BBA 66452

KINETIC PROPERTIES OF SUCCINYLATED AND ETHYLENEDIAMINE-AMIDATED δ-CHYMOTRYPSINS

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(Received June 15th 1971)

SUMMARY

Succinic anhydride reacts with 14 of the 15 amino groups of chymotrypsinogen A, giving a derivative from which succinylated δ-chymotrypsin was prepared. Upon succinylation, k_{cat}(lim) for specific ester substrates increases about 50–60%, and the apparent pK_a of the ionizing group on which k_{cat} depends, (probably histidine 57), shifts from 7.0 in the native enzyme to 8.0 in the succinylated enzyme, as expected for the excess of negative charges in the molecule. K_m(app) values are lower than those of the native enzyme and show a very small dependence on pH which can be accounted for by the ionization of an acid group (presumably the isoleucine 16 amino group) with a pK_a of 10 in the free enzyme and 10.6 in the E–S complex. The rate constant k_3(lim) for the deacylation of the indoleacryloyl-enzyme also increases about 50% upon succinylation, and a similar shift of the histidine pK_a from 8.0 to 9.0 is observed.

Direct succinylation of δ-chymotrypsin produces a rapid inactivation of the enzyme which is proportional to the disappearance of the isoleucine 16 amino group.

The kinetic properties of a derivative with an excess of positive charges were also studied. Reaction of ethylenediamine with δ-chymotrypsin in the presence of a water-soluble carbodiimide resulted in the amidation of 13 carboxyl groups of the enzyme. k_{cat}(lim) for the hydrolysis of specific esters and k_3(lim) for the deacylation of the indoleacryloyl-amidated-enzyme were the same as those of unmodified δ-chymotrypsin. Shifts in the pK_a of the histidine upon amidation, from 7.0 to 6.1 and from 7.8 to 7.1 were observed for the hydrolysis of *N*-acetyl-L-tryptophan methyl ester and deacylation of indoleacryloyl-enzymes, respectively.

In contrast to the native enzyme, K_m(app) values for the hydrolysis of *N*-acetyl-L-tryptophan methyl ester catalyzed by the ethylenediamine-amidated enzyme are strongly pH dependent. They increase sharply above pH 8.5, probably reflecting alterations in the tertiary structure of the protein as it becomes almost completely uncharged.

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INTRODUCTION

Our previous kinetic and equilibrium studies on δ-chymotrypsin-catalyzed reactions[1,2] have shown that this enzyme, in contrast to α-chymotrypsin, does not lose its ability to bind specific substrates when it is exposed to alkaline pH. K_m(app) for the δ-chymotrypsin-catalyzed hydrolysis of specific substrates[1] as well as K_i for competitive inhibitors[2] show a minor dependence on a group in the enzyme with a pK_a of 9.2–9.5, tentatively assigned as the isoleucine 16 amino group. As a consequence, in trying to explain the reversible inactivation of α-chymotrypsin at high pH, we have centered special interest not only in the amino group of isoleucine 16, but also in other groups, especially the alanine 149 amino group[1–3,6]. This group is present in α-chymotrypsin as the N-terminus of the C chain but is not present in δ-chymotrypsin where the C chain is tied to the B chain through the dipeptide threonylasparagine[4].

Continuing our studies on the relationship between the conformation and activity of chymotrypsins and the ionization state of these groups, it was desirable to prepare chymotrypsin derivatives soluble down to pH 3–4, in which the ε-amino groups of the lysine residues are blocked. The chemical modification of choice was succinylation[5] and succinylated derivatives have been used in our laboratory to study the reactivity of the amino group of isoleucine 16 in an acyl-enzyme intermediate[3] and the involvement of alanine 149 amino group in the reversible inactivation of α-chymotrypsin at alkaline pH[6].

Here we report the preparation and kinetic properties of succinylated δ-chymotrypsin. As succinylation involves the creation of an excess of negative charges in the molecule, it was of complementary interest to study the kinetic properties of an enzyme with an excess of positive charges. For this reason ethylenediamine-amidated δ-chymotrypsin was prepared and its kinetic properties compared with those of the unmodified enzyme.

