Vol. 80, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

January 13, 1978 Pages 7-13

A NEW METHOD OF DETERMINATION OF THE CARBOXYL-TERMINAL RESIDUE OF PEPTIDES

M.E. Parham and G. Marc Loudon*

Department of Chemistry
Cornell University
Ithaca, NY 14853

and

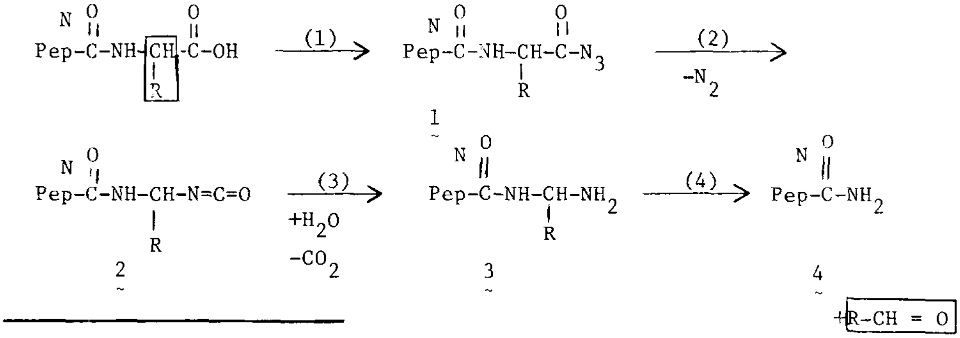
Department of Medicinal Chemistry
and Pharmacognosy
Purdue University
West Lafayette, IN 47907

Received October 28,1977

Summary. A method is presented for removal of the carboxyl terminal residue of peptides in high yield. The method appears to be suitable for extension to a sequential degradation of peptides and proteins.

Procedures for sequencing peptides and proteins from the carboxyl terminus would be a useful complement to the amino-terminal methodology now in use (1-3). Previous attempts to develop carboxyl-terminal sequencing methodology have met with limited success. Although we have also described a useful method for removing and identifying the carboxyl terminal residue from peptides, the yields were not sufficiently high for extension of the procedure to a sequential method (3-8). In this communication, we report a method of removal of the carboxyl-terminal residue from peptides in excellent yield. In the subsequent paper, we report the chemistry of a carboxyl terminal sequential peptide degradation.

The general plan of our degradation is shown in the reactions below.†



---

*Address correspondence to this author at the Purdue address.

0006-291X/78/0801-0007$01.00/0

A literature search showed that a plan similar to this one had been conceived over forty years ago by Bergmann and Zervas (9). Its successful execution in solution, however, was hampered by the large number of reactions required to synthesize the acyl azide **1** in only moderate yield, and the intermolecular reaction of the isocyanate **2** with amine **3** to produce a urea which was essentially intractable to further degradation. The key rearrangement, reaction (2), however, was found to proceed smoothly and quantitatively. We have found that the use of solid supports combined with modern reagents for the generation of the crucial intermediate **1** allows an efficient removal of the carboxyl-terminal residue.

Materials and Methods

Attachment of Peptides to Controlled Pore Glass.† Carboxylated CPG† (Pierce Chemical Co.) was suspended in a 1M solution of N-hydroxysuccinimide trifluoroacetate (10) in dry pyridine (Aldrich Gold Label), degassed, and then allowed to react at room temperature for 30-45 min. The CPG thus activated was washed twice with water and then with methanol before drying *in vacuo*. The peptide was applied to the activated glass according to the method of Williams and Kassel (6a) in a minimum volume of 0.2M phosphate buffer, pH 7.25, which was also 8M in urea. Typically incorporations of 3-15 μmol peptide/g of glass were obtained.

