the presence of two separate disulfide domains, a possibility also considered elsewhere (8). Accordingly, the need for a complete re-evaluation of NP disulfide pairing has been evident for some time.

From a structure–function perspective, knowledge of NP cystine pairing would place constraints on NP conformation and help to elucidate the structural relationship between the internally duplicated segments of NP. These segments, representing residues 12–31 and 60–77 (see Fig. 1) exhibit ≈60% similarity to each other (9) and are of unknown function. In preliminary reports (10, 11), we presented evidence for three NP disulfide bonds, Cys-21 to Cys-44, Cys-67 to Cys-85, and Cys-74 to Cys-79 and evidence suggestive of two separate disulfide domains. Here we confirm these results and assign the remaining disulfide pairs.

To assign disulfides we used sequential digestions with enzymes of decreasing specificity. The results of each digestion placed increasing constraints on potential pairs that, in turn, diminished potential errors from disulfide rearrangement. To additionally preclude disulfide exchange artifacts, each peptide assignment was deduced either from the analysis of a major digestion product or from the composition of different peptides. We also applied sequencing methods (D.W., S.B., and E.B., unpublished work) to the analysis of disulfide pairs within complex peptides containing multiple disulfides and used mass spectrometry to facilitate the analysis of peptides and peptide mixtures. Finally, we used data from both bovine NP-I and -II. This assumes identical disulfide pairing in the two NPs, an assumption supported by almost identical disulfide optical activity and chemical properties (3, 12, 13) and, here, by the isolation of comparable cystine-containing peptides from both proteins.

### EXPERIMENTAL PROCEDURES

Purified bovine NP-I and -II (13) were subjected to sequential enzymatic digestions as described (10, 11). After each digestion, the mixture was fractionated on Sephadex G-50, and the composition of released peptides was monitored, together with that of the residual protein core (the latter to become the substrate for the subsequent digestion). Peptide purification was achieved using ion-exchange and reverse-phase HPLC as described (10, 11). Mass spectrometric (MS) measurements on the <sup>252</sup>Cf fission fragment spectrometer (14, 15) and amino acid analyses were also done as described (10, 11).

Stepwise Edman degradation was performed on an Applied Biosystems gas-phase sequencer (model 470A) equipped with a model 120A PTH analyzer as generally described elsewhere (16). Typical quantities sequenced were 100–500 pmol for small peptides and up to 10 nmol for larger peptides containing multiple disulfides—the higher quantities facilitating detection of cystine for more cycles. Disulfides were not modified before sequencing, and reducing agents were omitted from direct contact with the peptide on the solid support during sequencing. Cystine was identified on the sequencer by the presence of the phenylthiohydantoin (PTH) derivative of Cys<sub>2</sub> and also as the dithiothreitol adduct of the PTH derivative of dehydroalanine (Dha); the latter is formed by the decomposition of cystine (and serine) during the Edman reaction (17).

Our strategy for assigning disulfide partners in peptides containing multiple disulfides applies the fact that, during sequencing, (PTH-Cys)<sub>2</sub> is seen in significant amounts only when the second half-cystine of a disulfide bond reacts with Edman reagent. However, (PTH-Cys)<sub>2</sub> elutes from the PTH analyzer at the same position as the PTH derivative of tyrosine (e.g., ref. 18). To distinguish between the PTH

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Abbreviations: NP, neurophysin; PTH, phenylthiohydantoin; Dha, dehydroalanine; S, protease, *Staphylococcus aureus* protease V8; u, unified atomic mass units.

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derivatives of cystine and tyrosine, in many of our studies, Tyr-49 was nitrated (19); the PTH derivative of nitrotyrosine elutes at a different position than (PTH-Cys)<sub>2</sub> (D.W., S.B., and E.B., unpublished data). It is relevant that low levels of (PTH-Cys)<sub>2</sub> can be seen at cycles where only the first half-cystine of a disulfide pair has been derivatized. This fact may reflect a low level of disulfide rearrangement during sequencing and can be recognized by the low yield of (PTH-Cys)<sub>2</sub> at this cycle relative to that obtained during subsequent sequencing steps (D.W., S.B., and E.B., unpublished data). Additionally the yield of (PTH-Cys)<sub>2</sub> relative to PTH-Dha can be shown to diminish with each cycle (18; D.W., S.B., and E. B., unpublished data). These considerations, derived from studies of proteins and peptides with established disulfide pairs (D.W., S.B., and E.B., unpublished data), are applied in our data analyses.

