Spectroscopic Studies of α-Chymotrypsin Catalyzed Reactions. I. Spectral Changes at 245 mμ

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Evidence is presented that the spectral peak and absorbancy changes in the 245 mμ region which accompany the deacylation of monoacetyl chymotrypsin are independent phenomena. The former is a component of the pH difference spectrum of the enzyme. The latter is due to light scattering resulting from pH dependent differences in the state of molecular aggregation of the monoacetyl-enzyme. Two monoacetyl-enzymes exist, AC-I and AC-A. Absorbancy changes at 245 mμ accompany only the deacylation of AC-A. At a pH where AC-A deacylates, an equilibrium between AC-A and an aggregate of AC-A, (AC-A)$_n$, is formed. Disaggregation of (AC-A)$_n$ precedes deacylation and is the rate limiting step in this reaction. The formation and disaggregation of (AC-A)$_n$ is responsible for the absorbancy changes.

Kinetic studies by Gutfreund and Sturtevant suggest that the α-chymotrypsin catalyzed hydrolysis of p-nitrophenyl acetate occurs in three steps.[2] These are: (1) the formation of the enzyme substrate complex; (2) the formation of a monoacetyl enzyme and liberation of p-nitrophenol; (3) the rate limiting step, the deacylation of the monoacetyl enzyme. Monoacetyl chymotrypsin was first isolated in stable form by Balls and Wood.[3] The hydrolysis of specific substrates for α-chymotrypsin probably involves the same three step mechanism as the hydrolysis of p-nitrophenyl acetate.[4,5] All the kinetic and structural evidence presented so far indicates that the formation of the monoacetyl enzyme involves, at least eventually, the acylation of the β-hydroxy group of a serine residue of chymotrypsin.[6] The over-all chymotrypsin catalyzed hydrolysis of p-nitrophenyl acetate, as well as the isolated monoacetyl chymotrypsin, have been used to study the deacylation of the monoacetyl enzyme. At the present no unique interpretation of the data is possible. The kinetic data of Gutfreund and Sturtevant[2] are consistent with the direct deacylation of the acetyl group from the β-hydroxy group of a serine residue of α-chymotrypsin. Experiments with monoacetyl-δ-chymotrypsin,[7] isolated according to Balls and Wood,[3] suggested a rapid shift of the acetyl group from the β-OH group of serine to an imidazolyl nitrogen and subsequent hydrolysis of the resulting N-acetyl-imidazolyl derivative.[7]

The N-acetyl-imidazole hypothesis arose from these observations[7]: (1) the difference spectrum of monoacetyl-δ-chymotrypsin at pH 8.9, where the

enzyme deacylates, versus pH 3.5 shows a spectral peak with a maximum near 245 mμ and (2) a rapid increase in absorbancy at 245 mμ followed by a slow decay is observed when the pH of monoacetyl enzyme solutions is raised from pH 3.5 to 8.9. The position of the spectral peak and the rate of decrease in absorbancy were considered characteristic of acetyl-imidazole and its rate of hydrolysis. The N-acetyl-imidazole hypothesis could also be reconciled with the kinetics of the chymotrypsin catalyzed hydrolysis of ethyl lactate.[8] Marini and Hess,[9] however, were able to isolate a monoacetyl-α-chymotrypsin derivative (AC-I) which did not show spectral changes at 245 mμ during deacylation. Kinetic data were presented[9] which indicated that AC-I is the true intermediate in the α-chymotrypsin catalyzed hydrolysis of p-nitrophenyl acetate and that it can rearrange to another monoacetyl enzyme (AC-A) under the conditions used by Balls and Wood.[3] This altered monoacetyl-enzyme (AC-A) shows the spectral phenomena first reported[7] for isolated monoacetyl-δ-chymotrypsin.

Spencer and Sturtevant observed[4] that when a lyophilized preparation of α-chymotrypsin was freshly dissolved in water and the pH was then raised from 4.8 to 8.2 by the addition of tris-(hydroxymethyl)-aminomethane–HCl buffer (Tris-HCl), a rapid decrease in absorbancy at 245 mμ was also observed. Although the rate constant for this decay was more than ten times as large as that observed with AC-A and although preincubation of the enzyme solution before raising the pH to 8.2 eliminated the absorbancy changes for α-chymotrypsin, but not for AC-A, it was assumed that these changes were of common origin and, therefore, not related to deacylation of the enzyme.