MATERIALS AND METHODS

Chemicals

1-Ethyl-3-dimethylaminopropyl carbodiimide · HCl was a Cyclo Chem. Co., Los Angeles, Calif. (lot H-2633) product. Succinic anhydride was a "Baker analyzed" reagent. Dinitrofluorobenzene and standard dinitrophenyl amino acids were purchased from Calbiochem, Los Angeles, Calif. Ethylenediamine · 2 HCl, obtained from Matheson Coleman and Bell, was recrystallized two times from aqueous ethanol before use. *N-trans*-cinnamoylimidazole, recrystallized four times from hexane (m.p. 132°, lit. m.p. 133–133.5° (ref. 7)) was a gift from Dr. Y. Nakagawa. *N-trans*-indoleacryloylimidazole, a gift from Dr. F. Wedler, was used as received (m.p. 198°, lit. m.p. 199–200° (ref. 8)). *N*-Acetyl-L-tryptophanyl methyl ester was a Cyclo product, lot 3-4735 and was recrystallized twice from acetonitrile before use (m.p. 153°, lit. m.p. 152.3° (ref. 9)). *N-trans*-(2-furyl)acryloyl-L-tryotophan methyl ester was a Cyclo product and was recrystallyzed twice from acetonitrile before use (m.p. 146°, lit. m.p. 145.5–146° (ref. 10)). *N-trans*-(2-furyl)acryloyl-L-phenylalanine methyl ester was synthesized from L-phenylalanine · HCl (Mann Research Lab., New York) and furylacryloyl chloride; the crude product was recrystallized twice from ethylacetate–

hexane (m.p. 100–101°, lit. m.p. 99–100.8° (ref. 7)). *N*-CBZ-glycylglycyl-L-tryptophan methyl ester was a Cyclo Chem. Co. product, lot C-1198, and was used as received. Stock solutions of the substrates were prepared in either acetonitrile (Mallinckrodt, nanograde) or dimethyl sulfoxide (Matheson Coleman and Bell). All buffer solutions were 0.1 M ionic strength and were prepared from analytical reagent grade materials.

Enzymes

All enzymes were purchased from Worthington Biochem. Corp., Freehold, N.J. Chymotrypsinogen A was a salt-free, five-times crystallized, electrophoretically homogeneous (lot CGC 8CC) preparation. δ-Chymotrypsin was salt-free, three-times crystallized (lot CDD-6032). This enzyme contains 0.85 ± 0.1 mole of isoleucine and 0.05 mole of serine, threonine and alanine as α-amino terminal residues per mole of active enzyme. Salt-free, two-times crystallized trypsin (lot TRL-6256) was used. Stock solutions of enzymes were prepared in 0.05 M acetate buffer (pH 5.0) immediately before use.

Modified enzymes

Succinylated δ-chymotrypsin was prepared by trypsin activation of succinylated chymotrypsinogen A, obtained by the general procedure of KLOTZ[11]. Solutions of 10 mg/ml of the zymogen were adjusted to pH 8 and a 750 molar excess of succinic anhydride was added slowly with stirring. The pH was maintained at 8.0 by the addition of 5 M NaOH and the temperature was kept at 5° by circulating cold water through the water-jacketed reaction vessel. When the pH was constant for 15 min the reaction was considered complete. Then, the solution was adjusted to pH 10 and incubated with 0.2 M NH_2OH for 10 min to afford the complete deacylation of *O*-succinyl-tyrosyl residues. After this period the pH was adjusted to 5.0 and the solution extensively dialyzed against distilled water at 4°. After dialysis the solution was lyophilized. This protein was shown to have 96% of the lysine ε-amino groups succinylated, as determined by the sulphonic acid method[12]. Succinylated chymotrypsinogen A was activated to succinylated δ-chymotrypsin by incubation with 5% (w/w) of trypsin in 0.05 M acetate buffer (pH 5.5), 0.01 M in $CaCl_2$ for 10 h at 4°. Active site titrations with *N-trans*-cinnamoylimidazole[14] indicated 85–90% of activation. After dialysis trypsin was removed by chromatography on CM Sephadex C-50 which had been equilibrated with water at 4°. Succinylated proteins passed through whereas non-succinylated ones, like trypsin, remained adsorbed on the column. After chromatography the protein was lyophilized. The isolated product was shown to contain 0.73 ± 0.1 mole of isoleucine and 0.03 mole of alanine and threonine as α-amino terminal groups.

