PROTECTED EDMAN DEGRADATION: A NEW APPROACH TO MICROSEQUENCE ANALYSIS OF PROTEINS

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Received 28 January 1974

1. Introduction

Automated techniques for sequential degradation by the phenylisothiocyanate procedure of Edman [1] have greatly accelerated progress in the structural analysis of proteins. Conventional procedures for sequenator analysis, however, require 100–500 nmoles of protein for an extended degradation. Since many proteins of great biological interest are available only in trace amounts, more-sensitive sequencing approaches are urgently needed.

We have developed a method for automated sequence analysis of nanomole quantities of proteins in the presence of a synthetic polyamino-acid carrier. The carrier protects the protein from chemical and mechanical losses during the degradation and allows longer degradations on smaller amounts of material than can be achieved when the protein is sequenced alone.

2. Experimental studies

2.1. *Synthesis of polyamino-acid carrier*

The carrier peptide H (Norleu–Arg)$_{27}$NH$_2$ was synthesized by the Merrifield [2] solid-phase procedure using the Beckman 990 synthesizer. The benzhydrylamine derivative of a poly (trifluoro-chloroethylene-*g*-styrene) resin (4.0 g) with 0.05 mmole-NH$_2$/g was used as the solid support [3]. An automated double-coupling procedure performed according to methods previously described [4] was used to assemble the peptide chain. *N*-α-tertiary-butyloxycarbonyl-L-N^G-tosyl arginine was coupled directly with the solid-phase and then alternately with *N*-α-tertiarybutyloxycarbonyl-L-norleucine for a total of 54 residues.

The peptide was cleaved from the resin with simultaneous removal of the tosyl protecting group by treatment with hydrogen fluoride in the presence of anisole for 1 hr at 0°C. After removal of excess hydrogen fluoride and anisole, the peptide was extracted into dilute acetic acid and lyophilised. The crude peptide was purified by gel filtration on Bio-Gel P-6 in 1 M acetic acid, yielding 0.65 g of peptide, which was used in the sequence studies without further purification.

2.2. *Automated Edman degradations*

A Beckman Sequencer Model 890C was used for the automated Edman degradations. Reagents and solvents were obtained from Beckman Instruments, Palo Alto, California. A single-coupling, double-cleavage program based on that of Edman and Begg [1] with minor modifications was employed. Identifications of the phenylthiohydantoin (PTH) amino acid derivatives were made by previously reported gas–liquid chromatography and thin-layer chromatography systems [5, 6].

In preliminary experiments, a series of separate sequenator runs was carried out on 300-, 100-, 25-, 10-, and 1 nmole samples of sperm-whale apomyoglobin. Repetitive yields calculated by gas chromatographic analysis of 'stable' PTH derivatives (valine, leucine) were regularly in the range 92%–94% for the 300 nmole and 100 nmole samples. Slightly lower yields (averaging 90%) were obtained at the 25-nmole level. Runs carried out on the 10 nmole samples gave more variable, but clearly much lower, repetitive yields (80%–86%); usually, identifications

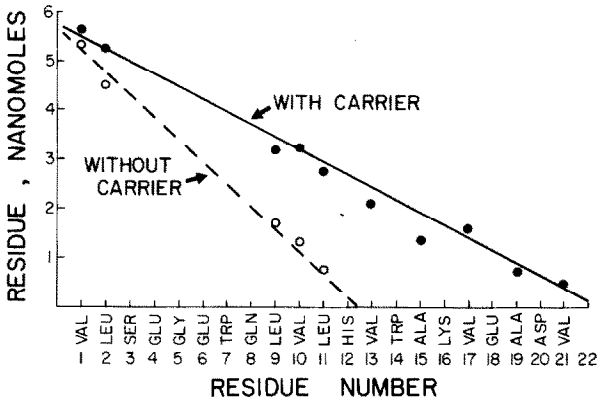

Fig. 1. Sequenator run on a 6 nmole sample of apomyoglobin with and without the addition of 150 nanomoles of synthetic poly(norleu–arg) carrier, showing repetitive yields obtained by gas chromatographic analysis of stable phenylthiohydantoin derivatives (valine, alanine, and leucine).

could not be clearly made beyond residue 11. Only the first one or two residues could be identified on the 1 nmole sample.

