MODIFICATION OF ε-AMINO GROUP OF LYSINE IN PROTEINS BY ACYLATION WITH PYROMELLITIC DIANHYDRIDE AND *o*-SULPHOBENZOIC ANHYDRIDE

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1. Introduction

A large number of acylating reagents have been used for modification of ε-amino lysine residue in proteins [1–10]. A good modifying reagent should be specific for a group under certain conditions and its attachment to a group should be reversible. Certain cyclic anhydrides of dicarboxylic acids conform to these conditions. Phthalic anhydride [11] has been found to be not very reactive in such modification reactions. Recently, the use of exo-*cis*-3,6-endoxo-Δ^4-tetrahydrophthalic anhydride [12] has been described which can selectively and reversibly acylate ε-amino-lysine group in proteins.

Pyromellitic dianhydride (PMDA) [13] and *o*-sulphobenzoic anhydride (SBA) have been used for quantitative acylation of hydroxy and amino compounds. Here, we have employed these two anhydrides for the modification of ε-amino function of the lysyl residue in proteins. The modification reaction has been used to differentiate lysine-type trypsin inhibitors from arginine-type. The reaction has also been used to modify tryptic digestion of lysine-containing proteins.

2. Materials and methods

Trypsin (bovine pancreas) and D,L-α-*N*-benzoyl arginine *p*-nitroanilide hydrochloride (BAPA) were procured from Aldrich (Milwaukee, WI). PMDA was obtained as a generous gift from Du Pont de Nemours (DE). Insulin (bovine) was obtained from M/s. Unichem (Bombay). SBA [15] and α-*N*-acetyl-lysine ethyl ester hydrochloride [16] were prepared in this laboratory.

2.1. *Modification of α-N-acetyl-lysine ethyl ester hydrochloride and subsequent tryptic digestion of modified derivative*

α-*N*-Acetyl-lysine ethyl ester hydrochloride, an esterolytic substrate for trypsin, is quantitatively ε-*N*-acylated by PMDA or SBA. With the former reagent, a solution of 0.23 g (0.001 mol) of α-*N*-acetyl-L-lysine ethyl ester hydrochloride in 5 ml water was stirred at room temperature, while 0.66 g (0.0030 mol) of PMDA solution in 5 ml DMSO were added gradually. The pH of the reaction mixture was maintained at 7–8 with sodium carbonate added from a microburette. After stirring for 2–4 h, the reaction was complete as indicated by the disappearance of ninhydrin-positive spot due to amino ester on TLC plate (solvent *n*-BuOH/H_2O/AcOH, 4:5:1).

In case of acylation with SBA, identical conditions were used, but a solution 0.28 g (0.0015 mol) of SBA in dioxane was employed.

The reaction mixture containing the above derivative (0.001 mol) in 10 ml 50% aqueous DMSO was treated with 2 ml trypsin solution (4.5 mg/10 ml in 50 mM Tris buffer (ph 8.2) and containing 1 ml 2 M $CaCl_2$/100 ml) for 30 min at 37°C. The quantitative determination of the unhydrolysed ester was as in [17,18].

2.2. *ε-N-Acylation of lysine residue in insulin B-chain and proteolytic action of trypsin upon modified ε-N-acylated insulin B-chain*

The B-chain of insulin was prepared and purified

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as in [19]. B-Chain of insulin (100 mg) and 5 ml distilled water were placed in a beaker. The solution was stirred magnetically. The pH was maintained at 7–8 throughout the course of reaction by addition of sodium carbonate from a microburette. A solution of 0.1 g PMDA in 5 ml DMSO or 0.1 g SBA in 5 ml dioxane was added to the stirred solution of insulin B-chain over 15 min. The reaction mixture was stirred for another 40 min and then dialysed against distilled water to remove low molecular weight impurities. The resulting solution was mixed with 1 ml trypsin solution (4.5 mg/10 ml in 50 mM Tris buffer, pH 8.2, and containing 1 ml 2 M $CaCl_2$/100 ml), and the solution incubated at 37°C for 2 h. A control experiment was simultaneously carried out under identical conditions using unmodified B-chain of insulin. TLC of the control and reaction mixture was performed using the solvent system (*n*-BuOH/AcOH/H_2O, 4:1:5).

2.3. *Acylation of lima bean trypsin inhibitor (LBTI) and esterolytic action of trypsin upon acylated inhibitor*

LBTI (1.0 mg) was dissolved in 1 ml distilled water and the pH of the solution was maintained at 7–8 with sodium carbonate added from a microburette. A solution of 0.1 g PMDA in 5 ml DMSO or 0.1 g SBA in 5 ml dioxane was added to the stirred solution of LBTI over 15 min. The reaction was allowed to continue at 37°C for 3 h, then diluted to 10 ml with water. In order to compare the selectivity of acylation of these anhydrides with acetic anhydride, LBTI was acylated with Ac_2O under identical conditions except that 0.1 ml Ac_2O in 5 ml dioxane was used. A control was similarly carried containing 1.0 mg inhibitor in 50% aq. DMSO or 50% aq. dioxane. The trypsin-inhibiting activity of the control was compared with the acylated inhibitors using BAPA and trypsin by the method in [15].

