SOLID PHASE SEQUENTIAL ANALYSIS: SPECIFIC LINKING OF ACIDIC PEPTIDES BY THEIR CARBOXYL ENDS TO INSOLUBLE RESINS†

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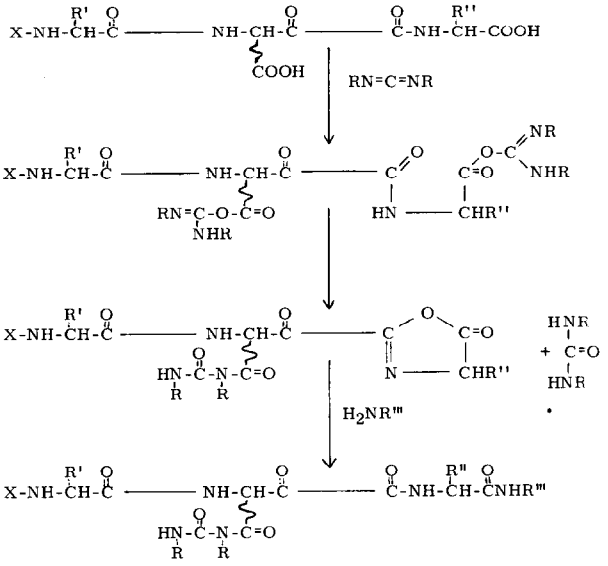
Received 19 April 1973

1. Introduction

The success of methods such as the Edman degradation for solid-phase sequential analysis of peptides depends on having efficient methods for attaching peptides to insoluble resins before degradation. Previously described [2, 3] attachment procedures using carbonyldiimidazole to couple the C-terminal carboxyl to an amino resin, suffer from the serious problem that side-chain carboxyls also become activated, leading to side reactions. In order to make the solid-phase Edman degradation generally applicable, it is essential to have a procedure for selective activation of the C-terminal carboxyl groups.

We wish to report here that a *N,N'*-disubstituted carbodiimide can effect both protection of side-chain carboxyl groups and activation of the C-terminal under suitable experimental conditions (scheme A).



Scheme A. General reaction of a disubstituted carbodiimide (RN=C=NR) with an N-protected (X-) acidic peptide. The *O*-acyl urea initially formed isomerizes to the inert *N*-acyl urea derivative at the side chain carboxyl, while the C-terminal remains activated as an oxazolinone which can subsequently react with a suitable nucleophile (H_2NR''').

2. Experimental

2.1. *Specific activation of the C-terminal group*

A solution of N-protected peptide (50 μmole/ml) was treated with EDC* (200 μmole/ml) in DMF, and

† Contribution No. 83 from the Research Group and the Department. A preliminary report of part of this work was presented at a Meeting held in Montpellier, September 1971, on "Recent Developments in the Chemical Study of Protein Structure" [1].

* *Abbreviations:*
EDC, *N*-ethyl,*N'*-(3-dimethylaminopropyl)-carbodiimide HCl; DMF, dimethylformamide, Pierce Sequenal Grade; Boc, *t*-butyloxycarbonyl; PTH-, phenylthiohydantoin-; PITC, phenylisothiocyanate.

Table 1
Condensation of Boc-peptides with amino acid esters (H_2N-R).

Peptides	H_2N-R	Yield (% of H_2N-R coupled per mole of dipeptide)	
		I	II
Boc-Gly–Leu	Ala OEt	75	75
Boc-Gly–Ala	Val OEt	82	78
Boc-Leu-Gly	Ala OEt	78	82
Boc-Glu–Phe	Val OEt	175	87
Boc-Asp–Leu	Val OEt	170	82
Boc-Glu–Val	Ala OEt	172	80
Boc-Asp–Phe	Ala OEt	170	82

I: direct condensation without pre-incubation; II: condensation after pre-incubation with EDC for 90 min at 40°C.

