Rate of Acetylation of α-Chymotrypsin by p-Nitrophenyl [1-¹⁴C]Acetate Studied by Isolation of [¹⁴C]-Acetyl Enzyme

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The rate of acetylation of α-chymotrypsin by p-nitrophenyl [1-¹⁴C]-acetate at pH 4.1, 8.0, and 10.0 has been studied with respect to the formation of ¹⁴C-labelled acetyl-enzyme. The reaction was interrupted after different times by the addition of 1 vol. of 10 % (w/v) trichloroacetic acid-0.004 M silicotungstic acid. After centrifugation, the precipitated protein was dissolved in an alkaline buffer solution containing sodium lauryl sulfate, and chromatographed on Sephadex G-25 in order to remove adsorbed substrate and products. The rate of acetylation at pH 8.0 is high enough to permit the isolated acetyl-enzyme to be a true intermediate in the catalytic hydrolysis.

Several investigators have studied the kinetics of the α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate.[1-6] The mechanism of catalysis involves the formation of an acetyl-enzyme with covalent bond between the active site serine-195 and the acetyl group and the concomitant release of p-nitrophenol. The deacylation of the acetyl-enzyme is the rate limiting step.

In all previous studies on the hydrolysis of PNPA * by chymotrypsin, the reaction has been studied spectrophotometrically by following the formation of p-nitrophenol. The steady state of the reaction is preceded by a rapid liberation of about one mole of p-nitrophenol per mole of enzyme.[7] It has been considered that this "initial burst" is reflecting the acetylation reaction, especially since Balls and Aldrich [8] were able to isolate an acetylated enzyme at low pH. This enzyme contained one acetyl group per enzyme molecule and was completely inactive, but could easily be reactivated by raising pH. Therefore, the rate constants for the acetylation reaction have been evaluated under the assumption that the p-nitrophenol is released simultaneously with the formation of the acetyl-enzyme formed. The acetyl-enzyme was isolated by gel chromatography after inactivation of the enzyme. The acetyl-enzyme was shown to be stable under the conditions used for its isolation. Rate of acetylation for the reaction with 1 mM substrate was measured at three different pH-values.

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* Abbreviations: PNPA, p-nitrophenyl acetate; ¹⁴C-PNPA, p-nitrophenyl [1-¹⁴C]acetate; TEE, ʟ-tyrosine ethyl ester.

EXPERIMENTAL

Materials. α-Chymotrypsin (EC 3.4.4.5) (type II) was purchased from Sigma. ^{14}C-PNPA was obtained from NEN Chemicals GmbH (Dreieichenhain, Germany) (1.21 mC/mmole) and unlabelled PNPA was an Eastman product. The unlabelled PNPA was recrystallised once from 50 % ethanol and once from hexane before use. DF^{32}P was purchased from the Radiochemical Centre (Amersham, England) (2.2×10^7 cpm/μmole). Sephadex G-25 and G-50 were obtained from Pharmacia Inc. (Uppsala, Sweden). All other chemicals were of reagent grade.

Analytical methods. Radioactivity of ^{14}C was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer, and Radioactivity of ^{32}P was measured on dried aliquots in aluminum cups, with a Philips instrument, type 111.611 A/10, equipped with an end-window Geiger tube No. 18546 and a sample changer, type PW 4001. The protein concentration of the chromatographic fractions was measured at 280 nm in a Zeiss PMQ II spectrophotometer. Chymotrypsin was assayed with TEE as substrate at pH 7,0, 25°, according to the method of Schwert and Takenaka.[9]

Substrate solution. A stock solution of the substrate was prepared by dissolving unlabelled and labelled PNPA separately in acetonitrile at final concentrations of 6×10^{-2} M. For the acetylation experiments, ^{14}C-PNPA was mixed with unlabelled PNPA in a molar ratio of 1:3. The specific radioactivity of this substrate solution was determined after removing the products of spontaneous hydrolysis by extraction according to the following. The substrate solution was diluted 60 times with 0.1 M Tris-HCl buffer (pH 7.0). A sample of the diluted substrate solution was extracted with an equal volume of chloroform. All PNPA was recovered in the chloroform phase. The aqueous phase was replaced with an equal volume of fresh buffer and the extraction was repeated. A portion of the buffer from each extraction was diluted 20 times with 0.4 M NaOH and the amounts of radioactivity and *p*-nitrophenol were estimated, the latter by absorbance measurement at 400 nm. After two extractions the absorbance at 400 nm as well as radioactivity were zero. Treatment of one volume of the chloroform phase with 20 volumes of 0.4 M NaOH for 5−10 min caused a complete hydrolysis of the PNPA. The radioactivity and amount of *p*-nitrophenol in the aqueous phase were estimated. The concentration of *p*-nitrophenol was calculated using a molar absorption coefficient of 1.83×10^4 M^{-1} cm^{-1} at 400 nm (Refs. 4, 10). The specific radioactivity of the substrate used was 5×10^5 cpm/μmole.

