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CHEMICAL MODIFICATION OR EXCISION OF NEUROPHYSIN ARGININE-8 IS ASSOCIATED WITH LOSS OF PEPTIDE-BINDING ABILITY*

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The role of neurophysin arginines in the binding of peptides to the principal hormone-binding site was evaluated by modification of neurophysin with cyclohexanedione and by limited proteolysis with trypsin. Binding affinities were reduced to values less than 3% of normal when both Arg-8 and Arg-20 were chemically modified or when residues 1-8 were excised by trypsin. Because residues 1-6 are non-essential to binding and residue 7 is not conserved in evolution, the results are interpreted in terms of a functional role for Arg-8.

The principal peptide hormone-binding site of neurophysin is largely undefined (e.g., 1). Evidence suggesting involvement of Glu-31 (2) and perhaps also of Tyr-49 (cf., 1,3) has been reported. No evidence has been presented implicating a neurophysin arginine either directly or indirectly in hormone-binding. Nonetheless, of the variable number of arginine residues found in different neurophysins, four are strictly conserved in evolution: Arg-8, 20, 43 and 66 (e.g., 1). The present studies were undertaken to evaluate the role of these residues.

EXPERIMENTAL PROCEDURE

Preparation of native and modified neurophysins: Bovine neurophysins-I and -II were prepared as described elsewhere (4). Modification of neurophysin arginine residues with cyclohexanedione (CHD) was carried out at pH 9, 37°C, 10 mg/ml protein, using established procedures (5). For studies in which binding affinities were to be quantitated, the native protein was first nitrated at Tyr-49 as previously described (6) and then treated with cyclohexanedione; peptide-binding affinities of the nitrated protein were then determined by CD as described elsewhere using L-phenylalanyl-L-tyrosine amide, which binds to the principal hormone-binding site, as the test ligand (6). Partial digestion of native neurophysin-II and -I (each 2 mg/ml) with trypsin was carried out at pH 7.6, 25°C, for 3 hours using 0.01 mg/ml bovine pancreatic trypsin (Sigma, chymotrypsin-inactivated) or for 1 hour using 0.02 mg/ml trypsin. After digestion, the trypsin was inactivated by treatment with tosyl-lysyl-chloroketone (0.5 mg/ml).

Separation and analysis of components of modified neurophysins: Protein that had been reacted to varying degrees of completion with cyclohexanedione was

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fractionated into components of differing peptide-binding ability using affinity chromatography as previously described (7) but including 0.1 M borate in the eluting buffer. "Non-binding protein" emerges at or near the void volume at pH 6.1 and has an affinity $\leq$ 1/40 that of native protein; "binding protein" is eluted in the void volume at pH 2.1 with an affinity >1/10 that of native protein. The different fractions were subjected to total amino acid analysis (7), adding thioglycollic acid during hydrolysis to protect modified arginines (5). Fractions were also analyzed for their trypsin-liberatable arginine residues by vigorous digestion with 2.5% by weight trypsin (pH 8 in borate, 37°C for 5 hours plus 25°C for 12 hours); released peptides were separated from the disulfide-linked residual protein core by gel-filtration on Sephadex G- 50 (7) and each fraction subjected to total amino acid analysis as described above.

Protein that had been modified by partial trypsin-digestion was also fractionated by affinity chromatography, followed by dialysis or gel-filtration to remove salt and peptides. The individual components were analyzed by polyacrylamide gel electrophoresis in the absence of SDS as previously described (7). Because the non-binding component of partially digested neurophysin-II was heterogeneous on polyacrylamide gel electrophoresis, the desired fraction (see text) was purified by isolation from analytical gels before staining. The different components from the partial digestions were analyzed for total amino acid composition and by SDS gel electrophoresis in the presence of mercaptoethanol (7). They were additionally treated with carboxypeptidase B to determine the number of arginine or lysine end-groups (7). CD studies were carried out using a Jobin-Yvon Mark 5 dichrograph.

