THE KINETICS OF THE AMIDASE AND ESTERASE ACTIVITIES OF TRYPSIN

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In previous reports from this laboratory investigations of the specific esterase activity of trypsin (1) and of the detailed kinetics of the hydrolysis of specific substrates by chymotrypsin and by carboxypeptidase have been reported (2, 3). In the present paper a reinvestigation of the kinetics of the action of trypsin on benzoyl-L-argininamide and the results of kinetic studies of the action of trypsin on a series of esters of benzoylarginine are presented.

EXPERIMENTAL

Enzyme—The trypsin used for this study (Tv) was prepared according to the procedures of Kunitz and Northrop (4) and of McDonald and Kunitz (5). The enzyme was recrystallized one time.

Substrates and Inhibitor—The preparation of α-benzoyl-L-argininamide has been described (1). α-Benzoyl-L-argininamide (BAA) and the methyl, ethyl, and isopropyl esters of α-benzoyl-L-arginine (BAME, BAEE, and BAIPE) were prepared according to the method of Bergmann, Fruton, and Pollok (6). The procedure of these authors was modified for the preparation of the cyclohexyl and benzyl esters of α-benzoyl-L-arginine (BACHE and BABE) in that the esterification was carried out only one time and the reaction mixture was heated on a boiling water bath for 3 hours. The reaction mixtures in all cases were concentrated under reduced pressure and ether was added to precipitate the esters as oils. BAEE, BAIPE, BACHE, and BABE crystallized on standing under ether in a cold box at −20°. These esters were recrystallized by dissolving them in the corresponding alcohol and adding ether to turbidity. As previously reported (1, 6), BAME could not be crystallized.

The analytical results for these materials are shown in Table I.

Methods—The methods for determining the amidase and esterase activity of trypsin have been described (1). The only modification in these procedures was the use of a small mechanical stirrer in place of hand stirring or stirring by nitrogen bubbles in the determination of esterase activity.

Enzyme concentrations were determined by the semimicro-Kjeldahl method.
Results

Action of Trypsin on Benzoylargininamide—Hofmann and Bergmann (7), Butler (8), and Schwert, Neurath, Kaufman, and Snoke (1) have reported that the hydrolysis of benzoylargininamide by trypsin follows first order kinetics. This result is surprising, since it has been generally found, in agreement with the concept established by Michaelis and Menten (9), that adherence to first order kinetics in enzyme systems is apparent rather than real. In recent studies from this laboratory it has been pointed out that the kinetics of enzymatic reactions can be generally treated according to the integrated Michaelis-Menten equation (2, 3).

Accordingly, the hydrolysis of benzoylargininamide by trypsin was reinvestigated. In Fig. 1 are shown the results obtained over a range of substrate concentration from 0.005 to 0.075 M plotted according to zero order kinetics. In this range of substrate concentration the initial reaction course is the same for all substrate concentrations used. This observation suggested that $K_m$ for this system is so much less than the lowest substrate concentration studied that the reaction follows zero order kinetics throughout this range of substrate concentrations and that deviations from zero order kinetics were caused by inhibition of the enzyme by one of the reaction products. The data shown in Fig. 1 could not be resolved by the Lineweaver and Burk equation for competitive inhibition (10), since the value of the ratio $K_m/K_I$ increased as the reaction progressed.1

1 $K_m$ is the Michaelis constant and is defined by $K_m = (k_r + k_t)/k_i$ where $k_i$ is the rate constant for the reaction of enzyme and substrate to form the enzyme-substrate complex, $k_r$ is the rate constant for the reverse reaction, and $k_t$ is the rate constant for the slowest reaction between the enzyme-substrate complex and the ultimate formation of free enzyme and reaction products. $K_I$ is the enzyme-inhibitor dissociation constant.
Since the addition of an equimolar quantity of ammonium chloride to the substrate resulted in no diminution of the reaction rate, it was concluded that inhibition must be due to benzoylarginine.²

Although the true solubility of benzoylarginine is very low, it was observed during the recrystallization of this substance that stable supersaturated solutions could be easily prepared by dissolving benzoylarginine at high temperatures and allowing the solution to cool. The small tendency of benzoylarginine to crystallize from supersaturated solutions probably accounts for the fact that enzymatic reaction solutions remain homogeneous even though the amount of benzoylarginine formed by the hydrolysis of a soluble derivative is much greater than the true solubility of this substance.

This observation was utilized in making the determinations shown in Fig. 2. It is apparent from this plot that in low concentrations of benzoylarginine the inhibition of the hydrolysis of BAA by trypsin is almost

² This result has been independently reached by Harmon and Niemann (11). We are indebted to these authors for access to these data prior to their publication.
entirely non-competitive and that the competitive nature of the inhibition is increased by increasing the concentration of benzoylarginine. Inhibition of this indeterminate type has been previously reported by Elkins-Kaufman and Neurath (12) for the action of butyric acid and chloroacetic acid on the carboxypeptidase-carbobenzoxyglycyl-L-phenylalanine system.