Rearrangement of the Carboxyl-Terminal Residue. The CPG-peptide was heated at 70° in a 0.2M $NaHCO_3$ buffer, pH 8.5, for 5 min, and washed twice with water and then with DMF. This treatment was found to increase substantially the yield in the subsequent degradation procedure. The CPG-peptide was then suspended in buffered DMF (11) for 10 min at room temperature. This solution was drained off and a 0.25 M DMF solution of di-*p*-nitrophenylphosphoryl azide (12) was added and allowed to react with the CPG-peptide at room temperature for 10 min. The solution was then made 25 mole % in *p*-methoxybenzyl alcohol (Aldrich) and the mixture was heated at 70° for 1 hr. Normally this procedure was repeated twice, after which a sample was taken for the usual 6N HCl-21 hr hydrolysis and amino acid analysis.

Apparatus. The reaction vessel is similar to that described by Williams and Kassel (6a) except that gentle agitation of the glass beads was accomplished by bubbling an inert, dry gas (Ar or N_2) up through the porous disc.

Acetylation of Lysines. The ε-amino group of lysine was acetylated by suspending the CPG-peptide in a DMF solution which was 1M in N-acetylimidazole (Aldrich) and 1M in triethylamine for 5 hr at room temperature.

---

†Pep = general peptide chain; Pep^N = amino-terminal peptide chain, Pep^C = carboxyl-terminal peptide chain; CPG = Corning^R Controlled Pore Glass; CPG-Peptide, = peptide immobilized as describing on CPG. DMF = dimethylformamide DAPA = 2,3-diaminopropionic acid DABA = 2,4-diaminobutyric acid.

Results and Discussion

Peptides were immobilized on CPG specifically at their amino terminus (6a, 13, 14). Although use of the Merrifield resins was attempted, these were found to be incompatible with some of the solvents used in this work. The carboxylic acid groups of CPG-peptides converted cleanly to acyl azides (reaction 1) in one step using the interesting reagent di-p-nitrophenylphosphoryl azide (12). Two successive treatments with this reagent were employed. In DMF solution, this reagent was found to bring about substantial racemization when used for peptide synthesis, but this difficulty is obviously of no concern in our procedure. Thermolysis of the acyl azide (reaction 2) yielded an isocyanate 3 which, in the DMF solvent used, was not converted to amine 4 until subsequent hydrolysis preparatory to amino acid analysis. Although direct identification of the aldehyde liberated in this procedure will be required for a sensitive assay of large peptides, we felt that development of the degradative methodology should receive first priority; therefore, the results given in Table 1 were obtained by subtractive amino acid analysis.

An examination of Table 1 shows that many of the problems encountered with our earlier degradation (7, 8) have been solved. For example, essentially all peptides have lost their carboxyl-terminal residues in better than 90% degradative yield. In the water-soluble version of our degradation reported earlier, glycine degraded in only 70-75% yield, and yields for degradation of aspartic and glutamic acids were poorer still. These residues evidently pose no problem in the work reported here. Our degradative methodology is applicable to peptides of reasonable size, as the insulin degradation shows.

It was found (entries 3a, b and 4a, b) that use of two cycles of the procedure significantly improved yields. The most reasonable explanation for this observation so far is that some base-labile linkage, perhaps an inter-chain anhydride, is being formed which deactivated the carboxyl-terminal carboxylic acid toward further reaction; this linkage is probably not an azlactone, since proline shows the same behavior. The mild base wash employed appears to

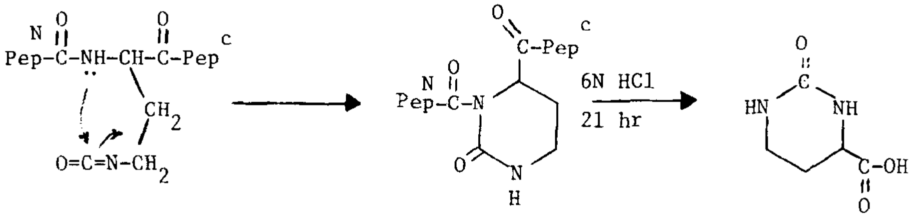
Table 1. Results of Carboxyl Terminal Residue Degradation in Peptides.

