EVIDENCE AGAINST THE OBLIGATORY FORMATION OF AN ACYL ENZYME INTERMEDIATE IN THE α-CHYMOTRYPSIN CATALYZED REACTIONS OF AMIDES

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Received August 18, 1969

The relative rates of chymotrypsin catalyzed reactions with water and with glycinamide differ for benzoyl-L-tyrosine ethyl ester and benzoyl-L-tyrosine glycinamide. These results indicate that a common benzoyl-L-tyrosine-chymotrypsin intermediate is not formed from both substrates.

The esterase and peptidase activities of trypsin can be affected differently by chemical modifications of the enzyme (1,2) suggesting possible differences in the mechanism of hydrolysis of the two classes of substrates. Formyl trypsin, for example,can exhibit 75% of the esterase activity of the native enzyme while showing no activity against peptides (1). Another difference between the amidase and esterase properties of a proteolytic enzyme is the sign of the Hammet rho constant for the chymotrypsin catalyzed hydrolysis of O-acyl phenols and anilides (3). The kinetic properties of trypsin and chymotrypsin are very similar, even to the extent of hydrolyzing nonionic acyl enzymes with the same rate (4). Chymotrypsin is the more thoroughly studied of the two enzymes and the formation of an acyl chymotrypsin as an intermediate in the enzyme catalyzed hydrolysis of esters has been well established (5).This mechanism is generally accepted to be applicable to all chymotrypsin catalyzed reactions, although some evidence against it has been found for the synthesis of hydroxamic acids (6). If amides, which are poorer acylating agents than esters, did not have to pass through an acyl enzyme intermediate during enzyme catalyzed hydrolysis, it could provide a basis for explaining the separation of esterase and peptidase activities in chemically modified proteolytic enzymes.

One of the properties of the acyl enzyme mechanism is that a common intermediate is formed from a series of substrates with the same acyl group. If the enzyme catalyzed reactions take place in the presence of water and a second nucleophile, the common acyl enzyme intermediate would give the same

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relative rates of reaction with water and with the added nucleophile. In agreement with the theory, an identical fraction of a series of hippurate esters is converted to hydroxamic acid in chymotrypsin catalyzed reactions in the presence of NH_2OH (7). Experiments using methanol or ethanol as nucleophiles also gave results consistent with the acyl enzyme theory (8). A special case of this type of experiment arises when one uses a nucleophile which is the same as the leaving group of the substrate. In this case the relative rates of reaction of the added nucleophile and water can be calculated from the inhibition of the rate of hydrolysis in the presence of the added nucleophile; provided that acylation of the enzyme is rate controlling. Using hydroxamic acid substrates, with NH_2OH as the added nucleophile, general agreement with the acyl enzyme hypothesis was found (9) although discrepancies were observed at high substrate concentration (10). It is the purpose of this work to test the acyl enzyme theory in a similar manner using an amide, benzoyl-L-tyrosine glycinamide (BTGA)[1], as substrate, which is more stable than a hydroxamic acid; and glycinamide as the added nucleophile, which has only one nucleophilic group. Even in the presence of the highest glycinamide concentration used (0,2 M) 95 % of the amide would be hydrolyzed at equilibrium at pH 7.90 (11). The chymotrypsin catalyzed reaction of BTGA with glycinamide has been demonstrated by isotope exchange studies (12) and the inhibition of the enzyme catalyzed hydrolysis of BTGA by glycinamide can be accounted for by this reaction (calculated from data in references 12 and 13). We wish to compare the relative rates of α-chymotrypsin catalyzed reactions using benzoyl-L-tyrosine ethyl ester (BTEE) and BTGA as substrates in presence of the nucleophiles water and glycinamide. In experiments with BTEE, enzyme concentrations and reaction times were chosen so that BTGA would be a final product. With more enzyme or longer incubation times the rate of hydrolysis of BTGA can be measured.

MATERIALS

α-chymotrpsin, 3x crystallized, lyophilized and salt free, type II (Sigma Chem. Co., Lot 858-1690) was purified by gel filtration on Sephadex G-25 and used within a few days (14). Results with this preparation were essentially the same as with the commercial preparation.

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1. Abbreviations: BTGA, benzoyl-L-tyrosine glycinamide; BTEE, benzoyl-L-tyrosine ethyl ester; BT, benzoyl-L-tyrosine.

Glycinamide hydrochloride (Pierce Chem. Co.) was recrystallized from hot methanol; neutralization equivalent, 110.3 (calculated 110.5).

Benzoyl-L-tyrosine ethyl ester (BTEE) purchased from Mann Research Labs. was recrystallized from methanol-water. Hydrolysing the ester at pH 7 in the presence of chymotrypsin gave a stereospecific saponification equivalent of 313 (calculated 313).

Benzoyl-L-tyrosine glycinamide (BTGA) was prepared by reacting BTEE with 2 M glycinamide at pH 7.9, 30 % methanol in the presence of α-chymotrypsin. A white precipitate formed on the addition of BTEE which later dissolved and subsequently another white precipitate formed. The BTGA was isolated as previously described (12); m.p., 216-217° (reported, 215-217° (11); N 12.2 % (calculated 12.3 %).

Benzoyl-L-tyrosine (BT) was purchased from Cyclo Chemical Company and recrystallized from hot water; neutralization equivalent 284 (calculated 285).

Acetonitrile was purified from a Matheson, Coleman and Bell practical grade product as previously described (15).

Tris buffer was prepared from Trizma Base, reagent grade (Sigma Chemical Co., Lot 188-5070).