RESULTS

Cyclohexanedione modification: Bovine neurophysin-I contains only the four evolutionarily conserved Arg residues -8, 20, 43 and 66. Treatment with CHD[1] for different time periods indicated that all four were modified at the same or overlapping rates; no amino acids additional to Arg were modified, as judged by amino acid analysis. Modification of all four Arg led to a reduction in peptide-binding affinity of the nitrated protein to a value 1/30 that of unmodified nitrated protein, if binding affinity was determined in the absence of borate. In the presence of 0.1 M borate, which forms an adduct with the cyclohexanedione conjugate (5), binding affinity was 1/1000 that of unmodified protein. Circular dichroism studies indicated little or no effect of CHD modification on neurophysin conformation. Reversal of CHD-modification by NH_2OH treatment (5) led to a return of binding affinity to a value 60% that of native protein.

To identify the Arg responsible for the CHD effect, protein treated with CHD to give varying degrees of modification was fractionated into "binding"

1/ Abbreviations used are: CHD, cyclohexanedione; CD, circular dichroism; SDS, sodium dodecyl sulfate, NP, neurophysin.

Table I. Distribution of Unmodified Arginine Residues in Different Components of CHD-modified Neurophysin-I[a/]

	Residues/mole	
	Binding fraction	Non-binding fraction
Total unmodified Arg	1.8 ± 0.1	0.9 ± 0.2
Unmodified Arg released by trypsin	1.0 ± 0.2	0.2 ± 0.1
Unmodified Arg-8	0.6 ± 0.2	0.1 ± 0.05
Unmodified Arg-20	0.4 ± 0.2	0.1 ± 0.05
Residual unmodified Arg after complete trypsin digestion	0.8 ± 0.1	0.7 ± 0.1
Unmodified Arg-43	0.25 ± 0.05	0.25 ± 0.05
Unmodified Arg-66	[0.55]	[0.45]

a/ Results are averages of three studies. The trypsin-releasable unmodified Arg were identified as described in the text; e.g., for each mole of trypsin-released Arg in the binding fraction, the composition of the released peptides and of the residual protein core indicated that only 0.6 mole of amino acids in the sequence 1-8 were lost (refer to Table II for sequence), leaving the residual 0.4 mole assignable to Arg-20 which is released as a Gly-Arg dipeptide. This dipeptide was also demonstrated by ion-exchange chromatography. Unmodified Arg-43 was determined by carboxypeptidase B digestion of the protein core left after trypsin-digestion; this quantitatively releases Leu-42 as the sole released leucine together with unmodified terminal Arg-43. Unmodified Arg-66 is estimated as the difference between the residual unmodified Arg content of the protein core and that ascribed to Arg-43.

and "non-binding" components by affinity chromatography and the degree of modification of each component determined. The most minimally modified non-binding component contained 2 modified Arg residues, suggesting that 2 Arg are involved in inactivation, but only more extensively modified non-binding protein was obtainable in good yield. Accordingly, protein that had been more extensively treated (to give 3 modified Arg/mole in the non-binding fraction) was similarly fractionated and analyzed. The results (Table I) indicated that the "binding" component contained one less modified Arg than the "non-binding" component. Arg residues contributing to the difference between the two components were identified as follows. Of the four Arg in neurophysin-I, two (Arg-8 and -20) can be released as small peptides by trypsin from the disulfide-containing protein while the other two remain in peptides that are disulfide-bonded to the protein core (8). Trypsin does not cleave at CHD-modified Arg residues (5). Accordingly, the binding and non-binding CHD-modified com-

