Eur. J. Biochem. 42, 237–243 (1974)

A Kinetic Study of the Deacylation of α-Benzamido-*trans*-cinnamoyl-chymotrypsin

Evidence for the Intervention of Non-Enzymic Species

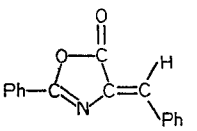
John DE JERSEY, Dianne T. KEOUGH, James K. STOOPS, and Burt ZERNER

Department of Biochemistry, University of Queensland, St. Lucia

(Received July 30/October 22, 1973)

The apparent first-order rate constant (k_{obs}) for the deacylation of α-benzamido-*trans*-cinnamoyl-chymotrypsin, determined by direct observation at 310 nm, pH 7.9 ($k_{obs} = 0.102\ s^{-1}$; K. Brockelhurst and K. Williamson [1967] *Chem. Commun.*, 666), differs from k_{cat} determined under the same conditions with $[S]_0 \gg [E]_0$ ($k_{cat} = 0.033\ s^{-1}$). This result is contrary to other evidence indicating that k_{cat} is a measure of the deacylation rate constant. The discrepancy has been shown to be due to the presence in commercial samples of α-chymotrypsin of impurities which catalyse the deacylation reaction under the conditions used for the determination of k_{obs} ($[E]_0 > [S]_0$). The impurities, which may be removed from enzyme solutions by gel filtration on Sephadex G-25 or G-50, have been shown to be autolysis products (presumably peptides) of α-chymotrypsin. Catalysis of deacylation results from attack of the free α-amino groups of these peptides on the acyl-enzyme, to give *N*-α-benzamido-*trans*-cinnamoyl-peptides. The consequences of these findings must be considered in interpreting any experiment in which deacylation is observed directly at high enzyme concentration, with $[E]_0 > [S]_0$. Particularly susceptible to error are experiments designed to determine the effect of pH on the deacylation rate constant, and literature results of some such experiments are critically assessed.

Oxazolinones were originally introduced into enzymology as activated derivatives of *N*-acylamino acids to simplify the preparation of acyl-enzymes and to allow a simple determination of deacylation rate constants [1]. The reaction of 4-*trans*-benzylidene-2-phenyloxazolin-5-one (I) with α-chymotrypsin



(I)

was initially studied to allow the determination of the deacylation rate constant for α-benzamido-*trans*-cinnamoyl-chymotrypsin [2,3]. The α-benzamido-cinnamoyl group contains the β-aromatic substituent and the α-acylamino group characteristic of good substrates for α-chymotrypsin [4], but the spatial arrangement of these groups with respect to the serine ester bond in the acyl-enzyme is restricted by the double bond.

Enzyme. α-Chymotrypsin (EC 3.4.4.5).

Several papers have appeared describing this reaction [2,3,5–7], but a number of discrepancies remain in the data which have been reported. For example, when the reaction of 4-*trans*-benzylidene-2-phenyloxazolin-5-one with α-chymotrypsin is followed under Michaelis-Menten conditions ($[S]_0 \gg [E]_0$) by observing the disappearance of the oxazolinone at 363 nm [3,6,7], $k_{cat} = 0.033\ s^{-1}$ and $K_m = 0.4\ \mu M$ (0.1 M phosphate buffer, 10% v/v acetonitrile, pH 7.93, 25 °C). The observation of a quantitative "burst" at pH 5.34, together with the very low value of K_m, strongly supports the conclusion that $k_{cat} = k_{+3}$ where k_{+3} is the deacylation rate constant [6]. However, conflicting results were obtained by Brocklehurst and Williamson [5] when the "deacylation" reaction was observed directly by the addition of *trans*-benzylidene-2-phenyloxazolin-5-one to an excess of enzyme, the reaction being followed by a decrease in absorbance at 310 nm. The apparent first-order rate constant, k_{obs}, was found to show a sigmoidal pH-rate profile with $k = 0.154\ s^{-1}$ and $pK_a' = 7.64$. Thus, at pH 7.93, the calculated value of k_{obs} is $0.102\ s^{-1}$, and since both k_{cat} and k_{obs} are purported to equal k_{+3}, a factor of 3 exists between

the different estimates of k_{+3}. Brocklehurst [7] has proposed a kinetic scheme to explain the discrepancy, which involves the reversible formation of a second covalent enzyme-substrate species.