Determination of apparent Michaelis constant. The rate of the hydrolysis of PNPA by chymotrypsin was measured by determining the appearance of *p*-nitrophenol at 400 nm. Correction for spontaneous hydrolysis of the substrate was made in parallel. The determinations were made at 25° in 0.1 M sodium phosphate buffer, pH 8.0 at PNPA concentrations ranging from 5 to 200×10^{-5} M (in all cases containing 1.7 % acetonitrile) and an enzyme concentration of 2×10^{-5} M.

Acetylation reaction. Acetylation at pH 8.0 was performed in the rapid mixing apparatus described by Wålinder, Zetterqvist and Engström.[11] By changing the polyethylene tubings connecting the syringes and the mixing chamber to tubings of inner diameters of 0.28 mm, incubation times from 30 to 160 msec were obtained.

For each experiment, the syringes were filled with 0.5 ml of 2×10^{-3} M PNPA in glass distilled water containing 3.3 % acetonitrile and 0.5 ml of 4×10^{-4} M chymotrypsin in 0.1 M sodium phosphate buffer, pH 8.0, respectively. The solutions were quenched in 1 ml of 10 % trichloroacetic acid containing 0.004 M silicotungstic acid.

After inactivation, the precipitated protein was collected by centrifugation at about 2000 g for 20 min, and dissolved in 3 ml of 0.1 M sodium phosphate buffer, pH 8.0, containing 0.5 % sodium lauryl sulfate. The colour of the solution indicated that totally less than 10 nmoles of PNPA or *p*-nitrophenol had coprecipitated with the protein. The solution was then chromatographed on a column (1.6×20 cm) of Sephadex G-25 in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.25 % sodium lauryl sulfate in order to separate ^{14}C-labelled enzyme from unreacted ^{14}C-PNPA, ^{14}C-acetate, and *p*-nitrophenol, which could have been in the precipitate. The radioactivity and absorbance at 280 nm of the different chromatographic fractions were determined and the amount of ^{14}C-acetate bound to the enzyme was calculated, assuming an absorption of 1.0 at 280 nm for a solution containing 20 nmole/ml of chymotrypsin (Ref. 5).

Acetylation studies at pH 4.1 and pH 10.0 were performed in a test tube with the enzyme dissolved in 0.1 M sodium acetate buffer and 0.1 M sodium carbonate buffer, respectively, and with the same substrate solution as for the experiment at pH 8.0. The enzyme solution (0.5 ml) was rapidly mixed with an equal volume of substrate at 25°. The reaction was terminated after appropriate time by the rapid addition of 1 ml of 10 % (w/v) trichloroacetic acid-0.004 M silicotungstic acid. The precipitated protein was collected, dissolved and chromatographed as described above. The acetylation per mole of enzyme was calculated from the specific radioactivity of the protein eluted from the columns and from the specific radioactivity of the ^{14}C-PNPA used.

Stability test of the ^{14}C-labelled protein. The stability of the inactivated ^{14}C-labelled protein at 50° was tested in the following way. A sample of the ^{14}C-acetyl-enzyme (0.5 ml containing about 1 mg) which had previously been chromatographed on Sephadex G-25 as described above was diluted with 1.5 ml of 0.2 M buffer to obtain pH 3 to 11, or with 4 M acetic acid to obtain pH 2. From pH 3 to pH 6 citrate-phosphate buffers were used. For pH 7, 8, and 11, phosphate buffers were used, and for pH 9 and 10 borate and carbonate buffers, respectively. The cation was in all cases sodium. The samples were kept at 50° for 1 h, cooled and then chromatographed on Sephadex G-25 as described above. The ratio of protein-bound radioactivity to total radioactivity of the sample was calculated. The pH of the incubation mixture was measured at 50° with a Radiometer pHM 28 pH-meter using a C glass electrode. Stability was also tested on labelled enzyme obtained after inactivation with 3 volumes of 0.4 M NaOH containing 0.5 % sodium lauryl sulfate, followed 15 sec later by 0.5 M phosphoric acid to neutral pH.

