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REACTION OF THE *p*-NITROPHENYL ESTER OF CARBOBENZOXY-*D*-PHENYLALANINE WITH CHYMOTRYPSIN

(Presented by Academician A. N. Nesmeyanov, 28 XI 1961)

The mechanism of the esterase action of chymotrypsin has attracted the attention of a wide circle of investigators. According to current concepts, an unstable chemical compound is formed between the enzyme and the substrate. Such a compound was first isolated by Balls in the hydrolysis of *p*-nitrophenyl acetate by chymotrypsin ([1]). Balls found that if the hydrolysis of *p*-nitrophenyl acetate by chymotrypsin is carried out at pH 5–5.6, then, despite an excess of nitrophenyl acetate, only an amount of nitrophenol equivalent to the chymotrypsin is rapidly formed, corresponding to a "burst"on the kinetic curve. The formation of nitrophenol is not accompanied by liberation of acetic acid; slow liberation of the latter occurs only when the pH is raised to 7.2. On the basis of these data Balls concluded that in the first stage of the reaction acetylation of the active center of the enzyme occurs. Since acetylchymotrypsin is stable at low pH, Balls succeeded in isolating it and demonstrating the presence of an ester bond in it. For this purpose the reaction mixture was treated with ether to remove the excess *p*-nitrophenyl acetate, and acetylchymotrypsin was isolated by lyophilization. It gave the hydroxamic reaction at low pH; in addition, at pH 5.5–6.2 the acetyl group could be transferred to ethanol hydroxyl and the ethyl acetate formed could be isolated.

Analyzing kinetic data on the hydrolysis of *p*-nitrophenyl acetate, Spencer and Sturtevant ([2]) established that acetylation of chymotrypsin also occurs at high pH values, i.e., acetylchymotrypsin proves to be a true intermediate compound in the hydrolysis of esters.

Subsequently ([3]), benzoyl-, salicyl-, and trimethylacetylchymotrypsin were isolated in the hydrolysis of esters of these acids. The stability of acylchymotrypsins depended to a considerable degree on the nature of the acyl residue. The

Fig. 1

Figure 1: Fig. 1

most stable was trimethylacetylchymotrypsin. At the same time, acylamino-acid derivatives of chymotrypsin decomposed so rapidly that they could not be isolated. Their formation could be established from the appearance of a "burst" on the kinetic curve, corresponding to liberation of an equivalent of nitrophenol. Such a burst was observed by Balls in the hydrolysis of *p*-nitrophenyl hippurate by chymotrypsin. Hammond and Gutfreund ([4]), in the hydrolysis of the *p*-nitrophenyl ester of carbobenzoxy-*L*-tyrosine, recorded a "burst" lasting less than 10^{-3} sec. On this basis they suggested that acylation is an intermediate stage also in the case of hydrolysis of specific substrates of chymotrypsin.

Esters of *D*-amino acids are inhibitors of chymotrypsin and are hydrolyzed considerably more slowly than esters of their *L*-antipodes. It could be assumed that the intermediate compound of chymotrypsin with the *D*-antipode of an acylamino acid would prove to be more stable.

In the present investigation the hydrolysis of the *p*-nitrophenyl ester of carbobenzoxy-*D*-phenylalanine by chymotrypsin was carried out.

The results are presented in Fig. 1. It follows from them that at pH 4.9 and 5.3 there is a "burst" in the liberation of nitrophenol—the stage of acylation of the enzyme, according to Balls. In the second stage the rate of hydrolysis slows—

it appears to correspond to the more slowly proceeding deacylation process. However, even at low pH values deacylation does not cease, i.e., the intermediate compound that is formed, as might have been expected, is much less stable than acetylchymotrypsin. Therefore the method proposed by Balls for isolating the intermediate compound proved unsuitable in this case.

We proposed a different scheme of treatment.