Action of Trypsin on Esters of Benzoylarginine—Although it has been observed (1) that the hydrolysis of BAME by trypsin follows zero order kinetics, no attempt has been made to determine whether the range of concentrations in which deviations from zero order kinetics occur is experimentally attainable. With the present method of determining esterase activity, concentrations of substrate below about $5 \times 10^{-4}$ M cannot be used conveniently. Fig. 3 shows the course of the reaction over the concentration range from 0.0007 to 0.03 M. Within this range the reaction apparently follows zero order kinetics through a very large part of the reaction course. From the concentration of substrate remaining when deviations from zero order kinetics occur, it can be estimated that $K_m$ must be smaller than $8 \times 10^{-5}$ M. This very low value of $K_m$, together with the relative inefficiency of benzoylarginine as an inhibitor in dilute solutions, must account for the difference in apparent order between the enzymatic hydrolysis of BAA and that of BAME.

In order to evaluate the effect of the size and nature of the alcohol group on the tryptic hydrolysis of esters of benzoylarginine, rate determinations

![Fig. 3. Hydrolysis of BAME by trypsin at 30°. 0.0334 mg. of trypsin N represents the amount of trypsin used for each determination. 2 ml. of 0.1 M phosphate buffer, pH 7.8, were added to each reaction solution. pH 8.00 was the null point for these measurements. The concentration of BAME and the volume of the reaction solution are shown by O, 0.0007 M, 100 ml.; ●, 0.003 M, 25 ml.; △, 0.03 M, 5 ml.](image-url)
with BAME, BAEE, BAIPE, BACHE, and BABE were made at identical enzyme and substrate concentrations. Fig. 4 shows the results of these determinations at two temperatures. It is apparent that replacement of the methyl group of BAME by a variety of alcohol groups results in no significant change in the hydrolysis rate.

A series of determinations at varying substrate concentrations was made with each ester to determine whether the kinetics of the hydrolysis of any of the esters studied could be characterized by a measurable $K_m$ value. In all cases the results were identical with those shown in Fig. 3 for BAME.

**Fig. 4.** Hydrolysis of esters of benzoylarginine by trypsin at 25° and at 3.3°. The volume of the reaction solution was 10 ml.; ester concentration 0.0075 M. 0.0227 mg. of trypsin nitrogen present in each reaction solution. The null point for these determinations was pH 8.00. For clarity many points have been omitted. The esters are designated by  O BAME,  • BAEE,  △ BAIPE,  ▲ BACHE,  □ BABE.

It is generally assumed that enzymatic reactions occur in two steps, a combination between enzyme and substrate and the subsequent activation and hydrolysis of the substrate. When zero order kinetics are found, it is usually postulated that the combination step is so rapid as compared to any subsequent process that the enzyme is always saturated with substrate. Since $K_m$ for the esters of benzoylarginine is so small that the two steps in the reaction cannot be distinguished experimentally, it was

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2 It is of interest that the $\alpha$-monoglyceride of benzoylarginine, a difficultly characterized oil, prepared by the reaction of the silver salt of benzoylarginine with $\alpha$-glycerol monochlorohydrin, is also hydrolyzed at a rate identical with that of the other esters.
reasoned that altering the character of the solvent might so alter the rates of the two processes that they could be measured separately. Although this expectation was not realized, the results obtained are of interest.

When the enzymatic hydrolysis of BAME is carried out in ethanolic solutions, the initial reaction rate, up to a limiting concentration of alcohol, is greater than that observed in water. In 16 volume per cent ethanol the initial rate is increased about 35 per cent over that in water, and in 32 volume per cent ethanol the increase is about 50 per cent. At an ethanol concentration of 54 volumes per cent the initial rate is identical with that in water. As the reaction in ethanolic solutions progresses, however, the rate decreases, the decrease being greater the higher the ethanol concentration. Analogous, but less marked, changes in rate were observed when BAA was acted upon by trypsin in ethanolic solutions. These observations are in agreement with the findings of Risley, Buffington, and Arnow (13) for the action of trypsin on bovine serum.

The failure of the esterase reaction to follow zero order kinetics in alcoholic solutions suggested that the enzyme might be inhibited by one of the reaction products in such solutions. Determinations in water and in 16 volume per cent ethanol indicated that the addition of 0.01 M benzoylarginine or of 0.01 M ammonium chloride causes no change in the rate of hydrolysis of 0.01 M BAME. It was also observed that 0.01 M benzoylarginine causes no inhibition in 50 per cent ethanol. Neither 0.01 M arginine hydrochloride nor 0.01 M guanidine hydrochloride causes any rate change in 16 per cent ethanol.