Determination of amount of active enzyme. The amount of active enzyme in the preparation used was determined by incubation with DF^{32}P (2.2×10^4 cpm/nmole) at a final concentration of 5.8×10^{-4} M for 20 min at pH 8.0 and 25°, followed by chromatography on Sephadex G-50 eluted with the same buffer as the G-25 columns. The amount of active enzyme was estimated from the total amount of ^{32}P bound to the enzyme. It was assumed that all the active enzyme molecules had reacted with the inhibitor.[12] The ^{32}P-labelling obtained, indicated that the enzyme preparation used was about 92 % active. This result is in good agreement with that obtained by another method.[13]
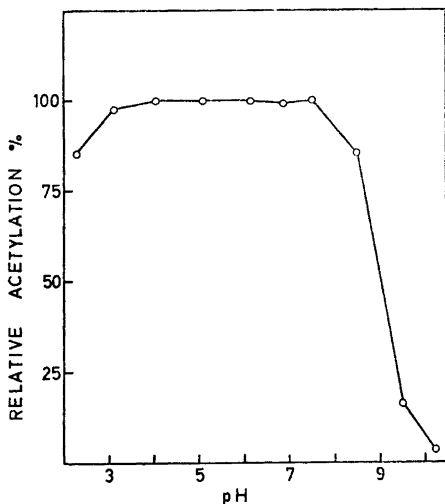

Fig. 1. Stability of ^{14}C-acetyl-chymotrypsin at different pH values. The acetylated enzyme was treated at 50° for 1 h, cooled and chromatographed on Sephadex G-25. For further details, see the text.
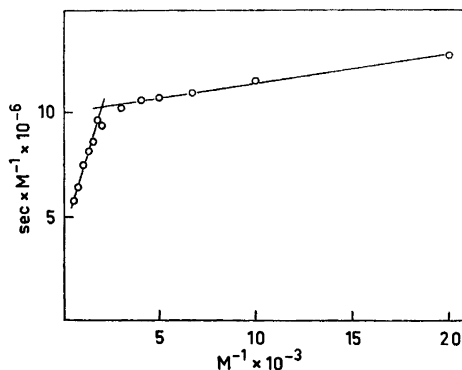
Fig. 2. Lineweaver-Burk plot of the steady-state hydrolysis of PNPA by chymotrypsin at 25° in 0.1 M sodium phosphate buffer, pH 8.0, containing 1.7 % acetonitrile. Enzyme concentration: 2×10^{-5} M.

RESULTS

Stability of the [14]*C-labelled enzyme.* Fig. 1 shows the pH stability of ^{14}C-acetyl chymotrypsin at 50°. The ordinate shows the relative amount of protein-bound ^{14}C-acetate in comparison to a sample which was chromatographed without treatment at 50°. The acetyl-enzyme obtained from alkaline inactivation was equally stable.

Determination of apparent Michaelis constant. Fig. 2 shows a Lineweaver-Burk plot of the steady-state hydrolysis of PNPA by chymotrypsin. The curve is seen to consist of two parts, from which two different apparent K_m-values could be calculated. At low substrate concentrations a value of 1.4×10^{-5} M was obtained, while the corresponding value for higher substrate concentrations was 7×10^{-4} M. This behaviour has been interpreted as a substrate activation of the hydrolysis.[4,5] The turnover at the conditions used in the acetylation experiment at pH 8.0, *i.e.* at a substrate concentration of 1 mM could be calculated to be 0.007 sec^{-1}.
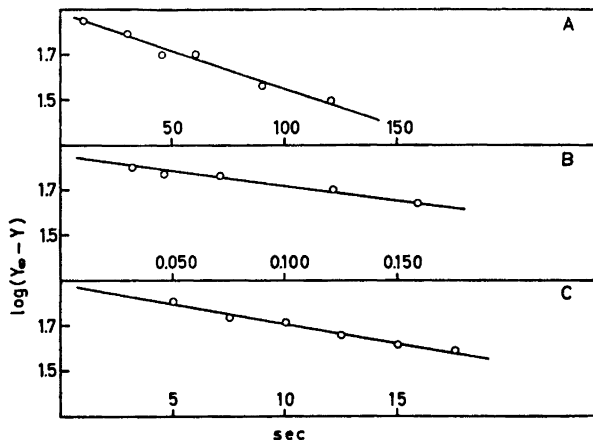

