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FORMATION OF A COVALENT INTERMEDIATE BETWEEN α-CHYMOTRYPSIN AND THE B-CHAIN OF INSULIN DURING ENZYME-CATALYZED HYDROLYSIS

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Summary

The reaction between α-chymotrypsin (EC 3.4.21.1) and the B-chain of bovine insulin was studied radiochemically, by using the ^{35}S-labelled sulfo B-chain. After incubation at pH 8.0, interrupted by the addition of trichloroacetic acid, a radioactive product was isolated from the reaction mixture. The labelled product was eluted in parallel with the enzyme in gel chromatography, and its properties at different H^+ concentrations indicated that chemically it was an ester, i.e. a covalent enzyme-substrate intermediate.

No interaction between sulfo B-chain and α-chymotrypsinogen or phenylmethyl sulfonyl fluoride-inhibited α-chymotrypsin was obtained during identical conditions.

Introduction

The mechanism of action of α-chymotrypsin (EC 3.4.21.1) has been more extensively investigated than that of any other enzyme. Although the natural substrates are proteins or fragments of proteins, most studies of the enzyme have been based on its hydrolase activity toward synthetic peptides, peptide amides, and esters (for a review see ref. 1). Kinetic measurements have indicated that the catalytic hydrolysis proceeds in three steps [1]. The first step is a rapid, reversible formation of an enzyme-substrate complex, and the second step is the formation of a covalent intermediate between the acyl moiety of the substrate and the active centre Ser-195 of the enzyme, with concomitant release of the alcohol or amine moiety. Finally, in the third step, the covalent intermediate is hydrolysed to native enzyme and acid. It has been found that the third step is rate-limiting during enzymatic hydrolysis of ester substrates,

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Abbreviations: PMSF, phenylmethyl sulfonyl fluoride; Tyr-OEt, L-tyrosine ethylester.

while the second step, i.e. the formation of a covalent intermediate, is the rate-limiting step during amide hydrolysis. However, no similar studies with polypeptide substrates have been reported so far.

In a previous communication from this laboratory the rate of formation of a covalent intermediate during reaction between chymotrypsin and *p*-nitrophenyl [1-^{14}C]acetate was studied by a rapid-mixing technique [2]. It was found that [^{14}C]acetyl-enzyme was formed sufficiently fast to be a true intermediate of the reaction. The rate constant obtained was in good agreement with that found by other investigators using spectrophotometric methods (see ref. 2 for references). It was therefore of interest to use a similar radiochemical method with a natural substrate which had been characterized with regard to chymotryptic digestion and which could be labelled fairly easily with a radioactive marker. The B-chain of insulin, obtained by sulfitolysis, fulfills both these requirements and was therefore used in the present investigation.

Materials and Methods

α-Chymotrypsin (Type II), α-chymotrypsinogen A and phenylmethyl sulfonyl fluoride (PMSF) were purchased from Sigma. Beef insulin (3 times crystallised) was a gift from Novo, Copenhagen, Denmark. $Na_2{}^{35}SO_3$ with a specific activity of 2.0—2.5 mCi/nmol was obtained from the Radiochemical Centre, Amersham, England. All other chemicals were of reagent grade. Sephadex G-50 and G-100 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Radioactivity was measured in an Intertechnique SL 30 liquid scintillation spectrometer using Instagel (Packard) scintillating liquid. The protein concentration of chromatographic fractions was estimated by measuring the absorbance at 275 nm in a Zeiss PMQ II spectrophotometer.

The substrate for the study was prepared by oxidative sulfitolysis of insulin according to the method of Leach et al. [3] (ratio of labelled to unlabelled sulfite: 1 : 3), with copper as the oxidant. The products of sulfitolysis were applied to Sephadex G-50 at 4°C and eluted with 0.05 M $(NH_4)_2CO_3$ buffer (pH 9.0) containing 0.1% Brij-35. The material of the first labelled major peak, containing the ^{35}S-labelled sulfo B-chain, was pooled and then precipitated by adjusting the pH to 5.0 with 2 M acetic acid. The precipitate was collected by centrifugation and kept overnight at 4°C under reduced pressure using P_2O_5 as a desiccant. Although a product of almost constant specific activity was obtained, the chromatographic procedure was usually repeated twice to ascertain that the substrate had a high degree of homogeneity. The specific radioactivity of the final product was $3.5 \cdot 10^3$ cpm/nmol of ^{35}S-labelled sulfo B-chain. This value was calculated from radioactivity and absorbance measurements at 275 nm, using a molar coefficient of absorbance of $3.1 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ [4].