## RESULTS

**Digestion with Trypsin and *Staphylococcus aureus* Protease V8 (*S. protease*); Initial Constraints on Disulfide Pairing.** Digestion of bovine NP-I and -II with trypsin released residues 1–8 and 19–20 from NP-I and -II and the peptide 87–95 from NP-II (10, 20). Additionally, complete cleavage at Arg-43 and Arg-66 occurred in NP-II (10) but only at Arg-43 in NP-I—cleavage at Arg-66 occurring in insignificant yield under the conditions used (see below). Points of trypsin cleavage are shown in Fig. 1. The failure to isolate a peptide containing residues 9–18 after trypsin treatment indicated that Cys-10 and Cys-13 are not mutually paired. [The possibility that this peptide was noncovalently bonded to the protein, similar to RNase S-peptide (21), was excluded when NP-II was precipitated by trichloroacetic acid after trypsin treatment and the peptide Gln-9–Lys-18 was not found in the supernatant. Additionally, mass spectrometric analysis of

trypsin-digested NP-I gave a measured mass of 8127.4 unified atomic mass units (u), confirming that the peptide was covalently attached to the core.] Digestion of trypsin-treated NP by *S. protease* resulted in complete cleavage at Glu-40 (and Glu-84 in NP-II) and partial cleavage at Glu-31, Glu-46, and Glu-47 (Fig. 1). Again, no cystine-containing peptides were isolated. Therefore, as described earlier (10), the pairs Cys-34 to Cys-44; Cys-34 to Cys-85; Cys-44 to Cys-85; and Cys-54 to Cys-61 were excluded. The NP-II “core” after trypsin and *S. protease* digestion was treated with thermolysin, leading to the quantitative release of several peptides, including two subsequently identified as representing the pairing Cys-21 to Cys-44 and Cys-67 to Cys-85 (10). Thermolysin digestion data also indicated complete cleavage after Cys-21, Cys-34, Ser-25, and Glu-31 (10). Identification of the latter two cleavage positions and the failure to isolate a peptide containing Cys-10, Cys-13, Cys-27, and Cys-28 accordingly indicate that these four half-cystines are not exclusively paired among themselves (see Fig. 1).

**Confirmation of the Structures of Peptides Containing Cys-67 to Cys-85, Cys-21 to Cys-44, and Cys-74 to Cys-79 by Peptide Sequencing.** Digestion of trypsin-treated NP-II with subtilisin released cystine-containing peptides. The isolation and brief characterization of several of these have been reported, the results suggesting the pairing Cys-67 to Cys-85, Cys-21 to Cys-44, and Cys-74 to Cys-79 (10, 11). We have now firmly established these disulfide pairs as follows:

(i) *Cys-67 to Cys-85.* Peptides containing this disulfide were previously assigned the structure(s) Cys-67-Ala, Cys-85-Arg and Cys-67-Ala-Ala, Cys-85-Arg by mass spectrometry and amino acid analysis (10, 11). Gas-phase sequencing confirmed the latter structure (Table 1, peptide 1). (PTH-Cys)<sub>2</sub> and a small quantity of PTH-Dha were observed at cycle 1,

