Chemical Modification of α-Chymotrypsin with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and Nucleophilic Reagents

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Chemical Modification of α-Chymotrypsin with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and Nucleophilic Reagents

by
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Abstract

Chemical modification of α-chymotrypsin with EDC[1] in the presence or absence of the nucleophilic reagents methylenediamine dihydrochloride or monomethylamine hydrochloride results in partial inactivation of enzyme activity. The modified enzyme showed the various hydrolytic activity according to the kinds of substrate.

It was suggested that the change of enzyme structure was caused around the substrate binding site of α-chymotrypsin by chemical modification.

Introduction

A study of chemical modification of α-chymotrypsin with EDC in the presence of nucleophilic reagents had been carried out to investigate the enzyme reaction mechanism (*1*). It is not yet discussed that whether the change of substrate specificity was caused by chemical modification. Therefore we attempted to modify substrate specificity of α-chymotrypsin by the chemical modification.

Materials and Methods

Materials—α-Chymotrypsin was purchased from Worthington Biochemical Corp.; EDC, Suc-Ala-Ala-Pro-Phe-pNA, and Bz-Tyr-OEt was obtained from Peptide Institute, Inc., Osaka; Ac-Phe-OEt, Bz-Tyr-pNA, and Z-Tyr-ONp was from Protein Research Foundation, Osaka; Congo red-elastin was from United State Biochemical Co.; Casein was a product of E. Merck, Darmstadt West Germany; Methylenediamine dihydrochloride was from Fluka AG, Switzerland. Other reagents were purchased from Wako Pure Chemical Industries.

Assay of Protease Activity—Proteolytic activity was measured by two methods. Caseinolytic activity was assayed by the method of Kunitz (*2*), with casein as a substrate.

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[1] Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; Suc, succinyl; pNA, p-nitroanilide; Bz, benzyl; OEt, ethyl ester; Z, benzyloxycarbonyl; ONp, p-nitrophenyl ester; Ac, acetyl; Tris, tris (hydroxymethyl) aminomethane.

Five μl of enzyme solution was added to 1 ml of a solution of 1% (w/v) casein in 0.02 M phosphate buffer, pH 7.3, at 37°C. After incubation for 30 min the reaction was terminated by the addition of 3 ml of 5% trichloroacetic acid. After standing for 30 min at room temperature, the resulting precipitate was removed by filtration through Toyo filter paper No. 5C and the absorbance at 280 nm of the trichloroacetic acid-soluble peptides formed was determined with Hitachi spectrophotometer 100–60.

The digestion of Congo red-elastin was assayed by David's method (*3*). Congo red-elastin (0.1%) suspension in 0.02 M sodium borate buffer, pH 8.8, was homogenized with a glass blender. The suspension was used as substrate. Digestion was allowed to proceed at 37°C and at regular intervals, centrifuges for 30 sec., and absorbancy at 495 nm of the supernatant was measured. This measurement solution was stirred, and allowed to proceed at 37°C for the next reaction.

Hydrolysis of 4-Nitroanilide—Rate of hydrolysis of 4-nitroanilide substrates were determined on the spectrophotometer in 50 mM Tris-HCl buffer, pH 7.5. The hydrolysis were followed by monitoring the release of 4-nitroaniline at 410 nm ($\varepsilon=8,800\ M^{-1}\cdot cm^{-1}$) for Suc-Ala-Ala-Pro-Phe-pNA (*4*); at 385 nm ($\varepsilon=17,200\ M\cdot cm^{-1}$ pH 8.0) for Bz-Tyr-pNA in same buffer containing 20% 2-propanol (v/v) (*5*).

Hydrolysis of Ester Substrate—Rates of hydrolysis of ester substrates were determined by spectrophotometric method (*6*) in 50 mM Tris-HCl buffer, pH 7.5. Rate of hydrolysis of Bz-Tyr-OEt were increase in absorbance at 256 nm ($\varepsilon=964\ M\cdot cm^{-1}$) in same buffer containing 15% dioxane (v/v). Hydrolysis rate of Ac-Phe-OEt determined at 230 nm ($\varepsilon=81\cdot M\cdot cm^{-1}$) in same buffer containing 8% ethanol (v/v). Rate of hydrolysis of Z-Tyr-ONp in same buffer containing 20% CH_3CN (v/v) were followed by monitoring the release of p-nitrophenylester at 400 nm ($\varepsilon=18,700\cdot cm^{-1}$, pH 8.0) (*7*).

Modification of α-chymotrypsin with carbodiimide (EDC) and amines—α-Chymotrypsin (0.4 mM) was incubated with 0.1 M EDC in 0.1 M N,N,N',N'-tetramethylethylenediamine-HCl buffer, pH 4.75. At various time intervals, an aliquot was withdrawn, added 4 M sodium acetate buffer, pH 3.5, and measured activity for casein, 4-nitroanilide, and ester as substrates. Methylenediamine dihydrochloride or monomethylamine hydrochloride were added in the modification reaction mixture to examine effect of nucleophilic reagents.