The reaction between α-chymotrypsin and ^{35}S-labelled sulfo B-chain was studied at 30°C using the following buffers: 0.1 M acetate buffer (pH 4.0), 0.1 M Tris · HCl buffer (pH 8.0) and 0.1 M ammonium acetate buffer (pH 10.0). The final concentrations of enzyme and substrate were 0.01 and 1 mM, respectively, in each incubation. The reaction was stopped after 1 min at pH 8.0 and after 3 min at pH 4.0 and 10.0 by the addition of 10% (w/v) trichloro-

acetic acid to a final concentration of 5%. The precipitated protein was collected by centrifugation at 2000 × *g* for 20 min and dissolved in 2 ml of 0.1 M Tris · HCl buffer (pH 8.0) containing 0.5% sodium dodecylsulfate. The mixture was then chromatographed at room temperature on a column (30 cm × 2 cm) of Sephadex G-100 in the same buffer (Fig. 1). The specific radioactivity of the labelled protein eluted in the position of α-chymotrypsin was calculated, using a coefficient of absorbance of $5 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [5], neglecting the possible contribution of the B-chain, since it was calculated that this did not exceed 5% of the total.

The stability of the labelling was tested at pH values ranging from 0.8—11.0, as follows: 3 ml of labelled protein solution, previously dialysed against 0.01 M Tris · HCl buffer (pH 8.0), was incubated for 1 h at 40 °C after mixing with 3 ml of appropriate 0.2 M buffer. The incubation mixture was then chromatographed on the Sephadex G-100 as described above. The specific radioactivity of the chromatographic fractions, in per cent of the original specific radioactivity, was taken as a measure of the stability and was plotted against the recorded pH-value (Fig. 3).

α-chymotrypsinogen A and PMSF-inhibited α-chymotrypsin were incubated with ^{35}S-labelled sulfo B-chain at the same pH values and under identical conditions as described above. The inhibition of α-chymotrypsin by PMSF was followed by measuring the hydrolysis of L-tyrosine ethylester (Tyr-OEt), according to the method of Schwert and Takenaka [6], and was allowed to proceed until the enzyme activity was completely abolished.

Results and Discussion

Fig. 1 shows Sephadex G-100 chromatograms of chymotrypsin (A), ^{35}S-labelled sulfo B-chain of insulin (B), and an incubation mixture of the two at pH 8.0 (C). The treatment of the samples prior to chromatography was identical, i.e. precipitation with trichloroacetic acid and collection of precipitate by centrifugation. As seen in Fig. 1C, a radioactive peak appeared at the position of the enzyme. Rechromatography of this labelled material in the same system gave only a loss of radioactivity corresponding to the amount of overlap between the two peaks. Identical results were obtained from incubation mixtures at pH 4.0 and 10.0. The amount of labelling at pH 8.0 indicates an incorporation of 0.20 mol of substrate per mol of enzyme, calculated with respect to ^{35}S. The corresponding values at pH 4.0 and pH 10.0 were 0.60 and 0.29, respectively.

Fig. 2 shows the structure of the B-chain of insulin according to Sanger and Tuppy[7], with the points of cleavage of α-chymotrypsin indicated by vertical arrows. When only the major sites of action were considered, it could be calculated that the maximal labelling obtainable amounted to 83%. The reason for this is that cleavage at the first major site leaves only one ^{35}S-group per molecule. This implies that the afore mentioned amount of labelling (0.20) actually means that there is 0.24 molecule of substrate per enzyme molecule.