activation of carboxyl groups was followed by the hydroxamate test [4] at pH 6 (fig. 1) or by reaction with an amino acid ester (table 1). In the latter case, aliquots of the reaction mixture were treated with a 10-fold excess of an amino acid ester, generated from the hydrochloride by addition of a stoichiometric amount of triethylamine. After 1 hr at 40°C, the solution was diluted about 10-fold with n-butanol, and this solution was washed with 0.1 M HCl to remove all unreacted amino acid ester. The butanol layer was evaporated under reduced pressure and the residue was

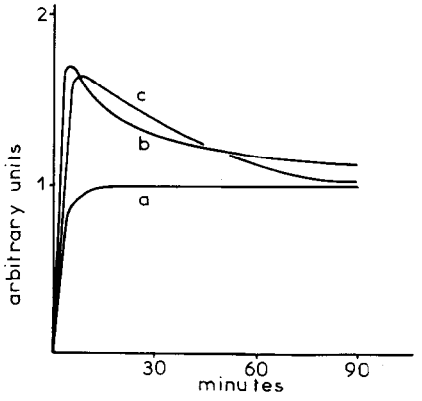


Fig. 1. Carboxyl activation of a) Boc-Ala–Gly, b) Boc-Glu–Val, and c) Boc-Asp–Val as a function of pre-incubation time with EDC at 40°C. The activation was followed by the hydroxamate test [4] at pH 6.

subjected to amino acid analysis after acid hydrolysis. The results obtained are reported in table 1.

2.2. *Attachment of peptide to insoluble resin*

The peptide (50–250 nmole) was treated with Boc-azide as described earlier [3] to mask the amino function, and was then dissolved in DMF (0.2 ml) containing 1–2 μl of *N*-ethylmorpholine. EDC (5 mg) was added and the reaction mixture was allowed to stand at 40°C. After 90 min (or 30 min at 50°C), 50 mg of sequencing resin (prepared by reaction of triethylenetetramine with chloromethyl polystyrene [3]; to be published), was added and this suspension was maintained at 40°C for at least 3 hr. To block excess resin amino groups, 1 ml of a solution of DMF:CH_3NCS:*N*-ethylmorpholine (8:1:1) was added to the resin and the suspension was allowed to react for additional 30 min at 40°C. The resin was then washed with methanol and dried [5].

2.3. *Edman degradation of insolubilized peptides*

2.3.1. Manual method

The resin-linked peptide was transferred as a suspension in DMF into a thermostatted cylindrical reaction vessel (1–2 ml volume) having sintered glass discs at the two ends. This apparatus permits shaking of the resin suspension, warming, and finally filtration to remove either excess of the reagents or products of the stepwise degradation. The resin linked peptide was submitted to the usual chemical treatment for solid phase Edman degradation [2, 3], except that the mixture, anhydrous DMF:*N*-ethylmorpholine: PITC (15:2:1) for 30 min at 40°C was used in the condensation step, rather than an aqueous buffer.

The PTH's of aspartic and glutamic acid residues were regenerated from their *N*-urea derivatives by acid hydrolysis (2 N HCl, 60 min, 100°C).

2.3.2. Automatic method

Automatic degradation of peptides was performed as described earlier [3, 5].

3. Results

Carbodiimide activation of peptide carboxyl groups as a function of time is shown in fig. 1. It can be seen that peptides containing a carboxyl group only at the

C-terminal position rapidly reach a degree of activation which remains constant, while peptides containing a side chain carboxyl show an initial maximum of activation which subsequently decreases to about one half of its value.

Analogous results were obtained when amino acid esters were utilized as nucleophiles instead of hydroxylamine. As shown in table 1, aspartyl and glutamyl peptides react with about 2 moles of an amino acid ester per mole of peptide by direct condensation with EDC, while the same acidic peptides are able to incorporate only one mole of amino acid ester after suitable incubation time with carbodiimide. The extent of condensation of amino acid esters with non-acidic peptides is about one mole of amino acid ester per mole of peptide and does not change during the incubation with carbodiimide.