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Bovine II	Ala	Met	Ser	Asp	Leu	Glu	Leu	Arg	Gln	<u>Cys</u>	Leu	PRO	<u>CYS</u>	GLY	PRO	GLY	GLY	LYS	GLY	ARG	<u>CYS</u>	PHE	GLY	PRO
Bovine I	—	Val	Leu	—	Asp	Val	—	Thr	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
Bovine II	SER	ILE	<u>CYS</u>	<u>CYS</u>	GLY	ASP	GLU	Leu	Gly	<u>Cys</u>	Phe	Val	Gly	Thr	Ala	Glu	Ala	Leu	Arg	<u>Cys</u>	Gln	Glu	Glu	Asn
Bovine I	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
Bovine II	Tyr	Leu	Pro	Ser	Pro	<u>Cys</u>	Gln	Ser	Gly	Gln	Lys	PRO	<u>CYS</u>	GLY	SER	GLY	GLY	ARG	<u>CYS</u>	ALA	ALA	ALA	GLY	ILE
Bovine I	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	
Bovine II	<u>CYS</u>	<u>CYS</u>	ASN	ASP	GLU	Ser	<u>Cys</u>	Val	Thr	Glu	Pro	Glu	<u>Cys</u>	Arg	Glu	Gly	Ile	Gly	Phe	Pro	Arg	Arg	Val	
Bovine I	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	

FIG. 1. Amino acid sequence of bovine NP-I and -II. The complete sequence of NP-II is shown, whereas for NP-I, only the substitutions are listed. Positions of 14 half-cystines are underlined. Residues 12–31 and 60–77 are in uppercase letters to show the internal duplication. †, Positions of cleavage by trypsin; ‡, positions of cleavage of trypsin-treated NPs by *S. protease* (10); and ‡a, position at which cleavage did not necessarily go to completion. See refs. 5 and 6 for representative sequence studies of the bovine neurophysins.

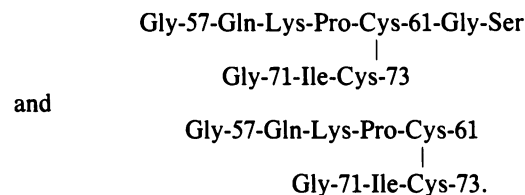
whereas PTH-Arg and PTH-Ala were identified at cycle 2. At cycle 3, PTH-Ala was the only residue identified.

(ii) *Cys-21 to Cys-44*. Amino acid and MS analysis of this peptide from NP-II (11) indicated the structure  $\overline{\text{Cys-21-Ser-25, Cys-44-Asn-48}}$ . The actual disulfide pairing was confirmed by sequencing (Table 1, peptide 2). At cycle 1, (PTH-Cys)<sub>2</sub> was observed in high yield with a small quantity of PTH-Dha. Residues at cycles 2–5 (Table 1) were as expected. We have now isolated the identical peptide from subtilisin digests of trypsin-treated NP-I and independently established its structure by MS and sequencing.

(iii) *Cys-74 to Cys-79*. Two peptides with this pairing were isolated from NP-II digests as closely eluting peptides on anion-exchange HPLC (10, 11). Amino acid composition and MS analysis of the major peptide indicated the sequence Cys-74–Glu-84 (10, 11). Sequencing results (Table 1, peptide 3) indicate a small quantity of (PTH-Cys)<sub>2</sub> at cycle 1; this is attributed to a limited rearrangement on the sequencer (see *Experimental Procedures*), because MS data (11) gave no evidence of a similar rearrangement. At cycles 2–5 and 7–8 the expected amino acids were obtained. At cycle 6, cystine was indicated by a clear increase in (PTH-Cys)<sub>2</sub>. The data uniquely define the peptide structure  $\overline{\text{Cys-74-Asn-Asp-Glu-Ser-Cys-79-Val-Thr}}$ . Sequencing of the minor component, from the anion-exchange column, also indicated Cys-74 to Cys-79 pairing (Table 1, peptide 4). The presence of aspartic acid at cycle 1, both asparagine and glutamic acid at cycle 2, and cystine at cycle 4 indicates that this peptide is derived from that above by subtilisin cleavage of the Asn-Asp bond.