ponents were analyzed for the number and identity of their trypsin-releasable Arg residues (Table I). The binding component contained 1 trypsin-releasable Arg while the non-binding component contained only traces of trypsin-releasable Arg. Analysis of the composition of the peptides released by trypsin and of the residual protein core indicated that the trypsin-releasable Arg in the binding component was not a single residue, but was essentially an equimolar mixture of Arg-8 and Arg-20. Preliminary analysis of the trypsin cleavage pattern at Arg residues in the protein core, using carboxypeptidase B to sequence residues adjacent to cleaved Arg positions revealed no significant difference in trypsin-sensitive core residues between binding and non-binding fractions (see Table I for details). These results are explained by a model in which modification of both Arg-8 and Arg-20 is necessary for the observed loss of binding ability. However, some diminution of activity associated with modification of either Arg-8 or -20 alone is not precluded since, in the affinity chromatographic procedure we use (7, Experimental Procedure) binding affinity of a component must be reduced to a value $\sim$1/10 that of the native protein before it can be distinguished from native protein.

Partial digestion by trypsin: To further investigate the role of Arg-8, we studied the effects of partial tryptic digestion on neurophysin activity. The object was to assess the effects of cleavage at Arg-8. While such cleavage would liberate residues 1-8, residues 1-6 of neurophysin-II are non-essential to binding (7). Fig. 1 shows the gel electrophoretic pattern of neurophysin-II after limited digestion compared to a standard of crude neurophysin. The crude NP standard is used as a calibrator of changes in net charge under our conditions (7). Components of the digest are numbered in order of their formation. On affinity chromatography, #1 was the only significant component retarded by the column (Fig. 1). It is relevant to the identity of the different electrophoretic components that neurophysin-II has three Arg residues additional to those found in neurophysin-I. These are Arg-86, 93 and 94, two of which (like Arg-8 and -20) can be released as peptides by tryptic attack on the native protein; i.e., tryptic attack at Arg-93 liberates the C-terminal Arg_{94}-Val_{95}