All other products used were analytical reagent grade and the water was distilled in a glass apparatus.

METHODS

The enzyme catalyzed reactions were studied in 0.10 M Tris buffer, pH 7.90; ionic strength, 0.25 M; 20 % acetonitrile at 25°.

A radiometer titrimeter was used for pH measurements and titrations. The instrument was standardized with buffer at pH 7.00. Ultraviolet absorption measurements were done with a Gilford Model 2000 spectrophotometer.

The fraction of BTEE hydrolyzed in the presence of glycinamide was determined by titrating the carboxyl group liberated. 1.5 ml of solution were reacted at pH 7.90 and the pH maintained within 0.1 units by the buffers. When the reaction was complete, as indicated by the failure to detect ester by the hydroxamic acid test and the fact that there was no further rise in carboxylic acid titer (at higher enzyme concentrations there was a slow rise in titer due to the hydrolysis of BTGA and a correction of less than 2 % was made for this in the calculations), the solution was adjusted to pH 4.6-5.2 by the addition of 5N HCl from a micrometer driven syringe. The pH was then lowered to 4.50 with 0.02 M HCl and the amount

of acid needed to lower the pH from 4.50 to 3.00 was then accurately recorded. Within these pH limits 71 % of BT is protonated as determined from the hydroysis product of BTEE in the absence of glycinamide as well as from BT itself. Blank runs, without substrate, took up 1.5 μmoles of acid and were independent of the presence of BTGA, BTEE or chymotrypsin. Separate blanks were run for each glycinamide concentration used and they varied by a few percent. The amount of acid used for the blank runs corresponded to about 20 % of that used in measuring the production of BT.

Rates of BTGA hydrolysis both in the presence and absence of glycinamide were measured by observing changes in the optical density at 257 mμ, which is the peak of the difference spectrum between BTGA and BT; BT having a 21 % higher extinction coefficient than BTGA at this wavelength. Beer's Law was obeyed in the concentration range studied. A substrate concentration of 5×10^{-4} M was used in both sample and blank cuvettes. At zero time, enzyme was added to the sample cuvette and initial velocities were obtained from the hydrolysis of about 10 % of the substrate.

TABLE 1

COMPARISON OF FRACTION OF ESTER HYDROLYZED WITH INHIBITION OF BTGA HYDROLYSIS IN GLYCINAMIDE SOLUTIONS
pH 7.90; ionic strength, 0.25 M; 20% acetonitrile at 25°

	0.10 M glycinamide	0.20 M glycinamide
Fraction of ester hydrolyzed	0.61±0.01	0.425±0.010
Ratio of rates of formation of BTGA/BT from ester	0.64±0.03	1.35 ±0.05
Ratio of rates of hydrolysis of BTGA in presence and absence of glycinamide	0.75±0.03	0.565±0.023
Ratio of rates of formation of BTGA/BT from amide	0.33±0.05	0.77 ± 0.07

RESULTS

The results presented (Table 1) are the means and their standard deviations calculated from five to ten runs made on at least three separate days using freshly prepared solutions. The rate of BTGA hydrolysis in water is 0.02 μmoles$(min)^{-1}(mg\ chymotrypsin)^{-1}$. The results were not affected by a change in the enzyme concentration using the range 0.05 to 0.3 mg/ml for the experiments with the ester and 0.2 to 1.3 mg/ml for the amide. The results in glycineamide solution were independent of the presence of Tris buffer.

DISCUSSION

The results in Table 1 clearly demonstrate that the ratio of the rates of α-chymotrypsin catalyzed reactions of an ester and an amide with glycinamide or water varies by about two fold. Since BTGA is much more slowly hydrolyzed than BTEE, enzyme acylation would be rate controlling for BTGA hydrolysis and thus these results are in direct disagreement with an obligatory formation of an acyl enzyme from amide substrates.

Several *ad hoc* explanations can be thought of within the framework of the acyl enzyme mechanism, however none of them are too convincing. One explanation is that the enzyme preparation is not completely homogeneous and if different molecular species were to preferentially attack the ester or the amide, differences in the ratio of rates could be observed. This explanation requires that the enzymes have different affinities for two similar substrates, whose binding features reside mainly in the benzoyl-L-tyrosine portion which is identical for both substrates. In addition, BTEE has been shown to be a specific substrate for chymotrypsin (16). A similar argument could be raised suggesting that α-chymotrypsin exists in more than one conformation. This would require that the rate of a chemical reaction, the deacylation of acyl chymotrypsin, be faster than the rate of change of conformation. One could also postulate a conformational change induced by glycinamide. However the low concentration of glycinamide used and the linear dependence of the ratio of rates on glycinamide concentration make this alternative unlikely. Finally one could suggest that glycinamide can bind to the acyl enzyme and accelerate the hydrolysis of the acyl enzyme. Such acceleration has been found on the binding of indole to acetyl chymotrypsin (17). However there is no precedent for such an effect with aromatic substrates or with non-aromatic activators. The fact that hydroxylamine (7) and alcohols (8) can be used as added nucleophiles in the

chymotrypsin catalyzed reactions with a series of esters and give precise agreement with the predictions of the acyl enzyme theory make the above alternative explanations even less likely. We are thus left with the conclusion that amides do not have to pass through an acyl enzyme intermediate when hydrolyzed by chymotrypsin.

Acknowledgements: The author wishes to express his gratitude to Drs. L.F.Leloir and R.Piras for making available space and materials to carry out this research and to all of the members of the Institute for helpful discussions.

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