**Isolation of a Peptide Containing Cys-61 to Cys-73; Demonstration of a COOH-Terminal Domain.** The peptide fraction obtained from subtilisin digestion of trypsin-treated NP-II was transferred to an anion-exchange HPLC column, and the nonbinding peak eluting at ≈2.35 min was further purified by reverse-phase HPLC. Amino acid analysis of a major peak, eluting at ≈17.0 min, suggested the presence of a peptide containing Cys-61 paired to either Cys-27 or Cys-73 as evidenced by approximately equimolar quantities of cystine,

lysine, glutamic acid (glutamine), and isoleucine. The disulfide pairing was established by MS and sequencing (Table 1, peptide 5), which demonstrated the presence of the following two related structure(s):



Sequencing yielded PTH-Gly at cycle 1 with an intensity approximately twice that of subsequent residues, whereas PTH-Ile and PTH-Gln were seen at cycle 2. Residues at cycles 3–6 were as assigned, including a good yield of (PTH-Cys)<sub>2</sub> and PTH-Dha at cycle 5. The lack of PTH-Ser at cycle 1 precluded the presence of Cys-27. MS analysis showed an intense peak corresponding to the measured mass 820.6 u and a much weaker mass peak of 964.7 u. The lower mass is in excellent agreement with that calculated (820.4 u) for the unextended peptide above, whereas the higher mass agrees with that calculated (964.5 u) for the extended peptide.

The pairing Cys-61 to Cys-73, Cys-67 to Cys-85, and Cys-74 to Cys-79 indicates that NP contains a COOH domain in which the six COOH-terminal half-cystines are paired among themselves; this was confirmed by independent determination of the pairing of the eight half-cystines in the sequence 1–54 (see below).

**Isolation of Peptides Containing Cys-10, Cys-13, Cys-27, Cys-28, Cys-34, and Cys-54 from NP-II.** In a preliminary study (11) the purified protein core from subtilisin digestion of trypsin-treated NP-II was analyzed by sequencing and MS. The results indicated the presence of a complex disulfide-linked set of peptides containing Cys-10, Cys-13, Cys-27, Cys-28, Cys-34, and Cys-54, providing preliminary evidence for two disulfide domains (11). A more complete analysis was precluded by the partial cyclization of Gln-9 to pyroGlu-9, resulting in a low yield of the sequence Gln-9–Lys-18 on the sequencer. Accordingly, a disulfide pairing study has now been done using bovine NP-I, which has threonine at position 9; as shown below this has permitted the pairing of Cys-10, Cys-13, Cys-27, Cys-28, Cys-34, and Cys-54.

**Pairing of Cys-10, Cys-13, Cys-27, Cys-28, Cys-34, and Cys-54.** These half-cystines could be paired among themselves in 15 possible combinations (22) shown in Table 2. Of these, combinations 1–3 are excluded because they would bridge Cys-10 to Cys-13. The actual pairs were established by

Table 1. Gas-phase sequencing of peptides isolated after subtilisin digestion of trypsin-treated NP-II

Peptide*	Cycle							
	1	2	3	4	5	6	7	8
1	Cys   Cys	Ala Arg	Ala					
2	Cys   Cys	Phe Gln	Gly Glu	Pro Glu	Ser Asn			
3	†	Asn	Asp	Glu	Ser	Cys   Cys	Val	Thr
4	Asp   †	Glu Asn	Ser	Cys   Cys	Val	Thr		
5	Gly‡   Gly‡	Gln Ile	Lys †	Pro	Cys   Cys	Gly		

\*Peptide 1 represents the bridge Cys-67 to Cys-85; peptide 2 represents Cys-21 to Cys-44; peptides 3 and 4 represent Cys-74 to Cys-79 and peptide 5 represents Cys-61 to Cys-73.

†Small quantities of (PTH-Cys)<sub>2</sub> and/or PTH-Dha appeared that were not significant for that cycle.

‡Intensity of the amino acid identified was higher than expected for 1 mol equivalent at that cycle.

Table 2. Possible disulfide pairing patterns of Cys-10, Cys-13, Cys-27, Cys-28, Cys-34, and Cys-54

Combination	(i)		(ii)		(iii)	
	Cys to Cys	Cys to Cys	Cys to Cys	Cys to Cys	Cys to Cys	Cys to Cys
1	10	13	27	28	34	54
2	10	13	27	34	28	54
3	10	13	27	54	28	34
4	10	27	13	28	34	54
5	10	27	13	34	28	54
6	10	27	13	54	28	34
7	10	28	13	27	34	54
8	10	28	13	34	27	54
9	10	28	13	54	27	34
10	10	34	13	27	28	54
11	10	34	13	28	27	54
12	10	34	13	54	27	28
13	10	54	13	27	28	34
14	10	54	13	28	27	34
15	10	54	13	34	27	28