We now report results which confirm our earlier conclusion that $k_{cat} = k_{+3} = 0.033\ s^{-1}$ at pH 7.93, and which suggest that the results of Brocklehurst and Williamson [5,7] have a simpler explanation. Further, these new results once again indicate the caution which is necessary in interpreting the results of enzymatic experiments (*cf.* [3]).

EXPERIMENTAL PROCEDURE

Materials

4-*trans*-Benzylidene-2-phenyloxazolin-5-one was prepared by the reaction of benzaldehyde with hippuric acid in the presence of acetic anhydride, and recrystallized from benzene, m.p. 165—166 °C, literature m.p. 167 °C [9]. The isomer of α-benzamido-cinnamic acid with m.p. 230 °C has been shown to have the *trans*-configuration by X-ray crystallography [8]. (It should be noted that the m.p. of this acid was listed incorrectly as 199 °C, K. Brocklehurst, personal communication). Therefore, the corresponding oxazolinone (m.p. 167 °C) may be assumed to be the *trans*-isomer.

4-*trans*-[2-^{14}C]Benzylidene-2-phenyloxazolin-5-one and 4-*trans*-[5-^{14}C]benzylidene-2-phenyloxazolin-5-one were prepared [9] using 0.5 mCi [1-^{14}C]benzoic acid and 0.5 mCi [1-^{14}C]glycine, respectively (Radiochemical Centre, Amersham). Both compounds had m.p. 165—166 °C. That labelled at C-2 had a specific activity of 0.195 mCi/mmol and that labelled at C-5 had a specific activity of 0.124 mCi/mmol.

N-Benzoyl-L-tyrosine ethyl ester was prepared from L-tyrosine ethyl ester hydrochloride [10] and recrystallized from chloroform-petroleum ether, m.p. 121—122 °C, literature m.p. 120—121 °C [11].

α-Chymotrypsin (three-times crystallized) was obtained from Sigma and Miles Seravac (Grade 2A, batch 385A). *p*-Nitrophenyl acetate was obtained from the Aldrich Chemical Co. and recrystallized from chloroform-hexane. Acetonitrile (Eastman Organic Chemicals, Spectro Grade) was used without further purification.

Buffers were prepared using analytical grade reagents. Measurements of pH were made on a Radiometer pH meter 4, standardized according to Bates [12], and are accurate to ± 0.01 pH units. Chymotrypsin solutions were prepared by dissolving a weighed amount of enzyme in buffer just before use, followed by centrifugation to remove cloudiness if necessary.

Methods

All kinetic data were obtained spectrophotometrically using a Cary 14 recording spectrophotometer equipped with 0—0.1 and 0—1.0 absorbance slide wires, and a cell compartment maintained at 25 °C. In a typical experiment, an aliquot (usually 25 or 50 μl) of 4-*trans*-benzylidene-2-phenyloxazolin-5-one in acetonitrile was added to 3 ml of enzyme solution equilibrated at 25 ± 0.1 °C ($[E]_0 > [S]_0$). The decrease in absorbance at 310 nm was followed, and thus, apparent first-order rate constants (k_{obs}) for the deacylation reaction were obtained. To minimize evaporation, cells were covered with close-fitting Teflon lids. Under these conditions, evaporation was found to make a negligible contribution to the absorbance change at 310 nm.