In a second series of experiments, the apomyoglobin degradations were repeated with different sample amounts but with the addition of 150–500 nmoles of poly (norleu–arg) carrier, which was mixed with the protein sample before transfer into the spinning cup of the sequenator. No significant effect of the carrier on repetitive yields was noted with amounts of apomyoglobin in excess of 50 nmoles, and a marginal effect was noted at the 25 nmole level. When the sample size was in the range 1–10 nmoles, however, there was a marked improvement in yield in the presence of carrier. This effect is illustrated in fig. 1 and table 1. Degradations were performed on 6 nmoles of apomyoglobin with and without the addition of 150 nmoles of carrier. In the absence of added carrier, identifications could not be made beyond residue 11. With the addition of carrier, positive identification of all residues was obtained for 21 cycles of degradation.

3. Discussion

When current sequenator methodology is examined in detail, it becomes apparent that there are two different kinds of limitations to the sensitivity of the analysis. The first relates to the methods used to identify the amino acid derivatives obtained at each cycle of degradation. The usual identification systems, based on gas chromatography, thin-layer chromatography, or amino acid analysis after regeneration of the free amino acid by hydrogen iodide [7], require approximately 5 nmoles at each cycle for reliable detection of all residues. The use of high-specific-activity ^{35}S-phenylisothiocyanate in the degradation [8] or the use of more sensitive thin-layer chromatographic procedures [9] helps to

Table 1
Thin-layer chromatography identification of PTH amino acids

Residue	No carrier	Carrier	Residue	No carrier	Carrier
1	VAL	VAL	11	–	(LEU)*
2	LEU	(LEU)*	12	–	(HIS)†
3	SER	SER	13	–	VAL
4	GLU	GLU	14	–	TRP
5	GLY	GLY	15	–	ALA
6	GLU	GLU	16	–	LYS
7	TRP	TRP	17	–	VAL
8	faint GLN + GLU	GLN + GLU	18	–	GLU
9	–	(LEU)*	19	–	ALA
10	–	VAL	20	–	ASP

* Identification of leucine obscured by norleucine from carrier; leucines were identified and quantitated by gas chromatography (see fig. 1).
† Pauly identification.

overcome this limitation and allows detection of PTH amino acids at the 20–50 pmole level. The second limitation becomes apparent when attempts are made to degrade trace amounts of protein in the sequenator. There is a rapid fall in yield due to mechanical losses of material in the solvent washes and probably also to chemical side reactions. Hence, only comparatively short degradations are possible.

In the approach described here, the protein is protected by the addition of a synthetic polyamino-acid carrier, poly(norleu–arg), which acts as a 'sink' for mechanical and chemical losses. This allows prolonged degradations to be carried out on less than 10 nmoles of purified protein.

It appears likely that the use of such carriers to achieve a 'protected' Edman degradation will be most helpful in structural work on proteins available only in nanomole amounts. Since the carrier methodology is quite compatible with the use of ^{35}S-phenylisothiocyanate, the combination of both approaches should allow even-further increases in sensitivity of analysis, and is currently being evaluated in our laboratory.

In this study, the choice of a repeating copolymer of norleucine and arginine was dictated by several considerations. To avoid interference with identification of the unknown protein sequence, a carrier composed of non-naturally-occurring amino acids must be employed. The carrier should be reasonably polar so that it will dissolve readily in the coupling and cleavage reagents. The guanidino group, which is protonated and therefore highly polar in both the alkaline coupling buffer and the anhydrous fluoro-acid used for cleavage, confers such properties. Finally, it seemed desirable to have a carrier that would give rise to phenylthiohydantoin derivatives to protect both organic-phase and aqueous-phase derivatives at the conversion step. The first choice for a carrier that would fulfill all these criteria was a repeating copolymer of norleucine and homoarginine. Since the appropriate tertiarybutyloxcarbonyl-blocked derivative of homoarginine for the solid-phase synthesis was not commercially available, it was decided to substitute arginine for homoarginine to allow the general approach to be rapidly evaluated. Synthesis of the norleucine–homoarginine copolymer is in progress, and, obviously, there are many other possibilities for design of a carrier molecule still to be investigated. The length of the present carrier molecule is probably also not optimal since, after 20–30 cycles of degradation, extractive losses of the shortened residual carrier start to become appreciable; use of a carrier of 80–100 residues could avoid this problem.

The principle of protected Edman degradation has a potentially broad application. Radioactive proteins, for example, may be sequenced at high sensitivity in the presence of nonradioactive carriers. Since the identifications are based on radioactivity, a natural protein can be used as the protecting agent. Using this approach, we recently determined the amino-terminal sequence of human proparathyroid hormone internally labeled with ^{3}H and ^{14}C amino acids, in the presence of a nonradioactive natural protein (apomyoglobin) as carrier [10].

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