The nature of the spectral changes which accompany the deacylation of AC-A and the relationship between the two monoacetyl-enzymes, AC-I and AC-A, are important considerations in attempts to understand chymotrypsin catalyzed reactions and are the subject of this paper. A preliminary report of a part of these investigations has appeared.[10]

(1) This work is part of a thesis submitted by J. F. Wootton to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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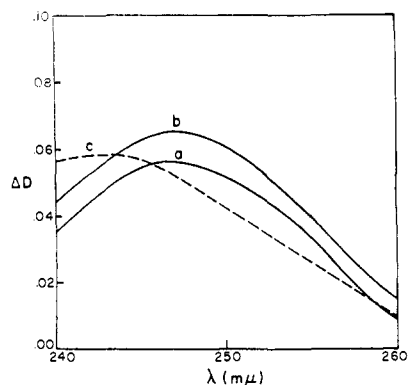(8) I. Tinoco, Jr., Archiv. Biochem. Biophys., 76, 148 (1958).

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Fig. 1.—Ultraviolet pH difference spectra, pH 9.0 (0.033 M Tris–HCl buffer) *versus* pH 3.5 (HCl); curve a, 6 × 10^{-5} M α-chymotrypsin or AC-I; curve b, 6 × 10^{-5} M AC-A; curve c, 2 × 10^{-5} M N-acetyl-L-tyrosine ethyl ester.

Results

Difference Spectra.—The difference spectra of AC-A, AC-I, α-chymotrypsin and N-acetyl-L-tyrosine ethyl ester at pH 9.0 *versus* pH 3.5 are shown in Fig. 1. The absorption maxima of the difference spectra of AC-A, AC-I, and α-chymotrypsin, at various pH's, 7.5–9.5 *versus* pH 3.5, increase with increasing pH in a manner which indicates that the principal component of this difference spectrum is due to tyrosyl ionization. The difference spectrum of N-acetyl-L-tyrosine ethyl ester at pH 9.0 *versus* pH 3.5, shown in Fig. 1, is strikingly similar to the above difference spectra of the monoacetyl-enzymes and α-chymotrypsin. The absorbancy near 245 mμ for AC-A is greater than the absorbancy for AC-I or α-chymotrypsin (Fig. 1).

The difference spectra of the enzyme solutions were taken at different pH's for comparison to data published previously by Dixon and Neurath.[7] A valid method of obtaining difference spectra of two enzymes differing only in the acetylation of the active site is to compare the monoacetyl enzyme to the deacylated control at the same pH, ionic strength, concentration and temperature. These conditions were used to obtain the difference spectrum of AC-A *versus* its deacylated control (Fig. 2). A 245 mμ peak cannot be observed, but an increase in absorbancy in this region is noted which disappears as the enzyme becomes deacylated. There appear to be two absorption peaks in the 290 mμ region which also disappear with time. When AC-I is compared to its deacylated control, one does not observe an increase in absorbancy at 245 mμ (Fig. 2). With AC-I one obtains a flat base line with the exception of the peaks in the 290 mμ region[11] (Fig. 2). The 290 mμ peak has been discussed in preliminary publications.[11,12]

Time Dependent Absorbancy Changes at 245 mμ. A. Absorbancy Changes Common to α-Chymotrypsin, AC-I and AC-A.—The absorbancy changes at 245 mμ, pH 8.0, 0.033 M Tris–HCl buffer containing no calcium chloride, 20°, of freshly prepared solution of monoacetyl-α-chymotrypsin prepared according to Marini and Hess
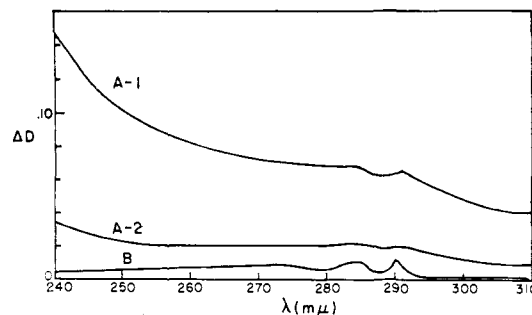
(11) G. P. Hess and J. F. Wootton, *Federation Proc.*, **19**, 340 (1960).
(12) J. F. Wootton and G. P. Hess, *Nature*, **188**, 726 (1960).