The hydrolysis of the ester was interrupted after a short interval by rapidly acidifying the solution to pH 3 and precipitating the protein with trichloroacetic acid. The precipitated protein was washed repeatedly with acetone. However, it was not possible in this way to wash out the adsorbed *n*-nitrophenol and the *n*-nitrophenyl ester of carbobenzoxy-*D*-phenylalanine. To remove them, reprecipitation of the protein from a 9 *M* urea solution was used. The character of the bond between carbobenzoxy-*D*-phenylalanine and chymotrypsin was demonstrated by two methods.

Fig. 1

1. By means of the hydroxamic acid reaction, which was carried out quantitatively, the formation of an ester bond in the acylated protein was established. Carbobenzoxy-*D*-phenylalanine hydroxamate was isolated chromatographically.

2. The ester bond in the acylated protein was hydrolyzed with 1 *N* alkali, and the carbobenzoxy-*D*-phenylalanine formed was isolated chromatographically, hydrolyzed, and the content of *D*-phenylalanine was determined quantitatively by the ninhydrin reaction. Both methods gave similar results (see Table 1).

Table 1

Acylation of chymotrypsin with the *n*-nitrophenyl ester of carbobenzoxy-*D*-phenylalanine

Experiment No.	Substrate, μM/ml	Chymotrypsin, μM/ml	Enzyme/substrate	pH	Duration of experiment, min	Amount of ester bond, % (by hydroxamic acid reaction)	Amount of phenylalanine, % (by ninhydrin reaction)	Chromatographic determination of CBZ-*D*-phenylalanine hydroxamate
1	0.1	0.04	1 : 2.5	4.7	20	17	17	–
2	0.4	0.08	1 : 5	6.0	10	12	12	–
3	0.2	0.04	1 : 5	6.5	5	30	26	+
4	0.2	0.04	1 : 5	6.5	4	19	25	+
5	0.4	0.08	1 : 5	6.2	1	23	17.5	+

Note. In experiments 1 and 2, reprecipitation from urea was not carried out. Therefore the percentages in the hydroxamic acid and ninhydrin reactions are given after subtraction of the amount of nitrophenol detected. In experiments 3, 4, and 5 nitrophenol was not detected.

Experimental Part

n-Nitrophenyl ester of carbobenzoxy-*D*-phenylalanine was synthesized by the method of Bodanszky (5), yield 88%, m.p. 125°, $[\alpha]_D^{20} + 22.6°$ (*C* 2, dimethylformamide). Literature data for the *n*-nitrophenyl ester of carbobenzoxy-*L*-phenylalanine: m.p. 126–126.5°, $[\alpha]_D^{20} - 24.7°$ (*C* 2, dimethylformamide) (6).

Chymotrypsin—crystalline, twice recrystallized Reanal preparation (Hungary). The molecular weight of chymotrypsin was taken as 25,000.

1. **Hydrolysis of the *n*-nitrophenyl ester of carbobenzoxy-*D*-phenylalanine by chymotrypsin** is presented in Fig. 1. A 1.6 ml

portion of an acetone solution of the *n*-nitrophenyl ester of carbobenzoxy-*D*-phenylalanine, $C = 0.5$ μmol/ml, 2 ml of 0.07 M phosphate buffer, and 0.4 ml of enzyme, $C = 0.2$ μmol/ml, were taken. The enzyme : substrate ratio was 1 : 10. Temperature 18°. The optical density at complete hydrolysis of the ester was 1.6. The optical density corresponding to the liberation of one equivalent of *n*-nitrophenol per equivalent of enzyme was 0.16. The amount of liberated nitrophenol was determined from the change in optical density of the solution at $\lambda = 330$ mμ on an SF-4 spectrophotometer.