When the rate of hydrolysis of BAME and of BAEE by trypsin was studied in a series of alcohols at the 16 volume per cent level, it was found that methanol causes a 15 per cent increase in rate, ethanol causes a 35 per cent increase, and n-propanol and tert-butanol cause a rate increase of about 40 per cent over that observed in water. No change in reaction rate was observed when the reaction was studied in 0.1 M glycine.

It has been suggested that the esterase activity of the proteases might be distinguished from the activity of the true esterases by the effect of fluoride upon this activity. Dry trypsin powder was dissolved in 0.5 M sodium fluoride solution and, after 15 minutes, a portion of this solution was added to BAME in the usual buffer. The concentration of fluoride in the reaction solution was 0.025 M. A rate identical with that observed in the absence of fluoride was found.

4 The possibility that the acceleration observed in the initial stages of the reaction in 16 and 32 volume per cent ethanol might be due to traces of some activating material was ruled out by using carefully redistilled absolute alcohol for comparative rate studies. The observed rate was independent of the source of the alcohol used.

6 By Dr. Frederick Bernheim.
DISCUSSION

The data presented in Figs. 1 and 2 show clearly that the hydrolysis of benzoyl-L-argininamide by trypsin cannot be interpreted by a single order of reaction, since one of the reaction products, benzoylarginine, exerts an inhibitory effect on the reaction. This inhibition is of an indeterminate type. It is initially non-competitive, but additional increments of benzoylarginine formed during the reaction cause disproportionately greater inhibition of the enzyme.

These results are in essential agreement with those of Harmon and Niemann (11) which have recently come to our attention. According to their data a value of $K_m = 0.0021$ fits the kinetics of the tryptic hydrolysis of BAA at 25° reasonably well provided values of $K_m/K_I$ varying between 1 and 2 are assumed, the higher value being required to approximate the reaction course, on the basis of competitive inhibition, at higher initial substrate concentrations.

Although the esters of benzoylarginine used for this study probably show some variation in their resistance to non-enzymatic hydrolysis, they are hydrolyzed by trypsin at identical rates. As enzymatic reactions are usually formulated, the possible limiting rates for a zero order reaction are (1) the rate of activation of the enzyme-substrate complex, (2) the rate of entry of water into the hydrolytic process, and (3) the rate of desorption of the products from the enzyme surface. The third of these possibilities is rendered improbable by the observation that only benzoylarginine, a product formed in the hydrolysis of both BAA and of the esters studied here, has been found to have inhibitory activity. Were the rate of desorption of the products the limiting step, the rates of hydrolysis of esters and amides should be identical. The other two possibilities can be distinguished only formally and may occur as one process.

If it is true that the rate being measured in the hydrolysis of esters by trypsin is the rate of activation of the enzyme-substrate complex, then it must be this rate which is increased by the addition of alcohols to the system. It is of interest that for the chymotrypsin-acetyl tyrosinamide system the addition of methanol causes no change in the rate of activation of the enzyme-substrate complex.6

The results obtained here at two temperatures for the five esters of benzoylarginine agree well with those previously reported (1) for BAME at three temperatures. Thus, over the range 0.5–42°, $\Delta E$, the Arrhenius activation energy for the rate-limiting step, is 11,200 calories per mole. The absolute reaction rate (moles of ester hydrolyzed per mole of enzyme per second) is 26.7 reciprocal seconds at 25°. With use of the theory of

7 The molecular weight of trypsin is assumed to be 36,000.
absolute reaction rates (14), $\Delta H^* = 10,600$ calories per mole, $\Delta F^* = 15,500$ calories per mole, and $\Delta S^* = -16.5$ entropy units. These values are of the same order as those previously reported for the hydrolysis of specific esters by chymotrypsin (2).

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SUMMARY

It has been found that benzoyl-L-arginine inhibits the tryptic hydrolysis of benzoyl-L-argininamide. This inhibition is of indeterminate type, being almost non-competitive in low concentrations of benzoyl-L-arginine and approaching competitive inhibition with increasing concentrations of this reaction product.

The methyl, ethyl, isopropyl, benzyl, and cyclohexyl esters of benzoyl-arginine are hydrolyzed at identical rates by trypsin. These reactions follow zero order kinetics and none is inhibited by benzoylarginine.

The effect of added alcohols, fluoride, arginine hydrochloride, and guanidine hydrochloride upon the rate of hydrolysis of benzoyl-L-arginine methyl ester by trypsin has been determined.

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