No.	CPG-Peptide[a]	Rearranged CPG-Peptide[b]	% Degradation
1	Gly(1.0)Asp(0.95)	Gly(1.0)Asp(0.06)	94
2	Ala(1.0)Ser(0.93)	Ala(1.0)Ser(0.09)[c]	91
3a	Gly(1.0)Glu(0.93)	Gly(1.0)Glu(0.03)[c]	
b		Gly(1.0)Glu(0.0)	100
4a	Ala(1.0)Pro(1.0)	Ala(1.0)Pro(0.17)[c]	
b		Ala(1.0)Pro(0.05)	95
5	Ala(1.0)Leu(0.97)Gly(0.98)	Ala(1.0)Leu(0.99)Gly(0.05)	95
6	Phe(1.0)Asp(0.97)Ala(0.97) Ser(0.86)Val(0.97	Phe(1.0)Asp(0.50)Ala(0.99) Ser(0.80)Val(0)DAPA(0.42)[1]	100
7	Ala(1.0)Ile(1.04)	Ala(1.0)Ile(0.01)	99
8	Gly(1.0)Met(0.78)	Gly(1.0)Met(0.09)	69-91[d]
9	Gly(1.0)Leu(0.85)Tyr(0.84)	Gly(1.0)Leu(1.01)Tyr(0.08)[c]	76-92[d]
10a	Glu(0.97)Gly(1.0)Phe(1.01)	Glu(0.15)DABA(0.60)Gly(1.0) Phe(0)[e]	100
b		Glu(0.18)DABA(0.83)Gly(1.0) Phe(0.10)	90
11	Pro(0.95)Phe(1.0)Gly(0.96) Lys[ε-acetyl](0.92)	Pro(1.0)Phe(1.01)Gly(1.0) Lys(0.03)	97
12	Gly(1.0)His(0.98)	Gly(1.0)His(0.01)	98
13	Thr(1.12)Lys(1.0)Pro(1.13) Arg(1.04)	Thr(0.93)Lys(1.0)Pro(1.1) Arg(0.08)	92
14	Bradykinin[Ser(0.96)Pro (2.94)Gly(1.00)Phe(2.06) Arg(1.82)] (C-terminal Arg)	Ser(0.92)Pro(2.40)Gly(1.00) Phe(1.93)Arg(0.88)	100
15	Insulin A Chain (C-terminal Asn)	Loss of 0.90 Asn (as Asp)	90

[a]Amino acid analysis prior to degradation [b]Amino acid analysis after degradation [c]Only one cycle of the azide procedure used; the normal procedure employs two cycles (see Materials and Methods) [d]Uncertainties in yield reflect uncertainties in amino acid analysis of the starting peptide [e]*p*-methoxybenzyl alcohol omitted.

reexpose the carboxyl groups for azide formation; omission of the base wash results in no improvement of the yield. The mild base procedure used does not appear to disrupt the linkage of the peptide to the glass, or to destroy the glass itself.

Functionalized side chains appear to offer no difficulties in this degradation; the detailed fate of these groups, of course, will have to be considered in further development of a sequential process. Side-chain carboxylic acid groups, of course, are also rearranged in this procedure to the corresponding amines. The less than quantitative rearrangement and recovery of _internal_ glutamic acid as Glu + DABA (entry 10a) is attributed to hexahydropyrimidone formation; the derivative 6 is stable to HCl hydrolysis which precedes amino acid analysis (15, 16).



5 6

Since this cyclization does not occur in the presence of an alcohol (17), addition of _p_-methoxybenzyl alcohol (18, 19) to the reaction mixture can be used to avoid the problem (entry 10b). Internal aspartic acid does not present the problem, probably because of the difficulties associated with the cyclization analogous to 5 which is "5-endo-trig" (20).

Preparation of the acyl azides of carboxyl-terminal arginine and lysine led to the corresponding lactams (21). The formation of arginine lactam is avoided by the use of "buffered DMF," (11), N-ethylmorpholine half-neutralized with acetic acid in DMF. The formation of lysine lactam is avoided by the simple expedient of acetylation.