Ethylenediamine-amidated δ-chymotrypsin was obtained by reaction of δ-chymotrypsin with ethylenediamine using the water-soluble carbodiimide method of HOARE AND KOSHLAND[15,16]. A 20 mg/ml solution of enzyme was made 1 M in ethylenediamine hydrochloride and adjusted to pH 4.75. Solid 1-ethyl-3-dimethylaminopropyl carbodiimide·HCl was then added to a concentration of 0.12 M and allowed to react at room temperature. The pH was maintained at 4.75 by the addition of 2 M HCl. Identical amounts of the carbodiimide were added after 1 and 2 h. After 3 h, the reaction was quenched with an excess of 1 M acetate (pH 4.75). The pH of the solution was then lowered to 3.5 and the reagents were removed by extensive

dialysis against $5 \cdot 10^{-4}$ M HCl at 4°. The number of ethylenediamine residues incorporated into the protein was measured by the trinitrobenzene sulphonic acid method of HABEEB[12]. It was found that each mole of modified protein contained an excess of 12 amino groups over the native enzyme, indicating that 12 carboxyl groups had been modified.

Methods

Kinetic runs were carried out in a Cary 14 recording spectrophotometer equipped with a thermostatted cell compartment.

The hydrolysis of *N*-acetyl-L-tryptophan methyl ester was followed at 300 nm as described before[1]. The same procedure was used for the hydrolysis of *N*-CBZ-glycylglycyl-L-tryotophan methyl ester.

The hydrolysis of *N-trans*(2-furyl)acryloyl-L-tryptophan methyl ester was followed at 335 nm. Absorbance data were converted into rate data using $\Delta\varepsilon = 1086$ $M^{-1} \cdot cm^{-1}$ as the difference in molar absorptivities between the ester and the acid[10].

The hydrolysis of *N-trans*(2-furyl)acryloyl-L-phenylalanine methyl ester was followed at 335 nm. A $\Delta\varepsilon = 1220$ $M^{-1} \cdot cm^{-1}$ was used to convert absorbance data into rate data.

K_m and v_{max} values were obtained using a one run digital computer program based on an unweighted least squares analysis of v *versus* v/S (Eadie plots)[17]. k_{cat} values were calculated by dividing v_{max} by the $[E]$ obtained by spectrophotometric titration with *N-trans*-cinnamoylimidazole[14].

Quantitative N-terminal group determinations were performed by the method of SANGER[18]. 3,5-Dinitrophenyl amino acids were measured spectrophotometrically after separation by thin-layer chromatography as described by LABOUESSE AND GERVAIS[19].

Free amino groups of proteins were determined by reaction with trinitrobenzene sulphonic acid as described by HABEEB[12]. The concentration of amino groups was calculated from a calibration curve obtained using L-alanine or α-chymotrypsin as sources of amino groups. A value of $1 \cdot 10^{-4}$ $M^{-1} \cdot cm^{-1}$ was obtained for the molar extinction coefficient of one amino group. This value is in good agreement with those reported in the literature using other protein substrates[12,13].

The pH of each reaction mixture was measured at the beginning and at the end of the reaction by using a Radiometer 4c pH Meter with a type B glass electrode.

All melting points were determined by the capillary tube method and are uncorrected.

RESULTS

Succinylated δ-chymotrypsin

The data presented in Table I indicate that under the present conditions succinic anhydride reacts with chymotrypsinogen A to form a modified protein in which 14 of the 15 amino groups are succinylated. This extensive degree of modification agrees with that obtained previously[20] with acetic anhydride and indicates that essentially all the lysine ε-amino groups of chymotrypsinogen A are exposed to the reagent in solution.

Succinylated chymotrypsinogen A was activated with trypsin under conditions

TABLE I

EXTENT OF MODIFICATION OF PRIMARY AMINO GROUPS AS DETERMINED BY THE TRINITROBENZENE SULPHONIC ACID METHOD

See MATERIALS AND METHODS for details. Expected values are based on the data of HARTLEY[22]. In the case of derivatized proteins complete modification is assumed. Observed values are means and standard deviations of 4 determinations.

