IMPROVED COUPLING OF PROTEINS TO THE SUPPORT FOR SOLID PHASE PROTEIN SEQUENCING

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1. Introduction

The solid phase sequence method which was originally designed by Laursen for the sequence determination of peptides [1] has now been applied to the investigation of partial sequences of intact proteins. By either automatic or manual degradations, N-terminal sequences of up to 15-35 residues of intact proteins have been obtained [2-4]. Although the solid phase method requires the extra work of attaching proteins to the solid support, it eliminates extraction losses and accumulative background. Also, considerable time was saved when employing a manual degradation because of the faster drying of the washed solid support [4].

As the coupling of the protein to the solid support is of prime importance during the solid phase sequencing, it is necessary to have a simple (needs no prior chemical modification of the protein) and efficient (high yield and less side reaction) coupling method. Among the conventional techniques, the phenylene diisothiocyanate attachment procedure [5] is the best to meet these requirements. However, we have reported recently that the attachment of reduced carboxymethylated histidinol dehydrogenase to glass beads by the phenylene diisothiocyanate method in the aqueous pyridine media suggested by Bridgen [3] resulted in extremely poor yield [4]. We resolved this problem by attaching the reduced histidinol dehydrogenase to the iodoacetamide activated glass beads in an 8 M urea aqueous solution and found the yield was over 70%. Some proteins, especially the modified proteins, were found to have limited solubility in the aqueous pyridine buffer. It is, therefore, advantageous to develop methods which would allow the coupling reaction to proceed under aqueous conditions.

This communication presents data which shows that much higher yields are obtained by the diisothiocyanate method when coupling is performed in aqueous alkaline media instead of the conventional aqueous pyridine buffer. A scheme of attaching proteins through tyrosine or histidine side chains to the diazotized glass beads is reported. This coupling procedure, which has been widely used in affinity chromatography [6,7], requires no pretreatment of proteins and is performed in an aqueous alkaline solution.

2. Experimental

Crystalline histidinol dehydrogenase was prepared as described by Yourno and Ino [8]. Bovine serum albumin, glucagon and lysozyme were obtained from Sigma. Ribonuclease was the product of Worthington Biochemicals. Amino glass was prepared by reaction of controlled porosity glass beads (Corning, CPG-250, 120-200 mesh) with 3-aminopropyltriethoxysilane according to Robinson et al. [9]. The coupling efficiencies of proteins onto the glass beads were determined from amino acid analysis (on a Beckman 120 C amino acid analyser) of the acid hydrolyzate of the support. The attached proteins were sequenated manually by the 4-NN-dimethylaminoazobenzene 4′-isothiocyanate method [4].

2.1. *Attachment of proteins to phenylene diisothiocyanate (DITC)-activated glass beads*

The DITC activated glass beads were prepared by

the reaction of excess DITC (50 fold) with amino glass according to the procedure described by Bridgen [3]. The protein was attached to the DITC-glass beads (about 1 nmole protein/mg glass bead) through lysine residues under the conditions of:

(1) 0.4 M NN dimethylallylamine (Pierce) buffer in pyridine/water (3:2, v/v) buffered to pH 9.4 with trifluoroacetic acid; room temperature 45 min; with or without the presence of 1% SDS.

(2) 0.2 M sodium bicarbonate aqueous solution buffered to pH 8.7 with 1 N NaoH; room temperature 45 min; with or without the presence of 1% SDS.

After the coupling reaction [3], the excess isothiocyanate groups were blocked by ethanolamine and the N-terminal residue which also attaches onto the glass support was cleaved by trifluoroacetic acid [4]. The protein attached glass support was washed extensively with 1 HCl, water and acetone and then subjected to amino acid analysis or sequence determination.

2.2. *Preparation of diazotized glass beads and their coupling to the tyrosine and histidine residues in proteins (scheme 1)*

Amino glass (1 g) was stirred with p-nitrobenzoic acid (55 mg) and dicyclohexylcarbodiimide (60 mg) in 25 ml of ethyl acetate at room temperature for 2 h. About 0.18 mmole of p-nitrobenzoic acid was covalently attached to the glass beads (1 g) as estimated by the decrease of absorption at 255 nm of p-nitrobenzoic acid in the solution. The diazotized glass support, after washing with excess acetone and drying, was suspended in 0.5 M sodium bicarbonate (30 ml) containing 0.2 M sodium dithionite, at 50°C for 1 h, to reduce the nitro groups to amino groups. After reduction, the glass beads were washed with excess water and acetone, dried and stored at −20°C.

