SODIUM/LIQUID AMMONIA REDUCTION OF PROLINE-CONTAINING PEPTIDES*

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It is desirable to have methods for splitting peptides at specific amino acid residues. A method for cleaving at proline residues can be based on the work of Birch et al. (1955) who studied the reduction of tertiary amides in solutions of sodium metal in liquid ammonia. These workers found that in the presence of protonic acids such as water, ethanol, or ammonium ion, tertiary amides were reduced in fair yields to hydroxyamines which decomposed in aqueous solution to yield the aldehyde corresponding to the acid portion of the original tertiary amide and presumably a secondary amine. This result suggested to us that sodium/NH_3 treatment of proline-containing peptides should result in cleavage of the peptide chain at the N-terminal side of proline residues. Other peptide bonds would not be expected to be reduced, but would instead, react with sodium to yield the anion of the amide, which, due to its negative charge, would resist the addition of electrons from the sodium. In this communication we wish to report the results of the successful application of this reaction to peptides.

While this research was in progress two other groups have reported on reductive cleavage methods specific for acyl-proline peptide bonds. Patchornik et al. (1964) found that solutions of lithium in methylamine at -70°C reduce C-terminal proline peptides to yield free proline, estimated by paper chromatograms, and that peptides which contained proline

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in the "interior" of the peptide chain yielded N-terminal proline residues. The extent of these cleavages ranged between 51-71%. (Presumably, this treatment would also reduce the benzene ring of phenylalanine residues to a cyclohexene ring since Patchornik and Wilchek (1962) observed reduction of the benzene ring of phenylalanine-containing peptides under similar conditions.) Ruttenberg et al. (1964) have reported on the reduction of proline peptides by lithium aluminum hydride in anhydrous tetrahydrofuran. Although the reaction was accompanied by some side reactions, they were successful in cleaving acyl-proline bonds in the cyclic peptides tyrocidin B and gramicidin S, as well as in some small linear peptides. In some cases the cleavage was essentially quantitative. In addition, they showed that the new C-terminal residue formed by cleavage of gramicidin-S was 2-amino-3-phenyl propionaldehyde.

Methods and Materials:

A heptapeptide, glycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-L-lysine, was kindly donated by Dr. F. H. Carpenter. Performic acid-oxidized B-chain was prepared from crystalline zinc beef insulin by the method of Craig et al. (1961). Other peptides were obtained from commercial sources and were checked for purity by amino acid analysis.

For reduction, a weighed amount of the peptide was placed in a 25 ml flask fitted with an empty cold finger condenser and then anhydrous ammonia was conducted into the flask through a drying tube filled with NaOH pellets. The outlet of the reaction vessel at the top of the condenser was fitted with a similar drying tube. Air was swept out with the ammonia and then a freezing mixture of isopropanol-CO_2 was placed in the cold finger. Liquid ammonia (15 ml) at -33°C was collected at the bottom of the flask. The resulting solution was stirred magnetically with a glass-covered stirring bar; (Teflon-covered stirring bars are attacked by Na/liquid NH_3). A weighed, freshly cut piece of Na metal was added to the rapidly stirred solution and the clock was started when the blue color permeated the solution. After

the desired period of time excess sodium was oxidized by the rapid addition of ammonium acetate crystals. The solvent was then allowed to evaporate through a drying tube filled with NaOH pellets, leaving a white residue in the flask. In experiments where large quantities of ammonia would interfere with the isolation of peptides, the reaction flask containing the residue of peptides and sodium acetate was evacuated in a vacuum desiccator for 24 hours over NaOH pellets and concentrated H_2SO_4 at room temperature and at 10-20μ of Hg pressure.

In experiments with glycyl-L-proline, and glycyl-L-prolyl-L-leucyl-glycyl-L-proline the residue was taken up in 20% $^{v}/_{v}$ aqueous acetic acid and the pH adjusted to 2.2 with concentrated HCl. Aliquots of the solution were diluted with 0.2 M sodium citrate buffer pH 2.2 and submitted for amino acid analysis.

Residues from reductions of heptapeptide were taken up in 0.2 M pyridine-acetate buffer pH 3.5 and adjusted to pH 3.0 by addition of 6 N HCl. Prolyl-lysine was isolated from the solution by chromatography on 50 A resin[1] (0.9 x 18 cm) using a linear gradient from pyridine (0.2 M)-acetate (pH 3.5) to pyridine (2.0 M)-acetate (pH 6.2) at 50°C. Ninhydrin positive material was located by the manual method of Moore and Stein (1954).

In experiments which tested for reductive losses in B-chain, the residue after reduction was taken up in 33% $^{v}/_{v}$ aqueous acetic acid and aliquots were hydrolyzed with an equal volume of concentrated HCl. Peptides were isolated from reduced B-chain as follows. The reduction residue containing about 9 mg peptide was suspended in 2.5 ml pyridine (0.1 M)-acetate (pH 3.5), the pH lowered to 2.5 with 1.0 N HCl and sufficient glacial HOAc added to produce a clear solution. The volume was then adjusted to 4 ml by addition of pyridine (0.1 M)-acetate (pH 3.5) and an aliquot of the solution was chromatographed at 53°C on 50 A resin[1] (0.9 x 17 cm) using a linear

[1]Beckmann Instruments, Inc., Spinco Division, Palo Alto, Calif.

gradient from pyridine (0.1 M)-acetate (pH 3.5) to pyridine (2.0 M)-acetate (pH 6.2). The peptide pro·lys·ala which was isolated in this chromatogram was found to be homogeneous on the basis of paper electrophoresis at pH 3.5 (pyridine (0.2 M)-acetate) for 1.5 hours at 27 volts/cm.