2.4. *Deacylation and subsequent tryptic digestion of acylated LBTI*

In order to establish the conditions for deacylation of monoamides of PMDA and SBA, a control experiment on acylation of *n*-butylamine with these anhydrides was carried under conditions as used in (2.1). This was followed by deacylation in aq. hydrochloric acid (pH 3). Complete hydrolysis of the amide was found to occur as detected by TLC. Acylated LBTI (1 mg), acylated by either PMDA or SBA, was brought to pH 3 by addition of 0.1 M HCl and the solution allowed to stand overnight at room temperature. Thereafter the pH of the solution was again raised to 8.2 and then subjected to trypsin digestion as in [17].

3. Results and discussion

Compared to other acylating reagents, the proposed reagents have an advantage, i.e., in case of PMDA each *N*-acyl group introduces three negative charges, while in case of SBA, the strongly ionised sulphonic group is introduced. Only one reagent [4] has been used earlier, i.e., β-sulphopropionyl chloride which introduces sulphonic acid group in lysine side chain on acylation.

3.1. *Modification and subsequent tryptic digestion of α-N-acetyl lysine ethyl ester hydrochloride*

Experiment 2.1 was designed to establish conditions for acylation of lysyl residue. It was found that complete disappearance of ninhydrin-positive spot due to α-*N*-acetyl lysine ethyl ester hydrochloride and development of a single slow moving spot due to diacyl derivative of the ester on TLC plate after ninhydrin spray confirmed the completion of the reaction.

In experiment 2.1 the modified diacyl derivative of lysine ethyl ester hydrochloride was subjected to esterolytic action of trypsin. Consequent to tryptic action, it was found that the ester content of the reaction mixture and starting material was the same. It clearly indicated that the substrate has been successfully modified and that the diacyl derivative no longer functions as an esterolytic substrate for trypsin.

3.2. *Modification of insulin B-chain and tryptic digestion of the modified derivatives*

The reaction mixture (2.2) when subjected to TLC (solvent system *n*-BuOH/AcOH/H_2O, 4:1:5) and ninhydrin spray, did not indicate liberation of alanine. However, in the control experiment under similar conditions with unacylated insulin B-chain, a distinct spot due to alanine was indicated. Thus acylation followed by tryptic digestion of this peptide offered a unique example of ε-amino modification by PMDA and SBA, and prevention of the cleavage of lysine–carboxy amide bond. Since this peptide has only one arginine[22] and one lysine[29] residue, the normal tryptic digestion liberated the C-terminal amino acid alanine and also exposed amino group of glycine after cleaving Arg^{22}–Gly^{23} amide bond. Thus, prevention of the tryptic

cleavage of lysine carboxy-end of ϵ-*N*-acylated insulin B-chain proved ϵ-*N*-acylation of the Lys^{29} in B-chain of insulin with PMDA or SBA.

3.3. *Modification of LBTI and subsequent tryptic digestion of acylated LBTI*

The modification of LBTI (2.3) with PMDA, SBA and Ac_2O was measured as the antitryptic activity left after their modification. The results are shown in fig.1. It has been observed that the extent of modification is comparable or even better both in case of PMDA and SBA than using Ac_2O.

The trypsin inhibiting activity on hydrolysis (2.4) with dilute HCl indicates that the ϵ-*N*-acyl-lysine groups have been hydrolysed.

4. Conclusion

The importance of using these newer reagent for acylation of ϵ-amino group of lysine in proteins lies in their high reactivity and the mild conditions under which they can acylate the lysyl side chain. Further, the modification is reversible since the modifying groups can be easily cleaved by hydrolysis which is facilitated by neighbouring group participation (cf. hydrolysis of phthalamic acid which is well known intramolecular process [20]).

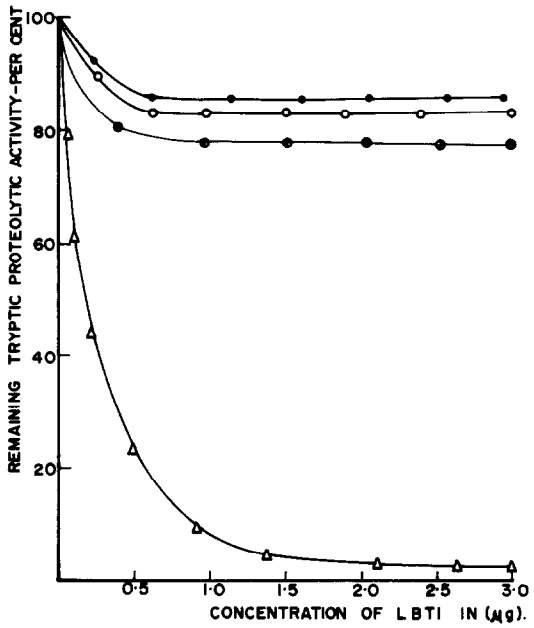

Fig.1. Inhibition of proteolytic activity of trypsin by LBTI and acylated LBTI. (△——△) Residual trypsin activity (T.A.); (●——●) T.A. after acylation with PMDA; (○——○) T.A. after acylation with SBA; (⊕——⊕) T.A. after acylation with Ac_2O.

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