It should be observed that the procedures to deal with the special problems just discussed (_p_-methoxybenzyl alcohol, buffered DMF, and acetylation) may be used generally and without prior knowledge of the _C_-terminal residue, although any or all may be omitted if amino acid analysis of the starting peptide indicates absence of the appropriate residues.

The success of this procedure is such that if a method can be found to continue a carboxyl-terminal degradation on the *amide* **4** produced by this process, a sequential degradation of several residues should be possible. Realization of this next objective is reported in the subsequent communication.

Acknowledgement

We are grateful for support of this work by the National Institute of General Medical Sciences, Grant GM 20315.

References

1. a. Edman, P. (1950), *Acta Chem. Scand.*, **4**, 277-282 b. Perham, R.N., ed. (1977), "Instrumentation in Amino Acid Sequence Analysis," Academic Press, New York.

2. Ilse, D., and Edman, P. (1963), *Aus. J. Chem.*, **16**, 411-6.

3. Edman, P., and Begg, G. (1967), *Eur. J. Biochem.*, **1**, 80-91.

4. Loudon, G.M., Parham, M.E., and Miller, M.J., in van Tamelen, E.E., ed., (1977), "Perspectives in Bioorganic Chemistry," v. 3, Academic Press, New York (in press).

5. Stark, G.R., in Hirs, C.H.W., and Timasheff, S.N., eds. (1972), *Methods Enzymol.*, **25**, 369-384.

6a. Williams, M.J. and Kassell, B. (1975), *FEBS Lett.*, 54, 353-7.
 b. Rangarajan, M., and Darbre, A. (1976), *Biochem. J.*, **154**, 307-315.

7. Miller, M.J., and Loudon, G.M. (1975), *J. Am. Chem. Soc.*, **97**, 5295-7.

8. Miller, M.J., DeBons, E.F., and Loudon, G.M. (1977), *J. Org. Chem.*, **42**, 1750-61.

9. Bergmann, M., and Zervas, L. (1936), *J. Biol. Chem.*, **113**, 341-357.

10a. Sakakibara, S., and Inukai, N., (1964), *Bull. Chem. Soc. Japan*, **37**, 1231-2.
 b. *Ibid.*, (1965) **38**, 1979-84.

11. Glass, R.D., Tolansky, A., Grzonka, Z., Schwartz, J.L., and Walter, R. (1974), *J. Am. Chem. Soc.* **96**, 6476-6480.

12. Shioiri, T., and Yamada, S. (1974), *Chem. Pharm. Bull.* **22**, 855-8.

13. Tarr, G.E., in Laursen, R.E., ed. (1975), "Solid Phase Methods in Protein Sequence Analysis," Pierce Chemical Co., Rockford, Ill., pp. 139-147.

14. Anderson, G.W., Zimmerman, J.E., and Callahan, F.M., (1964) J. Am. Chem. Soc., **86**, 1839-1842.

15. Ohira, T. (1942), Bull. Agr. Chem. Soc. (Japan) **18**, 74-76.

16. Kawasaki, H. (1960), J. Chem. Soc. Japan, **81**, 280-2.

17. LeQuesne, W.J., and Young, G.T. (1950), J. Chem. Soc., 1959-63.

18. Yajama, H., Fujii, N., Ogawa, H., and Kawatoni, H., (1974), J. Chem. Soc., Chem. Commun., 107-108.

19. Schröder, E., and Lübke, K. (1965), "The Peptides," v. 1, Academic Press, New York, p. 35.

20a. Baldwin, J.E. (1976), J. Chem. Soc., Chem. Commun., 734-6.
b. Baldwin, J.E., Culting, J., Dupont, W., Kruse, L., Silverman, Li, and Thomas, R.C. (1976), J. Chem. Soc., Chem. Commun., 736-8.

21. Schröder, E., and Lübke, K. (1965), ret. 19, p. 175.