On the basis of these results, a number of acidic peptides were incubated with EDC and then linked to an insoluble sequencing resin. The yields in coupling were nearly quantitative for shorter peptides and were about 70–80% for large peptides such as insulin A-chain and glucagon.

Stepwise degradation with the manual method was performed on the peptides Glu–Phe, Glu–Val, Asp–Phe, Asp–Leu and Phe–Asp–Ala–Ser–Val with approx. 90% yield per cycle. In each case aspartic and glutamic acid residues were released in their modified form during Edman degradation, showing that the side chain carboxyl groups were not linked to the resin. The peptides, Phe–Asp–Ala–Ser–Val, Gly–Leu–Asp–Gly–Ala–Glu–Lys, Asn–His–Leu–Ala–Arg, glucagon (19 cycles), and insulin A-chain (21 cycles) were sequenced by the automatic method [3]. In each case degradation proceeded through aspartic acid with little or no drop in yield, indicating that β-lactam formation, which blocks further degradation, did not occur. The PTH's found at the level of aspartic and glutamic acid regenerated PTH Asp and PTH Glu by acid hydrolysis.

3. Discussion

The difference in chemical reactivity between the C-terminal and the side chain carboxyl groups of a polypeptide is to be found in their neighboring groups rather than in other general properties. Actually the C-terminal carboxyl group is unique as it can be activated through an intramolecular dehydration with the adjacent peptide function to yield an oxazolinone. This reaction can be mediated by carbodiimide [6]. On the other hand, carbodiimides can be utilized to characterize aliphatic carboxylic acids as stable *N*-acyl urea derivatives [7]. Both these reactions are believed to possess a common intermediate, the active *O*-acyl urea derivative [7].

In the light of these precedents we reasoned that carbodiimide may affect the carboxyl groups of peptide chain in different ways depending on their environments. This proved to be the case and in practice, the same reagent can be used for both activating the C-terminal and blocking the side chain carboxyl groups in a single operation (scheme A).

Several factors influence the success of this EDC peptide attachment procedure. All nucleophiles (amino-resin, dimethylamine from the solvent DMF) must be absent from the activation mixture during the incubation period to prevent side reactions. The minimum amount of solvent (0.2 ml/50 mg) required to swell the resin should be used, and the dry resin should be added to the activation mixture. In this way the peptide is carried into the resin as the resin swells. Finally, the type of carbodiimide used is important; of several which were examined, EDC gave the best results.

This procedure seems to overcome a serious limit to the general applicability of the solid-phase Edman degradation. As described earlier [3], activation with carbonyldiimidazole resulted in glutamic acid side chains becoming attached to the resin; in the case of aspartic acid, a β-lactam was formed which prevented further degradation. Although other procedures have been devised to overcome these difficulties, they are either unsuitable for use with nanomole amounts of peptide [8] or are not generally applicable [5]. This proposed EDC procedure is theoretically impossible only in the rare case of a C-terminal proline peptide, which cannot form an oxazoline. In this case, however, as for all peptides which do not contain a side chain carboxyl, attachment can be accomplished by eliminating the pre-incubation period.

The manual procedure described here deviates from usual modifications of the Edman degradation in that a non-aqueous medium was used for the PITC condensation step. Since it is our general observation that

reactions of amino groups with reagents such as PITC are faster in non-aqueous solvents, it may be possible to shorten the time for each degradation cycle, and possibly to reduce the side reaction(s) which lead to progressively lower yields of liberated PTH during the course of degradation. Non-aqueous solvents, besides improving the resin swelling properties, and consequently the resin reactivity, should permit the use of a wide variety of other degradation reagents previously overlooked because of their poor reactivity in aqueous media.

Acknowledgements

This work was partly supported by grants from the National Science Foundation (GB27607), from Délégation Générale à la Recherche Scientifique et Technique (No. 72 70009), and from Fondation pour la Recherche Médicale Française. R.A.L. is a recipient of a USPHS Research Career Development Award (GM-17608) and an Alfred P. Sloan Fellowship (1972–74).

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