In order to investigate the nature of attachment of the labelling, a series of stability tests were performed. Fig. 3 shows the result of these tests. The profile of this curve is very similar to that obtained for the acyl enzyme [2]. This

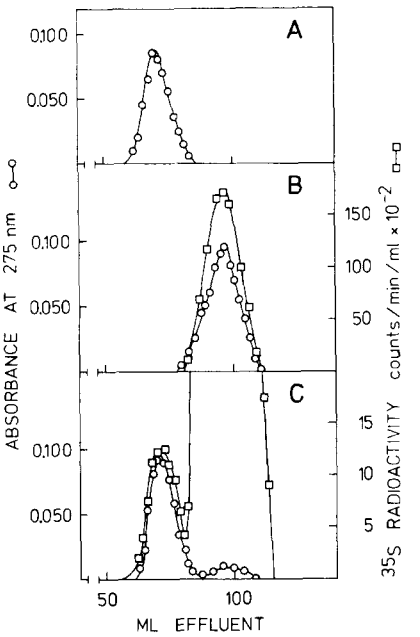

Fig. 1. Chromatograms on Sephadex G-100 of (A) α-chymotrypsin, (B) B-chain of insulin, and (C) incubation mixture (1 min, 30°C, pH 8.0) of α-chymotrypsin and B-chain of insulin. Samples were precipitated with trichloroacetic acid, and precipitates were collected by centrifugation before chromatography. Column dimensions: 30 × 2 cm. Elution was performed with 0.01 M Tris · HCl buffer (pH 8.0) containing 0.5% sodium dodecylsulfate. 2 ml fractions were collected every 2 min. ○———○: protein, absorbance at 275 nm; □———□ radioactivity, cpm/ml.

result suggests that the ^{35}S-labelled B-chain residue was bound to the enzyme with a covalent linkage, with stability properties similar to those of an acyl ester. It is therefore reasonable to assume that a covalent enzyme-substrate intermediate was in fact formed [8]. This assumption is furthermore supported by the fact that no interaction was seen between ^{35}S-labelled sulfo B-chain and α-chymotrypsinogen or PMSF-inhibited α-chymotrypsin.

The degree of acylation obtained at pH 8.0 provides some information concerning the relative rates of the acylation and deacylation reactions. It is interesting to note that the cleavage of a peptide chain more resembles the cleavage of an ester than of an amide substrate, since it was possible to isolate an acyl-enzyme intermediate. This means that the deacylation reaction is the

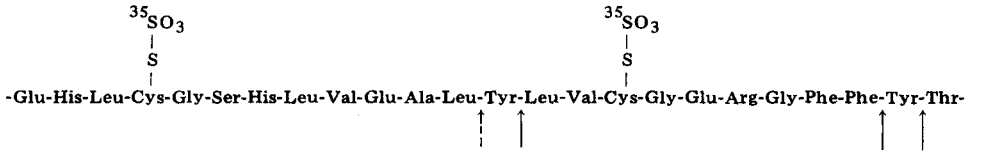

Fig. 2. The structure of the major part of the B-chain of insulin (residues 4-27) according to Sanger and Tuppy [7], with the ^{35}S-labels indicated. ↑ indicates major sites of action of α-chymotrypsin, and ↑ indicates another bond split by the enzyme [6].

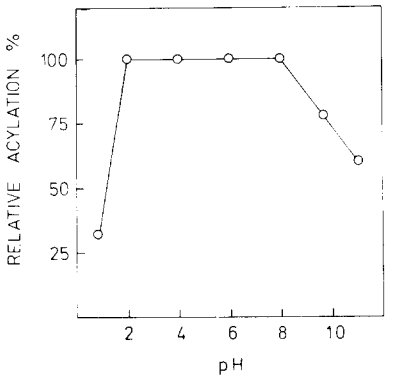

Fig. 3. Stability of ^{35}S-acyl-chymotrypsin at different pH values. The acylated enzyme was treated at 40°C for 1 h, cooled and chromatographed on Sephadex G-100 under the same conditions as the chromatographies in Fig. 1. For further details, see text.

rate-limiting step in the reaction with peptide substrates. One explanation for this may lie in the fact that a larger substrate has the opportunity of multiple point attachment to the surface of the enzyme molecule remote from the active site that results in a much faster acylation rate. The possibility of changed rate constants due to the steric interaction between the enzyme and different types of substrates has been pointed out by Hein and Niemann [8]. However, results from kinetic experiments may be consistent with the acyl-enzyme hypothesis but cannot prove this unless all other mechanisms are excluded. The results presented in this communication, together with earlier findings in this laboratory [2,9], give strong evidence for the view that a covalent enzyme-substrate intermediate is in fact formed with the acyl moiety of the substrate covalently linked to the active site of the enzyme.

Acknowledgements

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