Fig. 3. First order plots of the acetylation of chymotrypsin by 1 mM ^{14}C-PNPA in 0.1 M sodium acetate buffer, pH 4.1 (A), 0.1 M sodium phosphate buffer, pH 8.0 (B), and 0.1 M sodium carbonate buffer, pH 10.0 (C) at 25°. Y_∞ represents the maximal acetylation in per cent of total enzyme, and Y the acetylation obtained after varying time of incubation.

Rate of acetylation reaction. The rate of the acetylation reaction at different pH-values is shown in Fig. 3. Y_∞ denotes the maximal degree of acetylation, and amounted to 80 % at pH 4.1, 70 % at pH 8.0, and 100 % at pH 10.0. The acetylation rate constants obtained (corresponding, *e.g.*, to k of Ref. 5) were 0.01 sec^{-1} at pH 4.1, 3 sec^{-1} at pH 8.0, and 0.03 sec^{-1} at pH 10.0. The rate of acetylation at optimal pH consequently is high enough in comparison to the turnover rate at steady-state to allow the assumption that the acetyl-enzyme isolated is a true intermediate in the catalytic hydrolysis of PNPA.

Acetylation of native enzyme. In order to ascertain that the acetylation reaction was specific at pH 8.0, an experiment was designed to show that the native enzyme incubated with the labelled substrate was deacetylated by removing the substrate by gel chromatography at pH 8.0. Enzyme and substrate were mixed at the same final concentrations as in the acetylation experiments. After about 15 sec, when the acetylation should be maximal according to the previous experiments, the reaction mixture was chromatographed on a column (2.3 × 30.5 cm) of Sephaxed G-50 in equilibrium with 0.1 M phosphate buffer, pH 8.0. The flow rate was 0.5 ml/min. The effluent was analysed for protein content, radioactivity, and enzyme activity against TEE. The amount of ^{14}C-acetate remaining bound to the enzyme was in one experiment calculated to be 0.08 mole per mole of enzyme and 0.04 in another. The specific enzyme activity against TEE were in both cases the same before, as well as after the incubation with ^{14}C-PNPA.

Acetylation of DFP-inhibited chymotrypsin. Another control experiment to find out whether DFP-inhibited enzyme became labelled by ^{14}C-PNPA was also performed. 5 mg of chymotrypsin were completely inhibited at pH 8.0 by unlabelled DFP at a final concentration of 0.5 mM. Excess DFP was separated from the protein by chromatography on Sephadex G-50 at pH 8.0. The protein fractions were pooled and incubated with ^{14}C-PNPA at a final concentration of 1 mM. After 15 sec the reaction was interrupted, as described for the acetylation study, and protein analysed. From the amount of radioactivity it could be calculated that about 0.1 mole of ^{14}C-acetate per mole of enzyme was obtained in the protein fraction.

DISCUSSION

Chymotrypsin acetylated at pH 5 by PNPA was first isolated by Balls and Aldrich.[8] This suggested that the enzyme is intermediately acetylated during its action. Since that time several extended studies on the kinetics of the chymotrypsin-catalyzed hydrolysis of PNPA have been performed.[1-6] All these studies postulate the presence of an intermediate acetyl-enzyme.

With the method of enzyme inactivation used in the present work, it was possible to isolate an acetyl-enzyme even after short-time incubation at pH, which is optimal for the enzyme action. The fact that the rate of acetylation found in this investigation greatly exceeds that of the overall reaction, gives further strong support for the view that the enzyme is intermediately acetylated during its action.

Since the inactivated acetyl-chymotrypsin was stable over a wide range of pH, reaction of DFP-sensitive enzyme with compounds containing ^{14}C-acyl groups followed by inactivation of the enzyme, seems to be a possible way to label the active site of the enzyme for structural studies. The method to study the intermediate acetylation of chymotrypsin used in the present study, should be suitable for other DFP-reacting enzymes as well. This has been shown to be the case for two enzymes from *Streptomyces griseus* protease (Pronase), which are acetylated by PNPA (Ref. 14). It should, however, also be possible to use other substrates for similar studies.

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