Sample	*Amino groups per mole of protein*	
	Expected	*Observed*
Chymotrypsinogen A	15.0	14.4 ± 0.5
δ-Chymotrypsin	16.0	15.4 ± 0.4
α-Chymotrypsin*	17.0	17.0
Succinylated chymotrypsinogen A	0.0	0.8 ± 0.3
Succinylated δ-chymotrypsin	1.0	1.6 ± 0.4
Ethylenediamine-amidated δ-chymotrypsin	29.0	27.1 ± 1.0

* An affinity chromatography-purified sample[23] was used as standard for the method.

which are known to lead to the δ form of the enzyme[21], except that the optimum pH was found to be 5.5, significantly lower than that reported for the activation of unmodified chymotrypsinogen A[4].

The dependence of the succinylated δ-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester is presented in Table II. The K_m–pH and k_{cat}–pH profiles for the reaction are shown in Figs. 1 and 2, where they are compared with the profiles obtained previously for the hydrolysis of the same substrate by native δ-chymotrypsin[1]. From inspection of Fig. 1 it can be seen that the k_{cat}–pH

TABLE II

KINETICS OF THE SUCCINYLATED δ-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF *N*-ACETYL-L-TRYPTOPHAN METHYL ESTER

Conditions: 1.6% (v/v) acetonitrile–water at 25°; ionic strength, 0.1; $[E] = 2 \cdot 10^{-7}$ M; $[S] = 6 \cdot 10^{-4}$–$8 \cdot 10^{-4}$ M. The reported values are averages of two or three determinations which agreed within 5%.

pH	*Buffer*	k_{cat} *(sec⁻¹)*	$K_m \times 10^5$ *(M)*
6.50	Phosphate	3.3	6.5
7.23	Phosphate	14.5	6.6
7.55	Phosphate	25.0	6.2
7.96	Phosphate	34.0	6.4
8.36	Tris–HCl	51.0	5.5
8.63	Tris–HCl	53.0	5.0
9.04	Glycine	68.0	5.0
9.23	Glycine	72.0	6.3
9.47	Carbonate	70.0	5.7
9.67	Carbonate	74.0	8.0
9.84	Carbonate	72.0	8.5
10.05	Carbonate	74.0	9.0
10.32	Carbonate	67.0	10.6
10.65	Carbonate	66.0	14.6
10.85	Carbonate	68.0	19.7
11.29	Carbonate	58.0	20.5