Enzyme concentrations were determined at the beginning and end of each series of kinetic runs by titration with *p*-nitrophenyl acetate (1.04 mM) in 0.1 M phosphate buffer containing 9% (v/v) acetonitrile, pH 7.88 [13]. Decrease in enzyme concentration during a series of runs was at most 3%. The apparent molar absorption coefficient of *p*-nitrophenol at 400 nm was determined during each series of experiments using a standard solution of *p*-nitrophenol. The hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester by α-chymotrypsin was followed at 256 nm [14] in 0.1 M phosphate buffer, pH 7.88, where $\Delta\varepsilon = 880\ M^{-1} \cdot cm^{-1}$. Reactions were followed to completion ($[E]_0 \approx 0.037\ \mu M$), and k_{cat} and K_m determined using Henri plots and the method of least squares. Changing $[S]_0$ from 81.8 μM to 327 μM produced no change in k_{cat} and K_m.

Scintillation counting was carried out in Insta-Gel (Packard) using a Beckman LS 250 liquid scintillation system. Counting efficiency was determined by the use of [^{14}C]toluene as internal standard.

Gel filtration was performed at 25 °C on columns of Sephadex G-25 and G-50 (Pharmacia), equilibrated with 0.1 M phosphate buffer, 9% (v/v) acetonitrile, pH 7.88. Dimensions of Sephadex G-25 columns are given in the text. The Sephadex G-50 column was 34 × 2.5 cm.

RESULTS

Effect of Enzyme and Substrate Concentrations on the Deacylation Reaction

The effect of enzyme concentration in the range 2.1—230 μM on k_{obs}, the first-order rate constant for the decrease in absorbance at 310 nm, is shown in Fig. 1. The effect of the initial concentration of 4-*trans*-benzylidene-2-phenyloxazolin-5-one on k_{obs} at fixed enzyme concentration ($[E]_0 > [S]_0$) is given in Table 1.

Preparation of "Modified" Enzyme

The data of Fig. 1 are consistent with the reaction of a molecule of acyl-enzyme with a second enzyme molecule, in which the α-benzamido-*trans*-

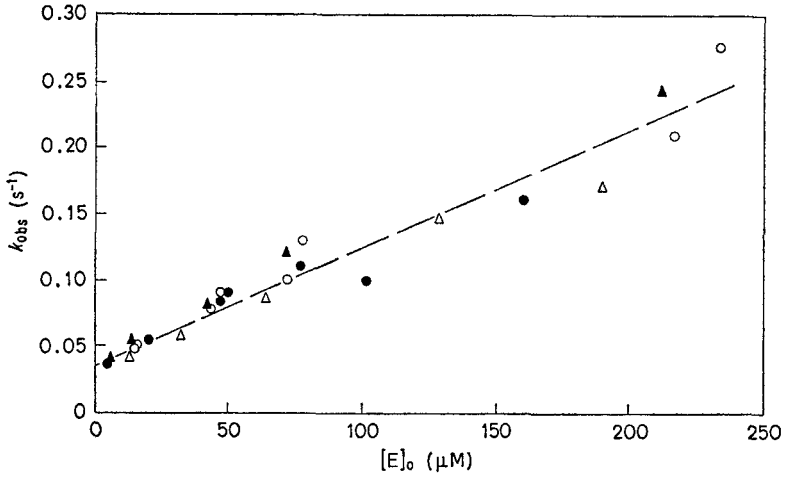


Fig. 1. *Effect of enzyme concentration on* k_{obs} *for the deacylation of α-benzamido-*trans*-cinnamoyl-chymotrypsin in 0.1 M phosphate buffer, 10.5% (v/v) acetonitrile, pH 7.90.* Conditions: (○) $[S]_0 = 10.69$ μM, Sigma enzyme; (△) $[S]_0 = 10.35$ μM, Miles Seravac enzyme; (●) $[S]_0 = 10$ μM, Sigma enzyme; (▲) $[S]_0 = 10$ μM, Miles Seravac enzyme

Table 1. *Effect of substrate concentration on* k_{obs} *for the decrease in absorbance at 310 nm*
The reaction was performed in 0.1 M phosphate buffer, pH 7.88, 10.5% (v/v) acetonitrile, at 25 °C. Substrate = 4-*trans*-benzylidene-2-phenyloxazolin-5-one