Table 3. Gas-phase sequencing of trypsin-treated NP-I

															Cycle									
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15										
Thr		Leu	Pro*	†	Gly	Pro	Gly	Gly*	Lys															
						Cys							Cys											
Cys	Phe	Gly	Pro*	Ser	Ile		†	Gly*	Asp	Glu	Leu	Gly		Phe										
						Cys				Cys			Cys											
Cys	Gln	Glu	Glu	Asn	Tyr	Leu	Pro	Ser	Pro		Gln	Ser	Gly	Gln										
										Cys														

Amino acids listed were those found as PTH derivatives in each cycle.

\*Intensity of the amino acid identified was higher than expected for 1 mol equivalent at that cycle.

†Small quantities of (PTH-Cys)<sub>2</sub> and/or PTH-Dha appeared that were not significant for that cycle.

sequential digestion of bovine NP-I with trypsin, *S. protease*, and thermolysin. The core was sequenced after each digestion to systematically exclude additional pairing patterns in Table 2 and establish the actual disulfides.

(i) *Sequencing of the trypsin-treated NP-I core.* The residual NP-I core from trypsin digestion was sequenced (Table 3). Cycle 1 yielded PTH-Thr and (PTH-Cys)<sub>2</sub>. At cycle 2, PTH-Phe and PTH-Gln were seen, indicating that the (PTH-Cys)<sub>2</sub> in cycle 1 is derived from NH<sub>2</sub>-terminal Cys-21 and Cys-44 such that the two are paired, as independently demonstrated above. No alanine was observed at cycle 2, indicating that, for NP-I, trypsin did not cleave after Arg-66; therefore, Cys-67 is not an NH<sub>2</sub> terminus. At all cycles, the residues identified confirmed that sequencing was occurring from positions 9, 21, and 44 (Table 3). The most significant results are as follows. At cycle 7, where Cys-27 is derivatized, PTH-Dha clearly increased, indicating that Cys-27 is paired to either Cys-10 or Cys-13. A peak eluting as (PTH-Cys)<sub>2</sub> or PTH-Tyr was also seen, but could not be assigned to (PTH-Cys)<sub>2</sub> because of potential carry-over of PTH-Tyr from cycle 6 (see *Experimental Procedures*). Accordingly, we sequenced an analogous core in which the PTH-Tyr contribution was avoided by nitration of Tyr-49. In this case, no significant PTH-Tyr was seen at cycle 6, whereas a chromatographically similar peak, accordingly identified as (PTH-Cys)<sub>2</sub>, increased at cycle 7, together with PTH-Dha. At cycle 8, Cys-28 was derivatized. However, PTH-Dha and (PTH-Cys)<sub>2</sub> did not increase, which means that Cys-28 is not paired to Cys-10, Cys-13, or Cys-27 but is paired to Cys-34 or

Cys-54, consistent with thermolysin-digestion constraints. At cycle 11, where Cys-54 is derivatized, (PTH-Cys)<sub>2</sub> increased, indicating that Cys-54 is paired to either Cys-28, Cys-10, or Cys-13, but not to Cys-34 or to Cys-27, because Cys-34 had not yet reacted, whereas Cys-27 is paired to either Cys-10 or Cys-13. At cycle 14, Cys-34 was derivatized and was expressed by increased (PTH-Cys)<sub>2</sub> and PTH-Dha, consistent with its pairing to Cys-10, Cys-13, or Cys-28. The sequencing results, therefore, exclude all pairing patterns in Table 2 except combinations 5, 6, 10, and 13.