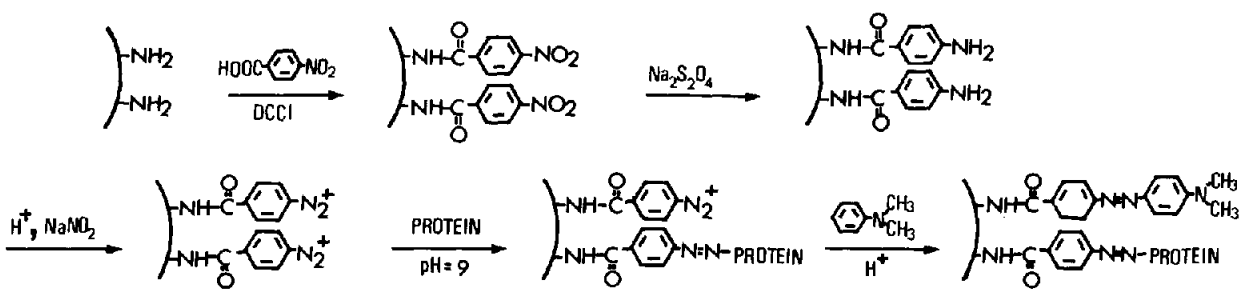
Coupling of protein to the support was carried out in the following way. The support (40 mg) was first diazotized in 1 N HCl (1 ml) and 0.2 M $NaNO_2$ (1 ml, added slowly) in an icebath for 15 min. The diazotized glass support was washed with 0.2 M $NaHCO_3$-NaOH buffer (pH 8.7) until the washing solution became alkaline. The same $NaHCO_3$ buffer (1.6 ml) and protein (40 nmole) were then added and the coupling reaction was allowed to proceed at 5°C for 8 h. After the coupling the supernatant was removed by gentle centrifugation. 1 N HCl (1.6 ml) and NN dimethylaniline (40 μl) were subsequently added and stirred for another 2 h to block the excess diazo groups. The supernatant was removed after the reaction period and the protein attached glass beads were washed extensively with 1 N HCl, water and acetone and then subjected to amino acid or sequence analysis.

2.3. *The attachment of protein to iodoacetamide activated glass beads and sequence determination of proteins attached onto the glass bead by 4-NN-dimethylaminoazobenzene-4'-isothiocyanate*

These were performed as described in our previous report [4].

3. Results and discussion

Table 1 gives the coupling efficiencies of five different proteins under different coupling conditions.



Scheme 1

Table 1
Coupling efficiencies of proteins to the glass support through different activated arms under the conditions specified

Protein \ Coupling conditions	DITC in pyridine/H_2O buffer	DITC in $NaHCO_3$ buffer	$-N_2^+$ in $NaHCO_3$ buffer	$-CH_2I$ in Tris buffer 8 M urea
Histidinol dehydrogenase	7%[a] 14%[a,b]	37%[a] 47%[ab]	41%[a]	88%[c]
Ribonuclease	82%[d] 84%[bd]	96%[d] 100%[bd]	49%[d]	89%[c]
Lysozyme	92%[d]	92%[d]	70%[d]	78%[c]
Glucagon	23% 23%[b]	74% 40%[b]	50%	–
Albumin	21%[a] 23%[ab]	45%[a] 34%[a,b]	–	84%[d]

[a]Carboxylmethylated protein
[b]In the presence of 1% sodium dodecyl sulfate
[c]Reduced protein (lyophilised)
[d]Native protein

In the DITC coupling procedure, with the exception of lysozyme and ribonuclease, remarkable increases in the coupling efficiencies were generally found when coupling was performed in aqueous solution. Quantitation of the released N-terminal during the sequence determination gave comparable data to that obtained from direct amino acid analysis.

The order of attaching DITC to both protein and solid support has been controversial. We prefer the attachment of DITC initially to the solid support because, apart from the simplicity (large amounts of DITC-solid support can be prepared for ready use), of the instability of the aromatic isothiocyanate group in alkaline solution. Under the coupling conditions suggested by Cavadore et al. [10], we found that about 20% of the isothiocyanate groups of 4-NN-dimethylaminoazobenzene 4′-isothiocyanate were hydrolyzed to amino groups. This indicates that there is a distinct possibility that 20% of the isothiocyanate groups of DITC which would be attached onto the lysine residues of the protein, could be hydrolyzed if DITC was attached to the protein in the alkaline solution first. The attachment of DITC to the solid suppport is, however, performed in nonaqueous media (DMF) which would be expected to preserve most of the isothiocyanate groups in an active form.

The attachment of proteins to the diazotized glass beads should result in the formation of covalent bonds between diazo groups and side chains of tyrosine, histidine and lysine. However, the diazo amino bond formed between lysine and the solid support is not stable. The treatment with NN dimethylaniline in strong acid solution would be expected to release the free lysine side chain. Under the proposed coupling scheme, the modification of tyrosine and histidine is by no means quantitative. From the amino acid analysis, the modified tyrosine was found to be less than 30–35% for the proteins coupled to the diazotized glass bead. By hydrolyzing a sample of Leu-Tyr attached to diazotized glass beads, the modified tyrosine (presumably aminated tyrosine) could be detected on the Beckman PA-35 column (column length, 13.5 cm; column temperature 52°C; sodium citrate buffer, pH 3.25; elution time: 85 min).

The attachment of reduced protein to the iodoacetamide activated glass support is probably the most useful technique in terms of yield. Since most proteins have to be denatured before the sequence determina-

tion can be commenced, it is irrelevant to regard the reduction and lyophilization as extra work. The coupling was performed in 8 M urea aqueous solution which would be expected to dissolve most intractable proteins. Moreover, the attachment was found to be specific for cysteine only, the histidine and lysine residues were recovered quantitatively after acid hydrolysis. Methionine residues could be identified during sequence determination without difficulty.

The great potential of the solid phase sequencing method was realized when we found that histidinol dehydrogenase, a protein with an extremely low solubility in the liquid phase coupling buffer, was able to be sequenated normally when attached on the glass support. This implies that proteins which cannot be sequenated satisfactorily by the liquid phase sequencing method due to their poor solubility in the coupling buffer could be attached to the solid support in the selected aqueous buffer to overcome the solubility problem.

The solid phase method, combined with the 4-NN-dimethylaminoazobenzene 4′-isothiocyanate stepwise degradation could be a very promising alternative for the sequence determination of proteins in the future because of its simplicity, sensitivity and efficiency.

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