E_0	S_0	k_{obs}
μM		s^{-1}
77.1	70.0	0.11
77.1	50.0	0.10
77.1	20.0	0.11

cinnamoyl group is transferred (in whole or in part) from the active-site serine of the first molecule to a different site on the second molecule. On this basis, repeated addition of 4-*trans*-benzylidene-2-phenyloxazolin-5-one to an enzyme solution should result in the conversion of all of the enzyme to the modified form, provided that this form was sufficiently stable under the conditions of the experiment. Four 50-μl aliquots of the oxazolinone were added to 3 ml of enzyme solution at intervals of 4 min; the solution contained 0.1 M phosphate buffer, 9% (v/v) acetonitrile, pH 7.88; $[E]_0 = 14.9$ μM; $[S]_0$ after each addition ≈ 10 μM. Titration with *p*-nitrophenyl acetate showed that repeated reaction with the oxazolinone as described had no effect on the concentration of active enzyme molecules. The reaction mixture was then passed through a column of Sephadex G-25 (20 × 1.5 cm). The enzyme solution so obtained was titrated with *p*-nitrophenyl acetate, and then reacted with the oxazolinone, with $[E]_0 > [S]_0$. Variation of enzyme concentration from 4.2–15.6 μM gave $k_{obs} = 0.033 \pm 0.001$ s^{-1} (9 determinations, $[S]_0 = 1.56$ μM).

*Attempts to Label α-Chymotrypsin by Reaction with 4-*trans*-[^{14}C]Benzylidene-2-phenyloxazolin-5-one*

In view of the foregoing results, attempts were made to label the enzyme with the oxazolinone containing ^{14}C at C-2 or C-5. As shown in Fig. 2, the enzyme was not significantly labelled (<10% of a stoichiometric amount) when the (2-^{14}C)-labelled oxazolinone was the substrate. Exactly similar results were obtained with the (5-^{14}C)-labelled compound.

Effect of Gel Filtration on α-Chymotrypsin

The possible presence of a low-molecular-weight impurity in commercial samples of α-chymotrypsin was checked by gel filtration on Sephadex G-25 and G-50. As shown in Fig. 3, gel filtration produces a dramatic decrease in the effect of enzyme concentration on k_{obs} for two different enzyme samples. Further, the effect is reduced to zero when the enzyme is treated with 4-*trans*-benzylidene-2-phenyloxazolin-5-one prior to gel filtration. Study of the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester by the three samples of α-chymotrypsin used in Fig. 3 (A) gave the values listed below for the steady-state kinetic constants: untreated enzyme (k_{cat}, 72.0 $\pm$ 0.5 s^{-1}; K_m, 17.6 $\pm$ 0.8 μM); enzyme gel-filtered on Sephadex G-25 (75.5 $\pm$ 0.4 s^{-1}; 19.9 $\pm$ 0.5 μM); enzyme treated with 4-*trans*-benzylidene-2-phenyl-