(ii) *Sequencing of the trypsin and S. protease-digested nitrated NP-I core.* The NP-I core from trypsin digestion was nitrated, treated with *S. protease* (10, 11), and fractionated. Sequencing of the residual core (Table 4) indicated that additional NH<sub>2</sub> termini (additional to those in the trypsin core) were generated in ≈50% yield at Leu-32, Glu-47, and Asn-48, reflecting partial cleavage by *S. protease* after Glu-31, Glu-46, and Glu-47, respectively (10). The significant features of the data are that, at cycles 2 and 3, Cys-10 and Cys-34 are derivatized respectively, but with no significant increase in (PTH-Cys)<sub>2</sub> and PTH-Dha; this indicates that Cys-10 is not bonded to Cys-34. At cycle 5, Cys-13 is derivatized, but (PTH-Cys)<sub>2</sub> again did not increase significantly, indicating that Cys-13 is not bonded to Cys-10 (as also

Table 4. Gas-phase sequencing of trypsin and *S. protease*-digested nitrated NP-I

										Cycle									
1	2	3	4	5	6	7	8	9	10										
Thr	*	Leu <sup>†</sup>	Pro <sup>†</sup>	*	Gly <sup>†</sup>	Pro <sup>†</sup>	Gly	Gly <sup>†</sup>	Lys										
						Cys	Cys												
Cys	Phe	Gly	Pro <sup>†</sup>	Ser <sup>†</sup>	Ile		Cys	Cys	Gly <sup>†</sup>	Asp									
						Cys													
Cys	Gln	Glu	Glu																
Leu	Gly	*	Phe	Val	Gly <sup>†</sup>	Thr	Ala	Glu											
Glu	Asn	NO <sub>2</sub> Tyr	Leu	Pro	Ser	Pro <sup>†</sup>	Cys	Gln	Ser										
Asn	NO <sub>2</sub> Tyr	Leu <sup>†</sup>	Pro <sup>†</sup>	Ser <sup>†</sup>	Pro	Cys	Gln	Ser	Gly										
						Cys													

\*Small quantities of (PTH-Cys)<sub>2</sub> and/or PTH-Dha appeared that were not significant for that cycle.

†Intensity of the amino acid identified was higher than expected for 1 mol equivalent at that cycle.

Table 5. Gas-phase sequencing of trypsin, *S. protease*-, and thermolysin-digested nitrated NP-I

							Cycle						
1	2	3	4	5	6	7							
Thr	*	Leu <sup>†</sup>	Pro <sup>†</sup>	Cys <sup>†</sup>	Gly	Pro							
				Cys									
Ile	*	Cys	Gly	Asp	Glu								
Leu <sup>†</sup>	Gly		Phe										
		Cys				Cys <sup>‡</sup>							
Asn	NO <sub>2</sub> Tyr	Leu <sup>†</sup>	Pro <sup>†</sup>	Ser	Pro								
						Cys <sup>‡</sup>							
NO <sub>2</sub> Tyr	Leu <sup>‡</sup>	Pro <sup>‡</sup>	Ser <sup>‡</sup>	Pro <sup>‡</sup>	Cys	Gln <sup>‡</sup>							
Leu <sup>†</sup>	Pro <sup>†</sup>	Ser	Pro <sup>‡</sup>	Cys <sup>†</sup>	Gln	Ser							
				Cys									

\*Small quantities of (PTH-Cys)<sub>2</sub> and/or PTH-Dha appeared that were not significant for that cycle.

†Intensity of the amino acid identified was higher than expected for 1 mol equivalent at that cycle.

‡For these residues it was not strictly possible to differentiate the observed peak from the carry-over from the previous cycle. However, the yields were higher than would be expected for such carry-over.

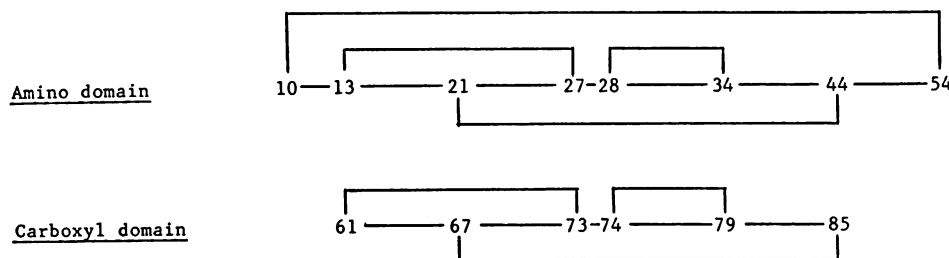


FIG. 2. Comparison of disulfide pairing in the two domains of the bovine NPs; only the positions of half-cystines are shown.

previously demonstrated) or to Cys-34; note that PTH-Dha cannot be used to quantitate cystine at this cycle because PTH-Dha has a contribution from Ser-25. The results further exclude all pairing patterns in Table 2 except 6 and 13 and indicate that Cys-34 must be bonded to Cys-28 (independently confirmed below). The release of (PTH-Cys)<sub>2</sub> at cycles 7 and 8 accords with these assignments.

