

## Preparation and Properties of Aminoacetylated $\beta$ -Casein

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### Abstract

A new protein aminoacylating agent, the hydroxysuccinimide ester of trifluoroacetyl aminoacetic acid (TFA-glycine) has been prepared. Lysine residues of  $\beta$ -casein A<sup>2</sup> have been trifluoroacetyl aminoacetylated in dimethylsulfoxide. Trifluoroacetyl groups were removed with molar piperidine at 0 C. The aminoacetylated  $\beta$ -casein A<sup>2</sup> was less reactive to trypsin than unmodified protein. Gel electrophoretic behavior of aminoacetylated  $\beta$ -casein A<sup>2</sup> reflected the difference in basicity between  $\epsilon$ -amino groups of lysyl side-chains and  $\alpha$ -amino groups of the  $\epsilon$ -glycyllysyl side-chains.

### Introduction

The modification of  $\beta$ -casein by acylation of amino groups with a variety of acid anhydrides has led to useful information on the role of alkyl groups in hydrophobic interaction dependent aggregation (6, 7). However, this type of modification invariably disturbs the charge of  $\beta$ -casein, because the protonatable amino groups of the protein are lost by conversion to amide groups. We have developed a method for the aminoacylation of  $\beta$ -casein which introduces a variety of modifiers onto the protein without altering the number of charged groups.

Aminoacylation of insulin and of ferredoxin has been performed with *p*-nitrophenyl esters of *t*-butyloxycarbonyl-(*t*-BOC)-amino acids (5, 9). However, in these cases, the insulin had one lysine residue and the ferredoxin had no lysine at all.

In our laboratory  $\beta$ -casein was successfully acylated with the *p*-nitrophenyl esters of *t*-BOC-L-alanine and of *t*-BOC-L-phenylalanine. Although acylation was complete, attempts to remove the *t*-BOC groups with anhydrous TFA were unsuccessful. These findings prompted us to look for another approach to the aminoacylation of  $\beta$ -casein and the results of that investigation are the subject of this paper.

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### Materials and Methods

**$\beta$ -Casein.**  $\beta$ -Casein A<sup>2</sup> was prepared from milk of a cow homozygous for the variant. The preparative method of Aschaffenburg (2) was used as previously described (6).

**TFA-Glycine.** This reagent was prepared according to Weygand and Geiger (14); mp 116-118 C [lit. 117-118 C (14)].

**TFA-Glycine, hydroxysuccinimide ester.** To 18 ml of redistilled, dry 1,2-dimethoxyethane held in a 25 ml glass stoppered flask placed in a salt-ice bath at -10 C (1), were added 0.805 g (0.005 mole) of TFA-glycine and 0.575 g (0.005 mole) of *N*-hydroxysuccinimide. To the cold, stirred solution was added 0.03 g (0.005 mole) of *N,N'*-dicyclohexylcarbodiimide. The temperature was maintained overnight at 0 to 4 C with stirring. The mixture was filtered and the solvent removed from the filtrate by vacuum distillation. The residue crystallized. Two recrystallizations from isopropyl alcohol and petroleum ether gave 0.47 g (37% yield)

TABLE 1. Amino acid molar ratios of  $\beta$ -casein and of aminoacetylated  $\beta$ -casein.<sup>a</sup>

	Casein A <sup>2</sup>	Amino-acetylated $\beta$ -casein A <sup>2</sup>
Asp	10.51 ± .09	11.20 ± .18
Thr <sup>b</sup>	9.27	9.04
Ser <sup>b</sup>	14.33	14.86
Glu	38.45 ± .66	39.53 ± .70
Pro	35.78 ± .77	34.46 ± .43
Gly	5.17 ± .18	16.85 ± .16
Ala	5.52 ± .09	6.09 ± .22
Val	18.04 ± .03	17.54 ± .22
Met <sup>b</sup>	2.31	2.55
Ile	9.05 ± .04	8.94 ± .12
Leu	21.23 ± .15	20.59 ± .31
Tyr	3.89 ± .04	3.66 ± .09
Phe	8.73 ± .08	8.50 ± .02
His	4.97 ± .21	4.88 ± .10
Lys	10.99 ± .08	11.17 ± .08
Arg	3.73 ± .04	3.68 ± .10

<sup>a</sup> Based on triplicate 24-hour hydrolyzates.

<sup>b</sup> No correction for acid destruction.

of product, mp 142 to 144 C. Theoretical ( $C_8H_7O_5N_2F_3$ , mol wt 268) C, 35.8; H, 2.6; N, 10.4; found C, 36.3; H, 3.3; N, 10.1. The structure was confirmed by mass spectrometry.

**TFA-Aminoacetylated  $\beta$ -casein.** Two hundred forty milligrams (10  $\mu$ moles) of  $\beta$ -casein A<sup>2</sup> were dissolved in 25 ml of purified dimethylsulfoxide. To the clear solution was added 45 mg (168  $\mu$ mole) of TFA-glycine hydroxysuccinimide ester. After stirring overnight at room temperature, the solution was diluted with 100 ml of water and adjusted to pH 7.0 with *N* NaOH. After dialysis against water at 4 C for three days (several changes), the protein was completely recovered by lyophilization.

**Aminoacetylated  $\beta$ -casein.** TFA groups were removed from 200 mg of TFA-aminoacetylated  $\beta$ -casein with *M* piperidine at 0 C by the method of Goldberger and Anfinsen (4). The protein was further purified by column chromatography on DEAE-cellulose (13) (final yield about 150 mg). The elution profile was similar to that for  $\beta$ -casein A<sup>2</sup>.