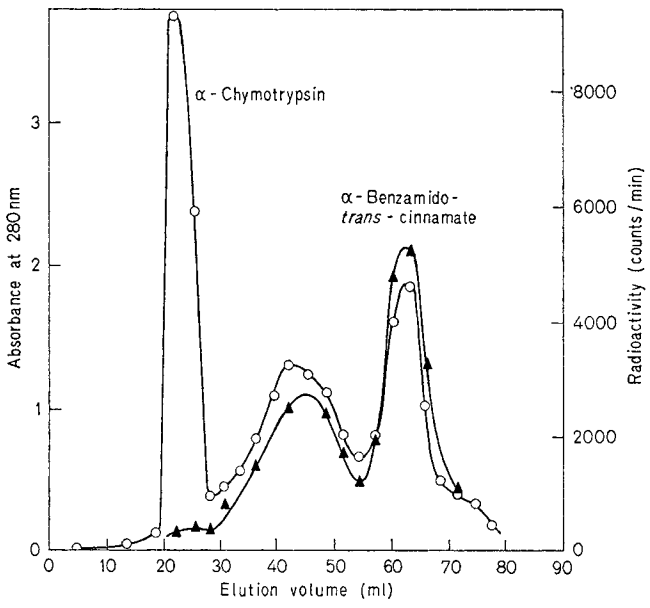


Fig. 2. *Chromatography on Sephadex G-25 of α-chymotrypsin previously reacted with 4-trans-[2-^{14}C]benzylidene-2-phenyloxazolin-5-one.* Ten 25-μl aliquots of (2-^{14}C)-labelled oxazolinone (10.97 mM in CH_3CN) were added at 3-min intervals to 3 ml of α-chymotrypsin (Sigma; 185 μM in 0.1 M phosphate buffer, 9% v/v CH_3CN, pH 7.90). The reaction mixture was then passed through a Sephadex G-25 column (21 × 1.7 cm); 3-ml fractions were collected: (○) absorbance at 280 nm; (▲) radioactivity of 200-μl aliquot

oxazolin-5-one, then gel-filtered ($74.5 \pm 0.4\ s^{-1}$; 20.9 ± 0.7 μM).

A sample of α-chymotrypsin (9 ml of 6 mg/ml) was subjected to gel filtration on Sephadex G-50. A 3-ml portion of the resulting enzyme solution (68.5 μM) was treated repeatedly with 20-μl aliquots of 4-*trans*-[2-^{14}C]benzylidene-2-phenyloxazolin-5-one and then rechromatographed on Sephadex G-25. A 3-ml portion of unchromatographed enzyme (71.7 μM) was treated with the radioactive oxazolinone and chromatographed in the same way. Fig. 4 shows the resulting plots of absorbance at 280 nm and radioactivity *vs* elution volume.

Effect of Autolysis on k_{obs}

Enzyme which had been chromatographed on a Sephadex G-25 column (35 × 2.3 cm), and which initially had a concentration of 89.1 μM showed a k_{obs} of $0.059_6\ s^{-1}$ ($[oxazolinone]_0 = 8.22$ μM). After 280 min at 25 °C, k_{obs} was $0.099\ s^{-1}$; after 20 h, k_{obs} had increased to $0.167\ s^{-1}$, and [E] had fallen to 65.3 μM.

DISCUSSION

The present results are in direct contrast to the observations of Brocklehurst [7]: "with $[S]_0 = 10$ μM the same value of k_{+3} at a given pH is obtained using