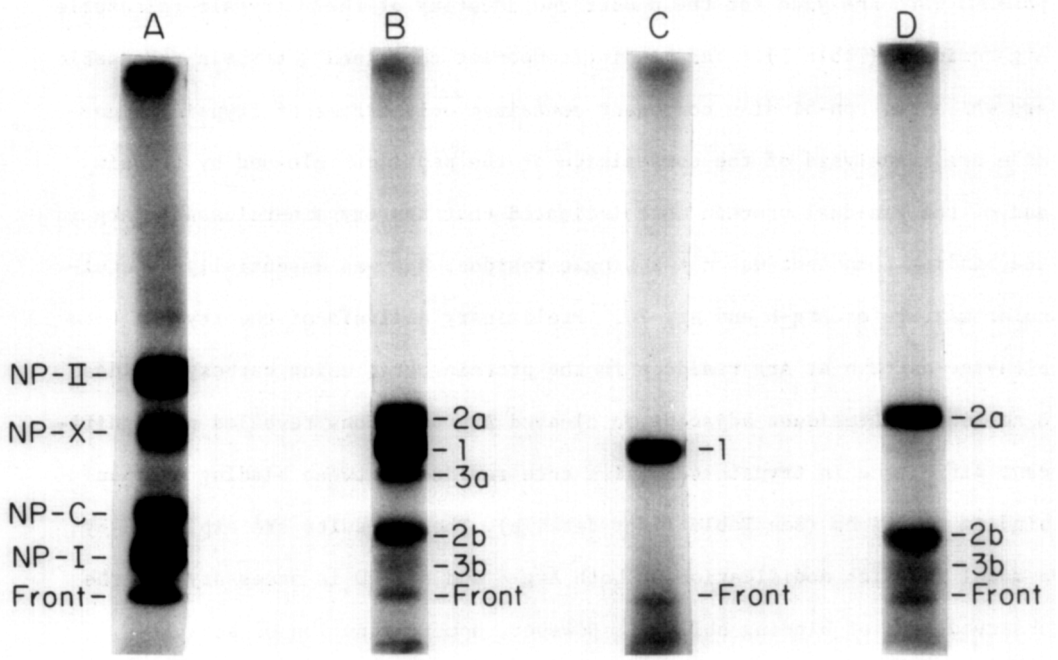

Figure 1. Polyacrylamide gel electrophoresis of components of trypsin-digested neurophysin-II. Conditions: running pH 9.5, no SDS; direction of migration (to the anode) is from top to bottom. For other details, see (7). Gel A: unfractionated crude bovine neurophysin used as electrophoretic standard (7); labels indicate different native neurophysin components (7). Gel B: purified neurophysin-II partially digested by trypsin; components are numbered in order of their appearance with time. Gel C: sample shown in Gel B, but after affinity chromatography to remove non-binding components; component shown (#1) is retained by the affinity column. Gel D: non-binding components separated by affinity chromatography from initial trypsin digest. Note that component 3a from the initial digest is not clearly evident in either Gel C or Gel D. Studies at higher concentration suggest that it is present as a trace component in the binding fraction.

dipeptide, while attack at Arg-86, liberates residues 87-93 (8). Amino acid analysis of #1 purified by this procedure (Table II) and its electrophoretic mobility relative to the standard (cf., 7) indicated that it represented attack at Arg-93 with loss of Arg-94 and Val-95, and, as such, was identical to the previously identified, fully active, first product of chymotryptic attack (7). The composition of the mixture of non-binding components indicated complete loss of residues 1-8 in addition to 94-95, and suggested 50% loss of 87-93 (data not shown). Consideration of the primary structure of neurophysin-II (1) indicates that loss of 1-8 will lead to a net gain of one positive charge (relative to #1) and is the only tryptic cleavage that can lead to a gain in

Table II
Compositions of Binding and Non-Binding Components from Partially Trypsin-digested Neurophysins vs. (Theory)[a,b]

	Residues/mole		Residues/mole deleted by trypsin							
	Native NP-II	Native NP-I	Binding NP-II		Non-Binding NP-II (2a)		Binding NP-I		Non-Binding NP-I	
Asp	5	7	--	(0)	1.1 ± 0.1	(1)	--	(0)	1.7 ± 0.2	(2)
Thr	2	2	--	(0)	0.3 ± 0.1	(0)	--	(0)	--	(0)
Ser	6	6	--	(0)	1.0 ± 0.1	(1)	--	(0)	--	(0)
Glu	13	9	--	(0)	2.6 ± 0.6[c]	(1.5)[b]	--	(0)	--	(0)
Pro	8	9	--	(0)	1.5[d]	(1)	--	(0)	--	(0)
Gly	15	14	--	(0)	N.D.[e]	(1)	--	(0)	--	(0)
Ala	6	9	--	(0)	0.9 ± 0.5	(1)	--	(0)	0.5[d]	(1)
Cys	14	14	--	(0)	--	(0)	--	(0)	--	(0)
Val	3.7[b]	3	1.2 ± 0.2	(1)	1.4 ± 0.1	(1.35)[b]	--	(0)	1.8 ± 0	(2)
Met	1	0	--	(0)	0.9 ± 0.1	(1)	--	(0)	--	(0)
Ile	2.3[b]	2	--	(0)	0.3 ± 0.3	(0.15)[b]	--	(0)	--	(0)
Leu	6	6	--	(0)	2.3 ± 0.5	(2)	--	(0)	2.0 ± 0.1	(2)
Tyr	1	1	--	(0)	--	(0)	--	(0)	--	(0)
Phe	3	3	0.2 ± 0.2	(0)	0.5 ± 0.3	(0.5)[b]	--	(0)	--	(0)
His	0	1	--	(0)	--	(0)	--	(0)	--	(0)
Lys	2	2	--	(0)	--	(0)	--	(0)	--	(0)
Arg	7	4	1.1 ± 0.1	(1)	2.75 ± 0.25	(2.5)[b]	--	(0)	1.0 ± 0	(1)

