THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS AND HYDROXYLAMINOLYSIS OF ACETYLTYROSINE p-NITROANILIDE*

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Received October 21, 1963

Acyl-enzyme intermediates probably occur in the α-chymotrypsin-catalyzed hydrolyses of p-nitrophenyl acetate (Hartley and Kilby, 1954; Gutfreund and Sturtevant, 1956), acetyl-L-tryptophan esters (Zerner and Bender, 1963), and hippuryl esters (Epand and Wilson, 1963). No direct indication that acyl intermediates are formed in hydrolyses of amide substrates has yet been given. Since such substrates are generally hydrolyzed much more slowly than the corresponding ester substrates (Hein and Niemann, 1961), it follows that if acyl intermediates are involved in these cases their formation must be rate-limiting, and they can be present during the steady state in only small concentrations so that their direct kinetic detection would be extremely difficult.

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*This investigation was supported by a grant (GM-4725) from the National Institutes of Health, U. S. Public Health Service.

[1]Contribution No. 1743.

In the case of ester substrates hydroxylamine has been shown to compete with water in the deacylation of the acyl-chymotrypsin intermediates, and to elevate significantly the overall rate of consumption of ester (Bernhard, Coles and Nowell, 1960; Bernhard and Gutfreund, 1960; Epand and Wilson, 1963; Caplow and Jencks, 1963). If the acylation step is required in the hydrolysis of an amide and is rate-limiting, hydroxylamine should lead to the formation of an hydroxamic acid as in the case of an ester substrate but should not cause any enhancement of the overall rate of consumption of the substrate. This paper reports the results of experiments performed to test this prediction.

Experimental

N-acetyl-D,L-tyrosine p-nitroanilide (ATNA) was synthesized according to the method of Bundy (1962) and was recrystallized five times from ethanol. M.p. 278.5-279° with decomposition (uncorr.); $[\alpha]_D = 0 \pm 0.8°$ (0.7% in dioxane). Analysis: calculated for $C_{17}H_{17}N_3O_3$, C 59.47, H 4.99, N 12.24%; found C 59.23, H 5.13, N 12.48%. Upon complete hydrolysis by chymotrypsin, the absorption spectrum of the product agreed with that for an equimolar mixture of ATNA, p-nitroaniline (NA) and acetyltyrosine, indicating that the D-isomer was not attacked by the enzyme. Na acetyl-L-tyrosinehydroxamide (ATH) was synthesized by the method of Hogness and Niemann (1953). After two recrystallizations from methanol-ether, the m.p. was 189.5-190° with decomposition (uncorr.). α-Chymotrypsin,

obtained from Worthington Biochemical Corp., was used without further purification. The molar concentration of the enzyme was determined spectrophotometrically, using $E^{280}_{1\%} = 20.0$ and taking the molecular weight to be 23,000 (Dixon and Neurath, 1957).

The overall rate of substrate consumption was determined by following the increase in absorption at 410 mμ. All experiments were performed at 25° in 0.2M phosphate buffer containing 10 or 33% dioxane to give sufficient substrate solubility. The concentration of ATH formed by the hydroxylaminolysis of ATNA was determined by a colorimetric method with ferric chloride described by Bernhard, et al. (1960), with minor modifications.

Results and Discussion

The initial rate of the chymotrypsin-catalyzed liberation of NA was determined in the absence of, and at various concentrations of, hydroxylamine, with the results shown in Table 1. The rate of non-enzymic liberation of NA was negligible in all cases.

The ratio of the amounts of ATH and NA formed is given in Table 2. It can be seen that the ratio increases with the hydroxylamine concentration, and that hydroxylaminolysis and hydrolysis are approximately equal in the presence of 1.6M hydroxylamine.

Table 1

Initial rates of the α-chymotrypsin-catalyzed NA formation from ATNA

(a) 10% dioxane, 0.2M KCl, initial substrate concentration 1.00×10^{-3}M

Hydroxylamine, moles per liter	Initial rate, sec^{-1}	Average dev. from mean	Number of determinations	pH range
0	0.0216	± 0.0002	4	7.94-7.96
0.1	.0208	-	1	8.00
0.2	.0219	.0001	3	8.05-8.20

(b) 33% dioxane, 1.6M KCl, initial substrate concentration 5.00×10^{-3}M

Hydroxylamine, moles per liter	Initial rate, sec^{-1}	Average dev. from mean	Number of determinations	pH range
0	0.0233	± 0.0003	6	8.27-8.30
0.4	.0232	.0006	3	7.78-8.35
0.8	.0238	.0002	3	8.30-8.45
1.2	.0230	.0005	3	8.30
1.6	.0219	.0005	5	8.27-8.40

It thus appears that hydroxylamine does not appreciably affect the overall rate even when the net rate of hydroxylaminolysis is comparable with that of hydrolysis. This indicates that a rate-limiting step precedes the step at which the interactions with hydroxylamine and water take place, and thus gives support to the view that acylation is an essential step in the hydrolysis of amide substrates.

Table 2

Ratio of hydroxylaminolysis to total consumption of substrate in the α-chymotrypsin-catalyzed hydrolysis of ATNA

33% dioxane, 1.6M KCl, initial substrate concentration 5.00×10^{-3}M

Hydroxylamine, moles per liter	Experiment number	Ratio of ATH to NA formed after 20 mins	40 mins	60 mins	90 mins
0.4	1	0.13	0.14	0.12	-
	2	.10	.10	.10	-
0.8	1	.24	.23	.26	-
	2	.29	.30	.31	-
	3	.19	.20	.20	-
1.2	1	.40	.40	.41	-
	2	.44	.46	.47	-
	3	.36	.37	.37	-
	4	.34	.34	.36	-
1.6	1	.42	.42	.42	.53
	2	.47	.48	.49	.53
	3	.44	.46	.49	-

Quantitative interpretation of the relative rates of hydroxylaminolysis reported In Table 2 is made difficult by the fact that ATH is itself a substrate for CT, as shown by Hogness and Niemann (1953). The kinetic parameters for ATH reported by these authors indicate that the actual rate of hydrolysis of ATNA and ATH in the latter stages of the experiments reported in Table 2 were of the same order of magnitude.

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