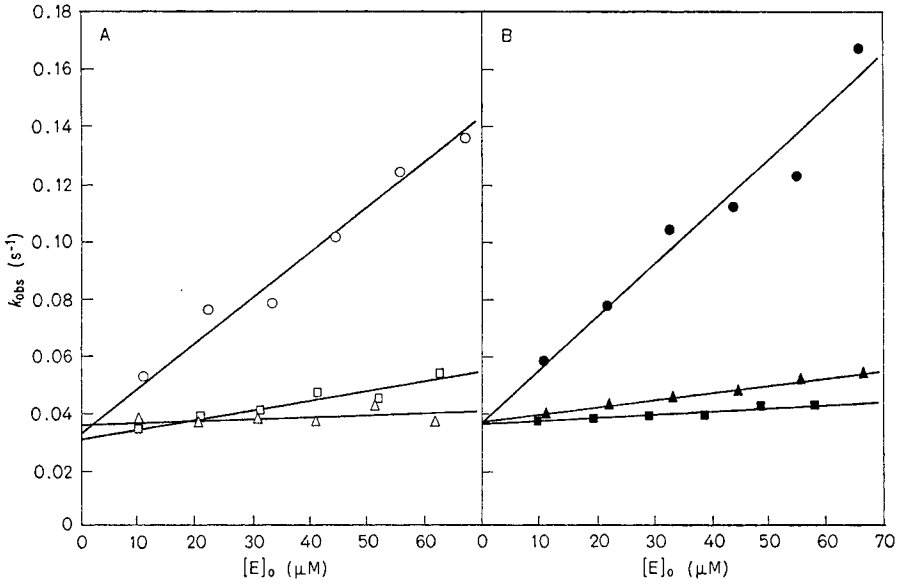


Fig. 3. *Effect of prior gel filtration on the dependence of* k_{obs} *on enzyme concentration in 0.1 M phosphate buffer, 9.7% (v/v) acetonitrile, pH 7.90.* Experiments in (A) and (B) were performed on different samples of Sigma enzyme. (A) $[S]_0 = 9.78$ μM; (○) untreated enzyme; (△) 9 ml enzyme (137 μM) was pretreated with ten 75-μl aliquots of 4-*trans*-benzylidene-2-phenyloxazolin-5-one (11.8 mM in CH_3CN) at 200-s intervals, then chromatographed on Sephadex G-25 (35 × 2.3 cm); (□) as above, but using CH_3CN without the oxazolinone. (B) $[S]_0 = 8.22$ μM; (●) untreated enzyme; (▲) 9 ml of 143 μM enzyme chromatographed on Sephadex G-25 (35 × 2.3 cm); (■) 9 ml of 143 μM enzyme chromatographed on Sephadex G-50

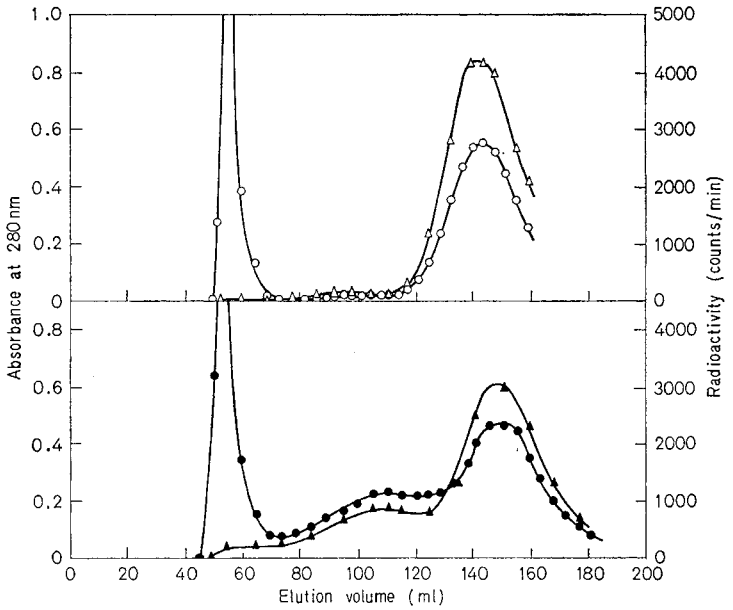


Fig. 4. *Effect of prior chromatography on Sephadex G-50 on the product of the reaction of α-chymotrypsin (Sigma) with 4-*trans*-[2-^{14}C]benzylidene-2-phenyloxazolin-5-one.* Ten 20-μl aliquots of (2-^{14}C)labelled oxazolinone (8.99 mM) were added to 3 ml of chymotrypsin solutions at 200-s intervals. Reaction mixtures were then chromatographed on a column of Sephadex G-25 (35×2.3 cm); (○, △) absorbance at 280 nm and radioactivity of a 400-μl aliquot, enzyme previously chromatographed on Sephadex G-50; (●, ▲) absorbance and radioactivity of a 400-μl aliquot, untreated enzyme

different enzyme concentrations in the range 50 to 500 μM". From Fig. 1, it can be seen that k_{+3}, determined under the same conditions and in the same manner, increases considerably with increase in enzyme concentration in the same range. Different samples of enzyme from two suppliers gave similar results. While the reason for the conflict between our results and those of Brocklehurst is not immediately apparent, it is possible that in their earlier report [5], they used a sample of enzyme which was extensively autolysed, whereas in the latter report [7], the enzyme sample was remarkably free of autolysis products (see below).