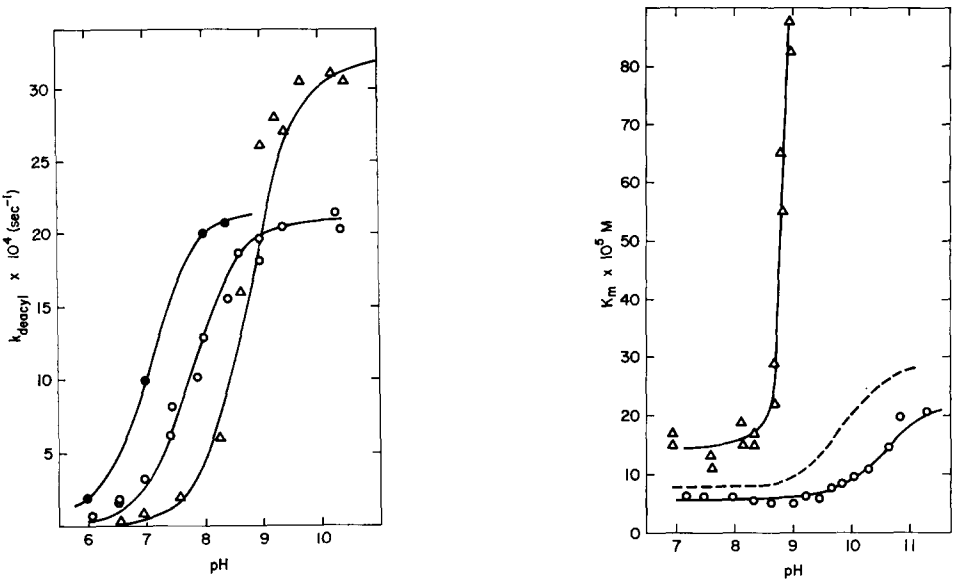


Fig. 1. pH dependence of k_{cat} for the hydrolysis of *N*-acetyl-L-tryptophan methyl ester catalyzed by different forms of δ-chymotrypsin at 25° and ionic strength 0.1. The solid lines are calculated using the equation: $k_{cat}(lim)/(1 + [H^+]/K^a)$ and the corresponding values of pK_a and $k_{cat}(lim)$. (△): succinylated δ-chymotrypsin, pK^a = 8.0, $k_{cat}(lim)$ = 74 sec^{-1}; (○): ethylenediamine-amidated δ-chymotrypsin, pK_a = 6.1, $k_{cat}(lim)$ = 50 sec^{-1}; (●): native δ-chymotrypsin[1], pK_a = 7.0, $k_{cat}(lim)$ = 47 sec^{-1}.

Fig. 2. pH dependence of K_m for the hydrolysis of *N*-acetyl-L-tryptophan methyl ester catalyzed by different forms of δ-chymotrypsin at 25° and ionic strength 0.1. (○): succinylated δ-chymotrypsin; (△): ethylenediamine-amidated δ-chymotrypsin; (— — —): native δ-chymotrypsin[1]. The solid lines are the estimated best fit to the data.

profile for the succinylated enzyme is a sigmoidal curve, significantly different from that of the native enzyme. Succinylation produces an increase of about 50% in the value of $k_{cat}(lim)$, that is, from 47 sec^{-1} found with the native enzyme to about 75 sec^{-1} found with the modified enzyme. The apparent pK_a of the ionizing group involved (probably histidine 57) has shifted from a pK_a of 7.0 in the native enzyme to a pK_a of 8.0 in the succinylated enzyme.

Changes in K_m were also found upon succinylation of δ-chymotrypsin. The pH dependence of K_m for the hydrolysis of *N*-acetyl-L-tryptophan methyl ester by the modified enzyme is shown in Fig. 2. K_m is independent of pH between 7 and 9, increases above pH 9.5; and seems to level off again near pH 11. The K_m values for the succinylated enzyme are lower than those of the native enzyme. When the data is plotted according to the method of DIXON[24], a pK_a^E value of 10.0 is obtained which shifts upon substrate binding to a pK_a^{ES} value of 10.6. These values are different from the values found previously for the native enzyme at the same ionic strength: pK_a^E 9.25 and pK_a^{ES} 9.75[1].

Similar variation of k_{cat} and K_m upon succinylation was also noticed with other specific ester substrates. The values of k_{cat} and K_m for the succinylated δ-chymotrypsin-catalyzed hydrolysis of *N-trans*(2-furyl)-acryloyl-L-tryptophan methyl ester, *N-trans*(2-furyl)-acryloyl-L-phenylalanine methyl ester and *N*-acetylglycyl-

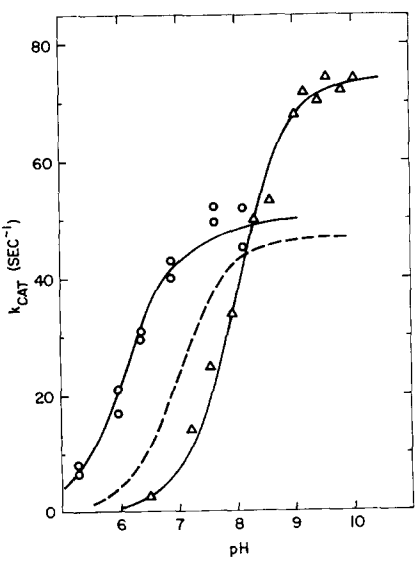
TABLE III

KINETIC PARAMETERS FOR THE HYDROLYSIS OF SPECIFIC ESTER SUBSTRATES BY SUCCINYLATED δ-CHYMOTRYPSIN AND NATIVE δ-CHYMOTRYPSIN

Conditions: temp, 25°; ionic strength, 0.1. The reported values are averages of three determinations which agree within 5%.