Fig. 2.—Ultraviolet difference spectra, monoacetyl-α-chymotrypsin *versus* deacylated enzyme, 6 × 10^{-5} M, pH 8.0 (0.033 M Tris–HCl buffer, 0.033 M CaCl₂), 25°. Times stated are for absorbancy at 310 mμ. Scanning speed 10 Å. per second; curve A-1, 1 minute after adjustment of pH of AC-A solution from 3.5 (HCl) to 8.0; curve A-2, 12 minutes after adjustment of pH of AC-A solution to 8.0; curve B, 1 minute after adjustment of pH of AC-I solution from 3.5 (HCl) to 8.0.
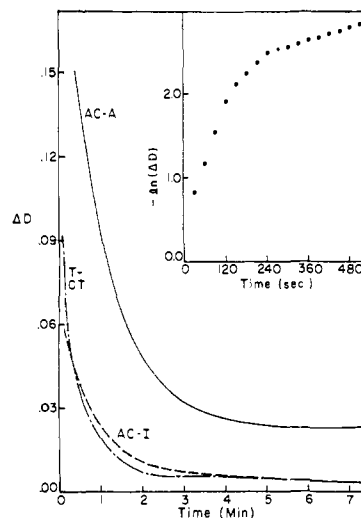

Fig. 3.—Absorbancy changes at 245 mμ of "treated" α-chymotrypsin (T-CT) and monoacetyl-chymotrypsins, AC-I and AC-A. 5 × 10^{-5} M enzyme, pH 8.0 (0.033 M Tris–HCl buffer), 25 ± 0.5°. Inset, plot of $-\ln (\Delta D)$ *versus* time for the decay of absorbancy of AC-A. Conditions as above.

(AC-I),[9] and "isolated" α-chymotrypsin, (T-CT), (see Experimental section) are shown in Fig. 3. The half times for the decay of AC-I and T-CT were of the order of 30 seconds, in agreement with the values reported by Spencer and Sturtevant[4] for lyophilized chymotrypsin. The observed half time for the decay of AC-A was about 200 seconds (Fig. 3). However, a plot of $\ln \Delta D$ *versus* time (inset Fig. 3) indicates that the decay of AC-A is due to two simultaneous events. The decay curve can be resolved in terms of two first order reactions with half times of 33 and 400 seconds. The rapid absorbancy change is therefore common to all three enzyme preparations, AC-I, AC-A and T-CT, and can be prevented by dissolving the enzymes in solutions 0.033 M in calcium chloride. In the presence of calcium chloride, time dependent absorbancy changes in the pH region examined, pH 5.5–9.0, could be observed only with AC-A, and not with AC-I, T-CT or α-chymotrypsin.
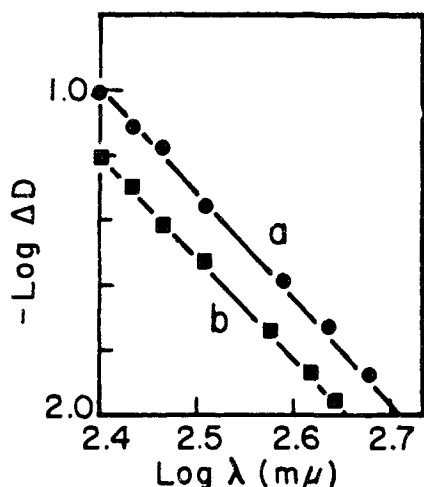
Fig. 4.—Ultraviolet and visible difference spectra of AC-A versus deacylated enzyme. 5 × 10⁻⁵ M enzyme, pH 6.3 (0.033 M Tris-HCl buffer, 0.033 M CaCl₂), 25 ± 0.5°. Times stated are for absorbancy at 500 mμ. Scanning speed 20 Å. per second; curve a, 1 minute after adjustment of pH of AC-A solution from 3.5 (HCl) to 6.3; curve b, 15 minutes after adjustment of AC-A solution to pH 6.3.
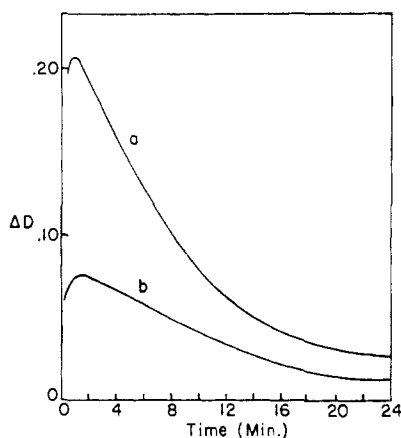