[a] Represents differences between compositions of untreated samples of each neurophysin and those of the different trypsin-generated components. Dashed lines indicate the absence of observed deletions. Values in parentheses are theoretical deletions for structures assigned in the text to the different components. For reference, the sequence 1-8 in NP-II is Ala-Met-Ser-Asp-Leu-Glu-Leu-Arg and the sequence 87-93 is Glu-Gly-Val(Ile)-Gly-Phe-Pro-Arg. The sequence 1-8 in NP-I is Ala-Val-Leu-Asp-Leu-Asp-Val-Arg. [b] Non-integral NP-II values reflect heterogeneity at position 89 (70% Val, 30% Ile) and assumed 50% deletion of 87-93 in non-binding fraction. [c] The high Glu content of NP-II makes difference calculations inexact. [d] Single determination. [e] Not determined because of residual glycine from electrophoresis buffer.

positive charge. Loss of 87-93 will lead to no change in net charge, while other potential tryptic cleavages, at Arg or Lys residues within the disulfide-linked protein core, will each generate a net negative charge under our electrophoretic conditions (7). By these criteria, we deduced that component 2a, which behaves as if it is more positive than #1, had lost 1-8 (in addition to 94-95) and possibly also 87-93. The more negatively charged non-binding components were identified a having lost these residues and having also undergone internal cleavage. Accordingly, component 2a was electrophoretically purified. Amino acid analysis (Table II) confirmed deletion of residues 1-8 (immediately apparent from loss of the single Met in position 2) and argued for 50% loss of residues 87-93. No evidence of cleavage at internal Arg or Lys residues was found by carboxypeptidase B digestion (cf., 7) which released 0.6 carboxy-terminal Arg residues per mole and no Lys or other residues (1 C-terminal Arg is predicted in the absence of internal clips).

Because bovine neurophysin-I has no Arg at position 86, ambiguities associated with effects of the possible loss of 87-93 in neurophysin-II, were removed by study of the effects of trypsin on neurophysin-I. Under the partial digestion conditions chosen, trypsin led to no discernible change in the electrophoretic behavior of neurophysin-I. However, on affinity chromatography approximately 50% of the partially digested protein was not bound by the column. The amino acid composition of the binding and non-binding protein fractions (from which all released peptides had been separated) indicated that the two differed only in the absence of residues 1-8 in the non-binding fraction (Table II). Carboxypeptidase B end-group analysis of the non-binding component indicated that only a fraction ($\sim$30%) contained internal clips. No significant unfolding was found by CD. We also used SDS gel electrophoresis in the presence of mercaptoethanol to look for internal clips in both component 2a from the neurophysin-II digest and the non-binding neurophysin-I component. No peptides migrating more rapidly than the native protein were found in either case, but trypsin-generated small neurophysin peptides behaved abnormally in this system.

DISCUSSION

The present results argue for a role for Arg-8 in both the cyclohexanedione-mediated and trypsin-mediated inactivation of neurophysin. The effects of trypsin merit additional comment. In view of the lack of a role in binding for residues 1-6 (7), effects of cleavage at Arg-8 can in principle reflect loss of either residue 7 or residue 8. Residue 7 is not strictly conserved in evolution (e.g., 1); we therefore assign most of the effects to loss of Arg-8. However, the fact that loss of residues 1-8 has a more profound effect on binding than the CHD-mediated modification of Arg-8 alone allows for some contribution of residue 7.

Circular dichroism studies indicate that the effects of excision or modification of Arg-8 are not mediated by major protein unfolding. The results therefore suggest that Arg-8 may be near the binding site, either participating directly in binding or in maintaining the local conformation necessary for binding. This conclusion is of added interest in light of the original suggestion (9) that the internally duplicated segments in neurophysin may be particularly important to binding. Arg-8 does not lie within a region of internal duplication.

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