Substrate	*Succinylated enzyme*		*Native enzyme*	
	k_{cat} (sec^{-1})	$K_m \times 10^6$ (M)	k_{cat} (sec^{-1})	$K_m \times 10^6$ (M)
N-Acetyl-L-tryptophan methyl ester*	72	60	47	148
N-trans(2-furyl)acryloyl-L-tryptophan methyl ester*	61	7	40	10
N-trans(2-furyl)acryloyl-L-phenylalanine methyl ester*	84	30	55	80
N-CBZ-glycylglycyl-L-tryptophan methyl ester**	140	25	85	35

* 1.6% (v/v) dimethyl sulfoxide–water.
** 6.9% (v/v) dimethyl sulfoxide–water.



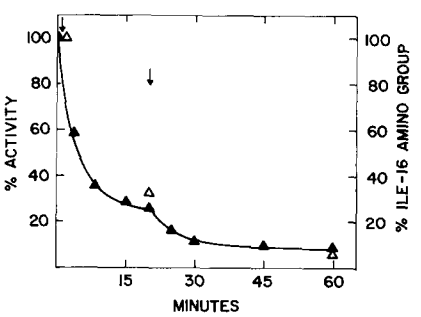


Fig. 3. pH dependence for the deacylation of indoleacryloyl-δ-chymotrypsins at 25° and ionic strength 0.1. The solid lines are calculated using the equation: $k_3 = k_3(\text{lim})/(1 + [H^+]/K_a)$ and the corresponding values of pK_a and k_3(lim). (△): indoleacryloyl-succinylated δ-chymotrypsin, $pK_a = 8.8$, $k_3(\text{lim}) = 32 \cdot 10^{-4}\ \text{sec}^{-1}$; (●): indoleacryloylethylenediamine-amidated δ-chymotrypsin, $pK_a = 7.1$, $k_3(\text{lim}) = 22 \cdot 10^{-4}\ \text{sec}^{-1}$; (○): indoleacryloyl-δ-chymotrypsin, $pK_a = 7.8$, $k_3(\text{lim}) = 21 \cdot 10^{-4}\ \text{sec}^{-1}$.

Fig. 4. Inactivation of δ-chymotrypsin by reaction with succinic anhydride. Enzyme, (10 mg/ml), was incubated at 4° in 0.1 M phosphate buffer (pH 7.6) and succinic anhydride (30 mg/ml) was added at the times indicated by the arrows. The pH was maintained at 8.0 by automatic addition of 5 M NaOH from a pH-stat. At convenient time intervals aliquots were taken and the enzymatic activity against *N*-acetyl-L-tryptophan methyl ester was measured after 60 min incubation in 0.1 M Tris–HCl–0.2 M NH_2OH buffer at 4°. Aliquots were also taken for quantitative N-terminal analysis. Closed symbols: enzymatic activity. Open symbols: N-terminal isoleucine amino group.

glycyl-L-tryptophanyl methyl ester at pH 9.50 are compared with those obtained with the native enzyme in Table III. It can be seen that succinylation produces a 50–60% increase in k_{cat} values and a significant decrease in K_m.

The effects of succinylation on the catalytic efficiency of δ-chymotrypsin was also investigated by looking at the deacylation of a chromophoric acylenzyme. For this purpose, indoleacryloyl-succinylated δ-chymotrypsin was prepared by reaction of succinylated δ-chymotrypsin with an excess of indoleacryloylimidazole at pH 4.8 followed by gel filtration on Sephadex G-25. The deacylation was followed spectrophotometrically at 340 nm[25] and the pH dependence of the first order deacylation rate constants was determined. The data are presented in Fig. 3. A clear difference between succinylated and native δ-chymotrypsins is again found. The k_3(lim) for the deacylation of the succinylated enzyme is about 50% higher than k_3(lim) for native δ-chymotrypsin. Also upon succinylation, the pK_a for deacylation has shifted from 7.8 to 8.8.

Direct succinylation of δ-chymotrypsin with succinic anhydride produces a rapid irreversible inactivation of the enzyme. This inactivation correlates well with the disappearance of the *N*-terminal isoleucine 16 amino group. The results of such an experiment at pH 8.0 and 4° are shown in Fig. 4. This effect is expected from the results of OPPENHEIMER *et al.*[20] using acetic anhydride.