Results and Discussion

Chemical modification of α-chymotrypsin with EDC was performed to search carboxyl groups concerned with substrate binding site on the enzyme molecule (Fig. 1). All enzyme activity for various substrates resulted in partial inactivation with the chemical modification. After the reaction for 6 hrs, 5–20% enzyme activity were maintained. The results indicated that some essential carboxyl groups, not always a fatal to the enzyme activity, arround active site were modified by EDC. Additionally, residual activity for Congo red-elastin was 29% after chemical modification for 6 hrs.

In the presence of nucleophilic reagents, monomethylamine, modification with EDC of α-chymotrypsin resulted in partial inactivation (Fig. 2). After modification for 6 hrs,

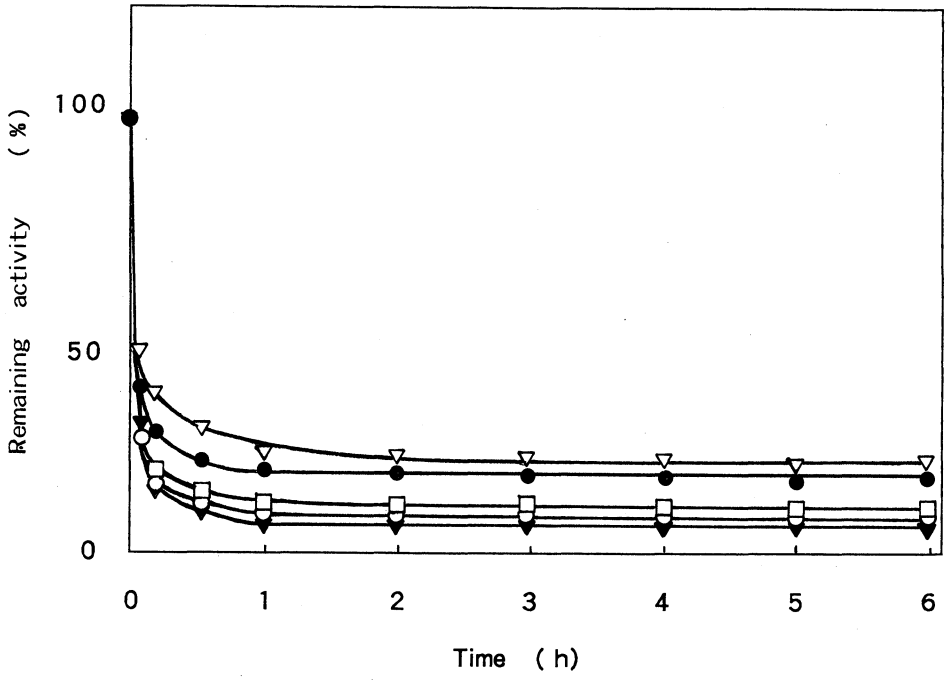

Fig. 1 Time course of the reaction of α-chymotrypsin with EDC. α-Chymotrypsin (0.4 mM) was incubated with 0.1 M EDC in 0.1 M N,N,N',N'-tetramethylethylenediamine-HCl buffer, pH 4.75. Aliquots (5 μl) were withdrawn from the incubation mixture and added 4 M sodium acetate buffer, pH 3.5. Chymotrypsin activity were assayed by the methods described in "Materials and Methods" for the indicated times using five substrates such as casein (●), Suc-Ala-Ala-Pro-Phe-pNA (○), Bz-Tyr-pNA (▽), Z-Tyr-ONp (▼), Ac-Phe-OEt (□).