(iii) *Sequencing of the trypsin and S. protease and thermolysin-digested nitrated NP-I core.* The trypsin and *S. protease*-digested nitrated NP-I was reacted with thermolysin, as described earlier (10) and fractionated. Sequencing of the core (Table 5) gave high yields of Thr-9, Ile-26, and Leu-32, at cycle 1, with lower individual yields of Asn-48, NO<sub>2</sub>Tyr-49, and Leu-50. At cycle 2, Cys-10 and Cys-27 were derivatized, but yields of (PTH-Cys)<sub>2</sub> and PTH-Dha were low relative to subsequent cycles, indicating that Cys-10 and Cys-27 are not paired to each other. At cycle 3, Cys-28 and Cys-34 were derivatized, and the yield of (PTH-Cys)<sub>2</sub> was good. Because the trypsin core data excluded the pairing of Cys-28 to either Cys-10 or Cys-27, the results independently confirm the pairing of Cys-28 to Cys-34. At cycle 5 and subsequent cycles, where Cys-13 and Cys-54 are derivatized, (PTH-Cys)<sub>2</sub> was observed in high yield, consistent with the pairing Cys-10 to Cys-54 and Cys-13 to Cys-27. Therefore, the data are all consistent with combination 13 in Table 2 and exclude all other arrangements.

## DISCUSSION

The two-domain pattern of NP disulfide pairing is summarized in Fig. 2. Related half-cystines in the two domains are homologically paired. Only the Cys-10 to Cys-54 pair of the NH<sub>2</sub> domain, which represents the additional two half-cystines not found in the COOH domain, is unique; this pair ties the two ends of the NH<sub>2</sub> domain together. The demonstration of homologous pairing in the two domains is of interest in view of the different methods used to assign disulfide pairs in each. Proteolytic cleavage of the COOH domain yielded small identifiable peptides containing single disulfides. Disulfides of the NH<sub>2</sub> domain were largely assigned by sequencing complex cores containing multiple disulfides and systematically following the release of (PTH-Cys)<sub>2</sub> from different proteolytically derived sequencing "start-points."

The presence of two disulfide domains accords with two earlier suggestions (7, 8), but the specific pairing departs markedly from previous suggestions for NP (4, 7, 8). The two-domain structure places the internally duplicated segments 12-31 and 60-77 in separate domains and invites attempts to split NP between domains. However, we have been unsuccessful so far in achieving enzymatic cleavage within this sequence without significant cleavage elsewhere. This result suggests that this region is not an ordinary hinge, but interacts with, or is hindered by, other regions of the molecule.

The Cys-10 to Cys-54 bridge clearly serves to help structurally organize the NH<sub>2</sub> domain. We suggest that this bridge represents the functionally critical but previously unassigned NP disulfide (3) that is highly vulnerable to reduction. There is reason to believe that the NH<sub>2</sub> domain plays the principal role in peptide binding. All NP residues proposed so far to

play a role at or near the binding site, Arg-8 (20, 23, 24), Tyr-49 (24, 25), and Glu-31 (26) fall within the NH<sub>2</sub>-terminal 54 residues. Although Glu-31 is now unlikely to represent the important (1) salt-bridge COOH group [Glu-31 is substituted by alanine in guinea pig NP (27)], the other *strictly conserved* carboxylic candidates for salt-bridge participation (e.g., Fig. 1) also lie within the NH<sub>2</sub> domain. This does not preclude a functional role for the COOH domain. Potential interactions between the two NP domains are indeed suggested (see above) and merit exploration.

**Note Added in Proof.** The two domains demonstrated here have recently been confirmed in human NP (28).

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