Ethylenediamine-amidated δ-chymotrypsin

The treatment of δ-chymotrypsin with an excess of ethylenediamine hydrochloride in the presence of a water-soluble carbodiimide results as shown in Table I, in the amidation of approx. 12 of the 14 carboxyl groups present in the enzyme.

TABLE IV

KINETICS OF THE ETHYLENEDIAMINE-AMIDATED δ-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF *N*-ACETYL-L-TRYPTOPHAN METHYL ESTER.

Conditions: 1.6% (v/v) dimethyl sulfoxide–water, 25°; ionic strength, 0.1; $[E] = 1 \cdot 10^{-7}$ M; $[S] = 8 \cdot 10^{-4}$ M. The reported values are averages of two determinations which agree within 7%.

pH	*Buffer*	k_{cat} (sec^{-1})	$K_m \times (10^5 M)$
5.30	Acetate	6.5	40.4
5.30	Acetate	7.2	48.4
5.97	Phosphate	17.3	31.1
5.96	Phosphate	21.5	43.3
6.39	Phosphate	29.8	28.4
6.37	Phosphate	31.0	29.9
6.90	Phosphate	40.0	17.3
6.90	Phosphate	43.0	15.5
7.65	Phosphate	49.5	11.6
7.62	Phosphate	52.2	13.9
8.15	Tris–HCl	45.2	15.1
8.17	Tris–HCl	50.2	19.3
8.34	Tris–HCl	43.1	17.2
8.34	Tris–HCl	41.9	15.1
8.70	Tris–HCl	37.0	22.5
8.72	Tris–HCl	40.0	29.1
8.85	Carbonate	—	65.0
8.84	Carbonate	—	55.2
9.00	Carbonate	—	82.0
8.99	Carbonate	—	85.0

This result agrees with that of CARRAWAY *et al.*[26] which shows that, in the absence of a denaturing agent, 13 carboxyl groups of α-chymotrypsin react with glycine methyl ester.

The kinetics of amidated δ-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester is shown in Table IV. The k_{cat}–pH profile obtained from these data is shown in Fig. 1 where it is compared with the profiles obtained with native and succinylated enzymes. No significant change in k_{cat}(lim) is found upon amidation. However, as expected, the apparent pK_a shifts from 7.0 to 6.1. Amidation results in an increase in K_m as shown in the K_m–pH profile of Fig. 2. The values show a minimum at pH 7.6. Above pH 8 K_m increases sharply and no reliable values can be obtained above pH 9 because of lack of saturation of the enzyme.

The deacylation of indoleacryloyl-ethylenediamine-amidated δ-chymotrypsin was also studied. The results obtained are illustrated in Fig. 3. Amidation does not change the value of k_3(lim) but shifts the pK_a of deacylation to a lower value, from 7.8 to 7.1.

DISCUSSION

It has been reported that several proteins undergo structural changes upon succinylation[5,10,27,28]. HABEEB *et al.*[5] have shown that succinylation of bovine serum albumin produces conformational changes detected by viscosity and sedimentation velocity measurements. The changes were attributed to expansion resulting from the high charge density present in the molecule.

The results obtained in this investigation indicate that the basic kinetic properties of δ-chymotrypsin do not change apreciably upon succinylation of the lysine residues. The observed changes in the pK_a of the imidazole group of histidine 57 and isoleucine 16 amino group are expected from a modification which involves an appreciable change in the net charge of the protein. The effects disappeared at high ionic strength as predicted by polyelectrolyte theory.

A rough calculation of the expected changes in pK_a can be attempted utilizing the equation introduced by Linderstrøm-Lang[29] written as $\Delta pK_a = 0.868\, w\, \Delta Z$, in which the change in pK_a is related to the change in electrical charge, ΔZ, and to an electrostatic factor, w, which depends on the ionic strength and size and shape of the molecule. From the extent of the modification, the amino acid sequence, the known pK_a values of the ionizable groups and the value of $w = 0.063$ calculated by KARIBIAN *et al.*[30] for δ-chymotrypsin, shifts of 1.3 and 1.1 pK_a units are calculated for histidine imidazole group at pH 7.5 and isoleucine amino group at pH 10, respectively. These values are somewhat higher than those obtained experimentally in this work which are 1.0 and 0.8 pK_a units, respectively. For ethylenediamine-amidated δ-chymotrypsin a shift of 1.2 is calculated for histidine at pH 7. A value of 0.8 is found experimentally. The discrepancies noted between theory and experiment can be tentatively attributed to changes in conformation upon modification, binding of salt ions or specific interactions between the charged groups of the protein[31].