The data of Fig. 1 are consistent with the equation:

$$k_{obs} = k_{+3} + k_1 [E]_0$$

where k_{+3} is the deacylation rate constant for α-benzamido-*trans*-cinnamoyl-chymotrypsin. The value of k_{obs} extrapolated to zero enzyme concentration is 0.033 s^{-1}, identical with the value obtained [3, 6, 7] for k_{cat} under the same conditions of pH *etc.* with $[S]_0 \gg [E]_0$. The data of Table 1 show that the variation of $[S]_0$ has no effect on k_{obs} at fixed enzyme concentration ($[E]_0 > [S]_0$).

It seemed possible that α-chymotrypsin was being chemically modified in its reaction with 4-*trans*-benzylidene-2-phenyloxazolin-5-one, and tyrosine-146 appeared to be a likely acceptor of the acyl group [15–17]. Fig. 2 shows not only that the enzyme is not labelled in the reaction with the ^{14}C-labelled oxazolinone, but also that a new radioactive compound is produced in addition to α-benzamido-*trans*-cinnamic acid. The elution profile argues that there may well be more than one new radioactive species.

From Fig. 3 it is clear that the anomalous effect of enzyme concentration on k_{obs} is due to an impurity in commercial samples of α-chymotrypsin (*cf.* [18, 19]) Further, the product of the reaction of the impurity with the acyl-enzyme is readily separated from the enzyme.

While k_{obs} is very sensitive to the concentration of the impurity (Fig. 1), the kinetic parameters of the specific substrate *N*-benzoyl-L-tyrosine ethyl ester are scarcely affected by removal of the impurity because of the low enzyme concentration used.

The results shown in Fig. 4 demonstrate unequivocally that the new radioactivespecies are produced by reaction of the acyl-enzyme (α-benzamido-*trans*-cinnamoyl-chymotrypsin) with impurities in commercial α-chymotrypsin, and that the increase in k_{obs} is due to this reaction.

Autolysis of α-chymotrypsin has a big effect on k_{obs}. Thus, after standing a solution of α-chymotrypsin (which had been largely freed of impurity by gel filtration) for 20 h at pH 7.9, k_{obs} had increased by a factor of 2.8, while the enzyme concentration hap fallen by 27%. Hence, it seems more than probable

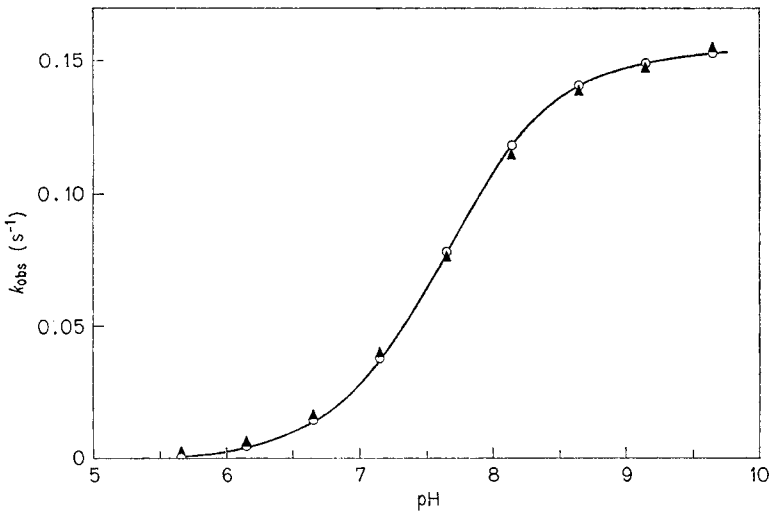


Fig. 5. *pH-Dependence of* k_{obs}. Theoretical curves calculated assuming a simple dependence of k_{obs} on a pK_a' of 7.64 (○), and according to Eqn (1) (▲) (see text)