**Amino acid analysis.** The method of Piez and Morris was used (12). Triplicate hydrolyses using redistilled 6 *N* HCl at 110 C for 24 hr were carried out in sealed evacuated tubes.

**Polyacrylamide gel electrophoresis.** Dr. R. F. Peterson kindly performed acid polyacrylamide gel electrophoresis (PAGE) at pH 3 (11). In addition, PAGE was performed at pH 9.2 (10), and at pH 7.0 using 0.05 *M* imidazoleacetate buffer, 10<sup>-3</sup> *M* in Na<sub>2</sub>EDTA.

**Dansylation.** The procedure of Gros and Labouesse was used (3).

**Tryptic digestion.** Peptides produced by tryptic digestion of  $\beta$ -casein A<sup>2</sup> and of aminoacetylated  $\beta$ -casein A<sup>2</sup> were examined by high voltage electrophoresis described by Kalan et al. (8).

## Results and Discussion

Aminoacetylation of  $\beta$ -casein in dimethylsulfoxide using the *N*-hydroxysuccinimide ester of TFA-glycine proceeded to completion at room temperature with only a slight molar excess of active ester. Amino acid analysis in Table 1 shows that glycine incorporation was extensive and matches the proportion of  $\epsilon$ -amino groups and of the *N*-terminal amino group, i.e., 12 moles of amino groups per mole of  $\beta$ -casein A<sup>2</sup>.

Dansylation of aminoacetylated  $\beta$ -casein produced only a trace of dansyl-lysine when compared to the amount of dansyl-lysine recovered from dansylated  $\beta$ -casein A<sup>2</sup>. As expected, a

TABLE 2. Polyacrylamide gel electrophoresis of aminoacetylated  $\beta$ -casein A<sup>2</sup>.

	Relative mobilities		
	pH 3.0 <sup>a</sup>	pH 7.0 <sup>b</sup>	pH 9.2 <sup>c</sup>
$\beta$ -Casein A <sup>2</sup>	1.00	1.00	1.00
Aminoacetylated $\beta$ -casein A <sup>2</sup>	1.00	1.07	1.21
TFA-Gly- $\beta$ -casein	.....	.....	1.29

<sup>a</sup> Acetic acid—formic acid pH 3.0 buffer (11).

<sup>b</sup> 0.05 *M* Imidazole—acetate pH 7.0 buffer, 10<sup>-3</sup> *M* Na<sub>2</sub>EDTA.

<sup>c</sup> Tris-borate—EDTA pH 9.2 buffer (10).

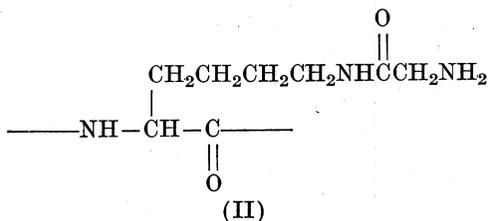
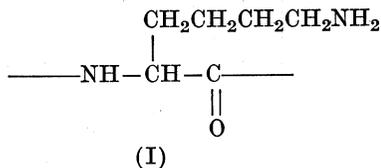
large recovery of dansyl-glycine was obtained from the aminoacetylated protein. This indicates that glycine incorporated into  $\beta$ -casein is indeed attached to the  $\epsilon$ -amino groups of the lysyl residues and that removal of TFA groups was complete. Thus, the reaction is specific for amino groups.

Aminoacetylated  $\beta$ -casein has the same relative mobility upon acid polyacrylamide gel electrophoresis at pH 3.0 as  $\beta$ -casein (Table 2). This supports the conclusion, from results of dansylation, that removal of the trifluoroacetyl groups was essentially complete. Furthermore, at pH 7.0 or 9.0 the aminoacetylated protein has a greater mobility than unmodified protein. This is expected because the  $\epsilon$ -amino groups of lysyl side-chains have been covered and replaced by *N*-terminal amino groups with lower pK<sub>a</sub> values.<sup>2</sup> Thus, at pH 7.0 or 9.0 the amino groups of aminoacetylated  $\beta$ -casein should be more deprotonated than untreated  $\beta$ -casein; this condition confers a greater net negative charge on the modified protein.

Trypsin had only limited hydrolytic action on aminoacetylated  $\beta$ -casein. Trypsin digestion of the modified protein at pH 8.2 produced five peptide bands by high voltage electrophoresis at pH 6.4. A similar hydrolysis of untreated  $\beta$ -casein produced 14 bands. Thus, the  $\epsilon$ -glycyllysyl residue is essentially resistant to trypsin action. This further delimits the specificity of trypsin, since the replacement of the amino group of the lysyl side-chain (I) by an aminoacetamido group (II) is sufficient

<sup>2</sup> Protein amino pK<sub>a</sub> values in 4.5 *M* urea, 6.5% polyacrylamide gel at 14 C are unknown but might be expected to be close to 8 for a terminal amino group and close to 10 for an  $\epsilon$ -amino group.

to make the lysyl residue resistant to trypsin action:



The procedure to aminoacetylate  $\beta$ -casein can be applied to other proteins that can withstand conditions for deblocking, i.e., *m* piperidine at 0 C. Woychik (15) removed TFA groups from  $\kappa$ -casein without detectable physical alteration of this protein which contains a disulfide bond. Active ester aminoacylation of proteins with peptides should also be possible. In the future controlled synthesis of nonlinear peptide chains appears feasible.

Since the hydroxysuccinimide esters of TFA-amino acids may be somewhat water soluble, proteins should be subject to aminoacylation in slightly alkaline solution. To date, aminoacylation has been carried out either in dimethylformamide (9) or in dimethylsulfoxide.

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