The increased k_{cat}(lim) of succinylated δ-chymotrypsin when compared with that of native enzyme is of interest. Our results indicate that at ionic strength 0.1, a shift of one pK_a unit is accompanied by an increase of about 50% in the value of k_{cat}(lim) toward specific ester substrates. A similar phenomenon has been observed

previously by GOLDSTEIN AND KATCHALSKI[32]. They have found that the activity–pH profiles of polyanionic derivatives (polyglutamyl-chymotrypsin) are shifted toward more alkaline pH values and are accompanied by an increase in the turnover number toward *N*-acetyl-L-tyrosine ethyl ester. Also related is the report indicating that carbamylation of chymotrypsin increases v_{max} for the hydrolysis of methyl hippurate and heptanoyl hexanoate[33]. GOLDSTEIN AND KATCHALSKI[32] have attempted to explain these phenomena in terms of a BRØNSTED[34] linear free energy relationship between the turnover number and the pK_a of the catalytic group on the basis of the general-base catalysis mechanism previously postulated for chymotrypsin[35].

The rate constant for the deacylation of indoleacryloylsuccinylated δ-chymotrypsin is also about 50% higher than that of the native indoleacryloyl δ-chymotrypsin. This indicates that succinylation affects the deacylation step of specific (rapid) ester substrates and non-specific (slow) ester substrates similarly and suggests that with both type of substrates the deacylation step occurs by the same mechanism.

At alkaline pH, K_m values for the succinylated δ-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester are also dependent on the ionization of an acid group, as found with the native enzyme[1]. Succinylation of the enzyme produces a significant decrease in K_m values at all pH values, very similar to that previously observed upon increasing the ionic strength of the medium[1]. In terms of the three-step mechanism established for chymotrypsin[35], for specific ester substrates $K_m = K_s \cdot k_3/k_2$ and the decrease in K_m may be a consequence of a decrease in K_s or an increase in k_2.

Contrary to a recent report by BLAIR and coworkers[36,37] and in agreement with the results of OPPENHEIMER *et al.*[20], succinylation of the α-amino group of isoleucine 16 abolishes the activity of the enzyme. This can be taken as further evidence for the essentiality of this amino group for the activity of the enzyme.

Amidation of the accessible carboxyl groups of δ-chymotrypsin with ethylenediamine results in the expected pK_a shift toward the acid pH region. No change in k_{cat}(lim) for the hydrolysis of *N*-acetyl-L-tryptophan methyl ester was found. The same is true for the deacylation of indoleacryloyl-ethylenediamine-amidated δ-chymotrypsin. This result is not expected from the work of GOLDSTEIN AND KATCHALSKI[32], which predicts a decrease in k_{cat}(lim). The reason for this discrepancy is not yet clear.

The pronounced change in K_m in the amidated enzyme above pH 8.5 can be related to the deprotonation of the lysine and ethylenediamine amino groups. As in the modified enzyme these are almost the only charged groups; the sharp increase in K_m is probably reflecting alterations in the tertiary structure of the protein which becomes completely uncharged and thus desolvated.

In conclusion, the results reported here agree with the general notion that the ε-amino group of lysines and the accessible carboxyl groups of chymotrypsin do not play a major role in the activity of the enzyme. They indicate, however, that these charged groups exert a strong influence on the dissociation of the ionizable groups of the active site. The knowledge of the nature and extent of this influence, revealed in part by this research, may be useful in rationalizing kinetic results obtained with chemically modified forms of succinylated δ-chymotrypsin[3].

ACKNOWLEDGMENTS

This research was supported by grant HEO-5726 of the National Institutes of Health. P.V. was a recipient of a Fulbright–Hays Travel Grant.

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