2. **Acylation of chymotrypsin with the *n*-nitrophenyl ester of carbobenzoxy-*D*-phenylalanine.** 175 mg of chymotrypsin were dissolved in 52.5 ml of 0.035 M phosphate buffer, pH 5.6. After standing for 30 minutes in a thermostat at 37°, the solution was filtered and, with stirring, 35 ml of an acetone solution of the *n*-nitrophenyl ester of carbobenzoxy-*D*-phenylalanine (concentration 1 μmol/ml) were added. After 60 sec, the solution was acidified to pH 3 with 20% HCl, 8 ml of 50% trichloroacetic acid (TCA) were added, and it was left in a refrigerator for 15 min at 0°. The precipitate that formed was centrifuged off, washed several times with acetone, and dissolved in 1.5 ml of 9 M urea solution. After 15 min, the protein was precipitated with 8% TCA and the precipitate was washed several times with acetone. Reprecipitation from urea solution was repeated two more times; the resulting precipitate was washed with acetone and ether and dried in a vacuum desiccator. The precipitate was a white, loose powder.

3. **Investigation of carbobenzoxy-*D*-phenylalanyl-chymotrypsin.**

A. **Quantitative determination of the ester bond.** To 25 mg of acylated chymotrypsin were added 1.2 ml of a solution of hydroxylamine hydrochloride adjusted to pH 11 with 3.5 N NaOH. After 50 min, 0.9 ml of water, 0.3 ml of hydrochloric acid (1 : 3), 1.2 ml of 8% TCA, and 0.9 ml of a solution of ferric chloride in 0.1 N HCl (16.6 g in 100 ml) were added. The solution was filtered and the optical density was measured on an SF-4 at 534 mμ. Nonacylated chymotrypsin treated in the same way was used as the standard. The percentage content of hydroxamate was calculated from a calibration curve for the absorption of carbobenzoxyphenylalanine hydroxamate (7) (see Table 1).

B. **Chromatographic isolation of carbobenzoxy-*D*-phenylalanine hydroxamate.** To 50 mg of acylated chymotrypsin were added 1.2 ml of a solution of hydroxylamine hydrochloride adjusted to pH 11 with 3.5 N NaOH. After 50 min, the solution was extracted several times with ether, acidified to pH 5, and again extracted with ether. The residue after distillation of the ether was chromatographed in 60% ethanol with a marker—carbobenzoxy-*DL*-phenylalanine hydroxamate. After drying, the chromatogram was developed with a solution of ferric chloride in 0.1 N HCl; the R_f of the dark cherry-colored spot detected was 0.86, and the R_f of the marker was 0.86.

4. **Quantitative determination of *D*-phenylalanine.**

A. **Alkaline hydrolysis of acylated chymotrypsin.** To 25 mg of the substance was added 1 ml of 1 *N* NaOH, and it was left for 2 hours. The solution was then acidified to pH 5, extracted six times with ether, the ether extract was evaporated to dryness in vacuo, the residue was dissolved in a drop of alcohol, and quantitative chromatography was carried out in the system butanol–2 *N* NH_3 with markers–*n*-nitrophenol and carbobenzoxyphenylalanine. After drying for 30 minutes at 50-60°, the chromatogram was cut into two strips: A–with the markers, and B–with the test solution. Nitrophenol on strip A was detected as a yellow spot, R_f 0.57; to detect carbobenzoxyphenylalanine, strip A was developed with a solution of KJ + KJO_3 + starch, R_f 0.73. On strip B, a section was cut out,

corresponding to R_f0.57, it was eluted with 4 ml of water, and the nitrophenol content was determined by measuring the optical density at 330 mμ.

b. Acid hydrolysis of carbobenzoxy-*D*-phenylalanine. The part of band **B** corresponding to R_f0.73 was cut out and eluted with alcohol. After the alcohol had been distilled off in vacuum, several millimeters of 20% HCl were added to the dry residue, and hydrolysis was carried out for 2 hours at 110–120°. After removal of HCl in vacuum, the residue was dissolved in several drops of water and quantitatively chromatographed in the system butanol–water–acetic acid (4 : 5 : 1). The quantitative determination of phenylalanine was carried out by the method of G. N. Zaitseva ([8]). The results of the experiments are presented in Table 1.

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Note: Figure translations are in progress. See original paper for figures.