Amino acid hydrolyses were carried out as described by Crestfield et al. (1963). In some experiments with B-chain a crystal of phenol was added; recovery of tyrosine was poor otherwise.

Results and Discussion:

The extent of cleavage of acyl-proline bonds and the methods used for estimating this extent are shown in Table I along with the conditions used for reduction. The reduction of heptapeptide under the conditions shown in Table I resulted in large losses of phenylalanine and similar losses

TABLE I

Extents of Cleavage of Proline-Containing Peptides

Peptide reduced	Conditions*				Method of determination of extent of cleavage	% of cleavage
	weight (mg)	Na (mg)	time (min)	temp. (°C)		
gly-pro	17.1	62	90	-33	proline analysis	88**
gly-pro	17.2	50	3	-78	proline analysis	95#
gly-pro-leu-gly-pro	6.96	51	90	-33	proline analysis	64
heptapeptide	4.04	53	90	-33	aa analysis of isolated pro-lys	57
B-chain	7.77	5.8	0.67	-78	threonine analysis	91+
B-chain	8.62	6.9	0.67	-78	aa analysis of pro-lys-ala	78+

*All reductions were performed in 15 mls liquid NH_3.

**Under these conditions L-alanyl-L-valine was cleaved to a small extent (5.3%).

#There was no observable cleavage of L-alanyl-L-valine.

+The peptide was dried in vacuo, over P_2O_5 at 60° overnight before reduction; ammonia was distilled over Na.

occurred on reduction of B-chain under such conditions. That these losses could be greatly reduced was indicated by using the conditions shown for B-chain in Table I. The recovery of other amino acids from acid hydrolysates of unfractionated reduced B-chain was satisfactory as shown in Table II. The adjustment to pH 6 of a 33% aqueous acetic acid solution of reduced B-chain

TABLE II

Amino Acid Analysis[§] of B-chain and its Reduction Products

amino acid	Residues found* (molar ratios) B-chain	reduced B-chain	pH 6 precipitate	pro lys ala	theory for B-chain
asp	1.02	1.03	0.95		1
thr	0.91#	0.094#	0.05#		1
ser	0.82#	0.83#	0.86#		1
glu	2.97	3.14	2.97		3
pro	1.10	1.01	-	1.07	1
gly	3.01	3.05	3.00		3
ala	2.00+	2.00+	1.06	1.00+	2
1/2 cys	-	-	-		-
val	3.07	3.14	3.00		3
met	-	-	-		-
isol	-	-	-		-
leu	4.10	4.23	4.00++		4
tyr	1.98	1.93	1.81		2
phe	2.97	2.60	2.68		3
lys	1.02	1.02	0.05	1.23	1
his	1.98	2.12	1.80		2
arg	1.01	1.07	1.03		1
NH_3	2.26	5.42	2.98		2

[§]Peptides hydrolyzed 24 hrs, 110°, 6 N HCl.

*Cysteic acid was not determined.

#Not corrected for hydrolytic destruction.

+Taken as the basis for calculation of molar ratios.

++Leucine was taken as the basis for calculation of molar ratios since the threonine and lysine contents indicated a contamination (ca. 5%) of this peptide by unreacted B-chain, which results in a high value for alanine.

gave a precipitate which had the amino acid analysis expected for the N-terminal peptide produced by reductive cleavage at the threonyl-proline peptide bond (see Table II). Performic acid oxidation of unfractionated reduced B-chain produced no increase in the amount of threonine found after acid hydrolysis, and this may indicate that the new C-terminal residue is not threonine aldehyde.

Further experiments on this potentially useful reaction are currently being conducted in this laboratory, including its application to proteins containing several proline residues and identification of the new C-terminal residues produced on reduction. The behavior of methionine and tryptophan containing peptides under this reductive treatment has not yet been studied but such studies are now under way. It might be anticipated that partial reduction of rings in tryptophan and phenylalanine and reductive cleavage of the carbon-sulfur bond in thioethers would occur as side reactions. However, even at this stage of development this technique would clearly be of value in the structural study of peptides (and occasionally proteins) which contain few phenylalanine residues, and no methionine or tryptophan residues.

References

Birch, A. J., Cymerman-Craig, J. and Slaytor, M. (1955). Austr. J. Chem. 8, 512.
Craig, L. C., Konigsberg, W. and King, T. P. (1961) in Biochemical Preparations, Vol. 8, A. Meister, Ed. John Wiley and Sons, Inc. New York, London, p. 70.
Crestfield, A. M., Moore, S., and Stein, W. H. (1963). J. Biol. Chem. 238, 622.
Moore, S. and Stein, W. H. (1954). J. Biol. Chem. 211, 907.
Patchornik, A. and Wilchek, M. (1962). J. Am. Chem. Soc. 84, 4613.
Patchornik, A., Wilchek, M. and Sarid, S. (1964). J. Am. Chem. Soc. 86, 1457.
Ruttenberg, M. A., King, T. P. and Craig, L. C. (1964). Biochemistry 3, 758.