that all of the observed effects are due to the nucleophilic attack of free α-amino groups of peptides released by autolysis on α-benzamido-*trans*-cinnamoyl-chymotrypsin, to form the corresponding amides. Further support for this explanation is provided by observations not included in the Results section, *viz.* (a) the absorption spectrum of the new products is consistent with their being amides of α-benzamido-*trans*-cinnamic acid [6], and (b) the fact that ΔA_{310} corresponding to deacylation is smaller when k_{obs} is large (*i.e.* mostly formation of amides) than when k_{obs} is small (*i.e.* formation of α-benzamido-*trans*-cinnamate), as expected from the spectra of the acid anion and amide [6].

The pK_a' of 7.64 determined by Brocklehurst and Williamson [5] for the deacylation of α-benzamido-*trans*-cinnamoyl-chymotrypsin seems anomalously high [20]. In view of the foregoing discussion the pH dependence of k_{obs} must involve both the normal pK_a' for the deacylation of an acyl-chymotrypsin (pK_D') and the pK_a' of the α-amino groups of the peptides from autolysis (pK_A'), unless the enzyme sample used is essentially free of autolysis products. A detailed analysis of the data of Brocklehust and Williamson [5] has been made according to Eqn (1):

$$k_{obs} = \frac{\bar{k}_3}{1 + \frac{[H^+]}{K_D}} + \frac{\bar{k}_2 [A]_t}{1 + \frac{[H^+]}{K_A}} . \qquad (1)$$

This allows for the catalysis of deacylation by free amine, using the values $\bar{k}_3 = 0.037\ s^{-1}$, p$K_D' = 7.15$ [6,20], p$K_A' = 7.80$, and $\bar{k}_2\ [A]_t = 0.117\ s^{-1}$ (where $\bar{k}_2$ is the second-order rate constant for catalysis of deacylation by free amine A, and $[A]_t$ is the total concentration of amine; from $\bar{k} = 0.154\ s^{-1}$ [5]). A calculated curve has been obtained which is experimentally indistinguishable from a simple pH dependence for k_{obs} with a p$K_a' = 7.64$ (Fig. 5). Moreover, this specific criticism may be applied to any direct determination of the effect of pH on the deacylation of acyl-chymotrypsins, where $[E]_0 > [S]_0$. Indeed, many data which have to this time been difficult to rationalize find a ready explanation in terms of the catalysis of deacylation by autolysis products. Specifically, Bernhard *et al.* [21] found pK_a' values of 6.70, 7.32 and 7.68 for the deacylation of 3,5-dinitrobenzoyl, cinnamoyl and β-(3-indole)acryloylderivatives of chymotrypsin. It is significant that the deacylation constants for these acyl-enzymes were reported to be 4.56 min^{-1}, 0.793 min^{-1} and 0.113 min^{-1}, respectively. The pK_a' for the deacylation of 3,5-dinitrobenzoyl-chymotrypsin should be least susceptible to perturbation by amines and that of β-(3-indole)acryloyl-chymotrypsin, most susceptible because of the variation in k_{+3}, as is found experimentally. Indeed, Bernhard's value of 7.32 for the pK_a' for the deacylation of cinnamoyl-chymotrypsin is significantly higher than that (7.15) determined by Bender *et al.* [20], and establishes the proposition that his measured values do not necessarily represent true pK_a' values for deacylation.

Finally, it is clear that while the system described in this paper is a very sensitive detector of autolysis, in many other systems the effect of autolysis products will be less obvious [6]. The only safe approach, therefore, in dealing with high concentrations of α-chymotrypsin is to free it of catalytic impurities using one or other of the procedures described in this paper.

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B. Zerner, J. de Jersey, and D. Keough, Department of Biochemistry, University of Queensland, St. Lucia, Queensland, Australia 4067

J. K. Stoops' present address: Department of Biochemistry, Baylor College of Medicine, Houston, Texas, U.S.A. 77025