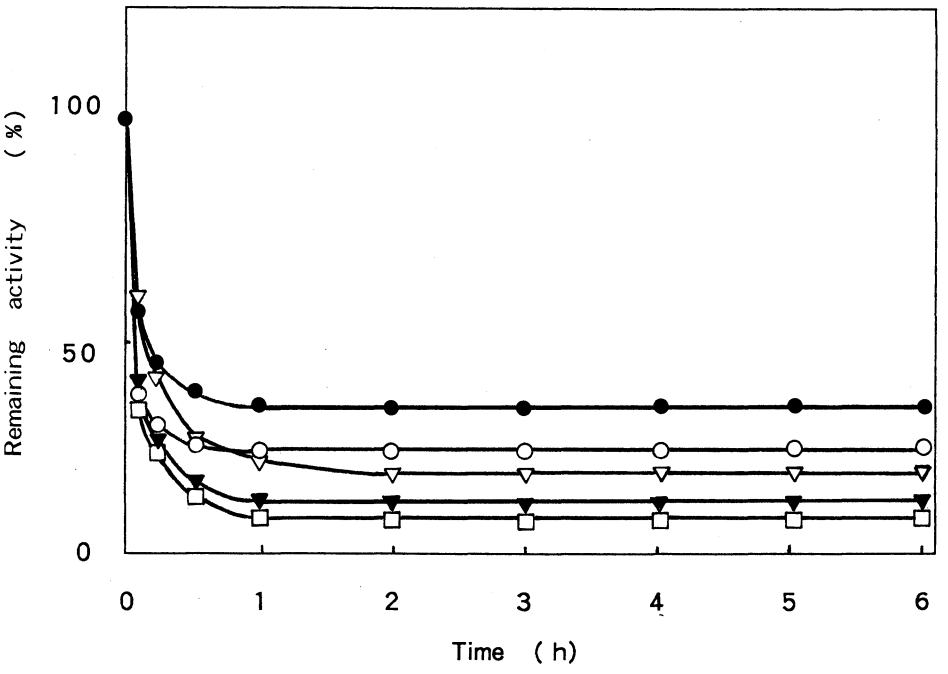

Fig. 2 Time course of the reaction of α-chymotrypsin with EDC and monomethylamine. The conditions were the same as those used to investigate in Fig. 1 without addition of 1 M monomethylamine.

caseinolytic activity was 25% higher than the absence nucleophilic reagents modification. The results suggested that the modified enzyme took on a favorable structure for protein substrate than small substrate as peptide amides.

α-Chymotrypsin obtained some amino groups and its surface charge considerably changed positive by chemical modification with EDC and methylenediamine. But the experiment was almost same result in case of monomethylamine (Fig. 3). Increments of positive charge on the surface of α-chymotrypsin did not affected the proteolytic activity. Caseinolytic activity was slightly higher than activity for other substrates. It seems that substrate specificity is not changed satisfactorily by the chemical modification with EDC.

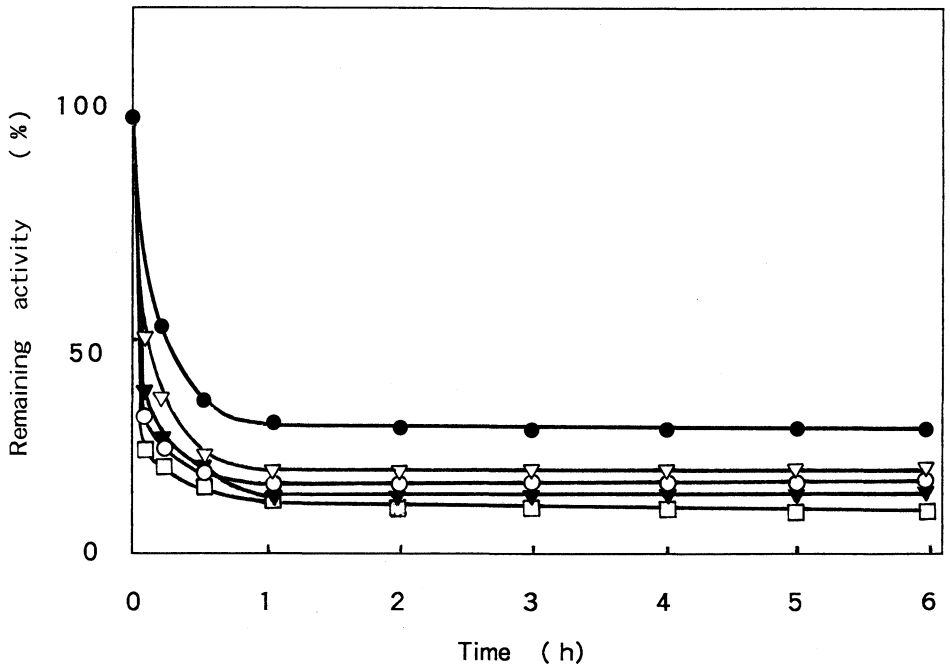

Fig. 3 Time course of the reaction of α-chymotrypsin with EDC and methylenediamine. The conditions were the same as those used to investigate in Fig. 1 without addition of 1 M methylenediamine.

References

[1] Phyllis, E. J., James, A. S., & Kenneth, G. D. A. (1976) *J. Biol. Chem.*, **251**, No. 8, pp2353–2362
[2] Kunitz, M. (1947) *J. Gen. Physiol.*, **30**, 291
[3] David, M. S. (1970) in *Methods in Enzymology* (Perlmann, G. E. & Lorand, L., eds.) Vol 19, pp 113–140, Academic Press, New York
[4] Erlanger, B. F., Kokowsky, N., & Cohen, W. (1961) *Arch. Biochem. Biophys.*, **95**, 271–278
[5] Bundy, H. F. (1962) *Anal. Biochem.*, **3**, 431–435
[6] Schwert, G. W., Takenaka, Y. (1955) *Biochem. Biophys. Acta,* **16**, 570–575
[7] Walsh, K. A. & Wilcox, P. E. (1970) in *Methods in Enzymology* (Perlmann, G. E. & Lorand, L., eds.) Vol 19, pp 31–41, Academic Press, New York