Fig. 5.—Effect of concentration of AC-A on the absorbancy changes at 245 mμ, pH 8.0 (0.033 M Tris-HCl buffer, 0.033 M CaCl₂), 25°; curve a, 9.4 × 10⁻⁵ M AC-A; curve b, 4.7 × 10⁻⁵ M AC-A.

B. Absorbancy Changes Accompanying Deacylation of AC-A.

The following experiments were performed to investigate the possibility that the absorbancy changes accompanying the deacylation of AC-A result from light scattering due to pH dependent differences in the state of molecular aggregation of AC-A.

A log-log plot of ΔD versus λ for AC-A versus deacylated AC-A at pH 6.3 is shown in Fig. 4. An absorption peak cannot be seen in the 245 mμ region. The absorption at 245 mμ extends linearly into the visible region where α-chymotrypsin is known not to absorb. The rate of decrease in absorbancy at 245 mμ and in the visible region is the same during the deacylation of AC-A (Fig. 4).

The dependency of the absorbancy at 245 mμ on enzyme concentration does not obey Beer's Law, as is shown in Fig. 5. Furthermore, changing the concentration of the enzyme affects not only
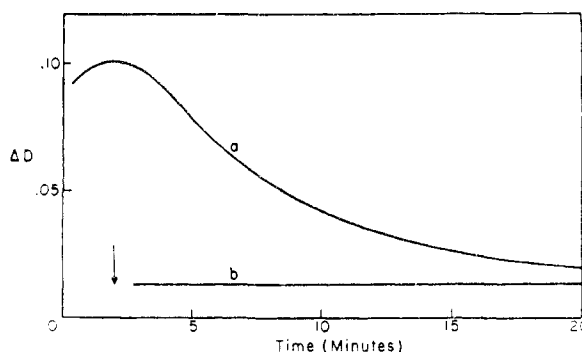

Fig. 6.—Effect of 8 M urea on the absorbancy changes at 245 mμ of AC-A at pH 8.0. Final enzyme concentration 5 × 10⁻⁵ M, 25°. Solution in reference cell identical to solution in sample cell except for omission of enzyme; curve a, recording of absorbancy change after adjustment of pH of AC-A solution from pH 3.5 (HCl) to 8.0 (0.033 M Tris-HCl buffer, 0.033 M CaCl₂); curve b, AC-A solution adjusted to pH 8.0 as above and at time indicated by arrow, solution made 8 M with respect to urea.

the absorbancy disproportionately but also the rate of increase in optical density. The approximate half time for the increase in absorbancy is 9 seconds, when the enzyme concentration is 9.4 × 10⁻⁵M, and 14 seconds when the enzyme concentration is 4.7 × 10⁻⁵M.

Curve a in Fig. 6 shows the normal progress curve of the changes at 245 mμ when a solution of AC-A is brought from pH 3.5 to 8.0. The maximum absorbancy is obtained in about 30 seconds. Curve b is obtained when a solution of AC-A is brought to pH 8.0 and after 30 seconds is added to a urea solution of pH 8.0, so that the final concentration is 8 M with respect to urea. The absorbancy of this solution decreases almost instantaneously to the value obtained in aqueous solution after deacylation of AC-A at this pH (Fig. 6, Curve b). This decrease is not due to a rapid deacylation of the enzyme, since the hydroxamate test indicates that the enzyme is still 90% acylated.

C. Effect of pH on Absorbancy Decay of AC-A.

The pH dependency of the rate of decay of AC-A at 25° is recorded in Table I. The apparent first order rate constants (k') at pH 8.0 and 8.5 are in excellent agreement with those reported by Spencer and Sturtevant[4] and by Dixon and Neurath.[7] The results shown in Table I demonstrate that the decay in absorbancy at 245 mμ is pH dependent below pH 8.0 and apparently pH independent above this pH. While this behavior does not conform to the known properties of N-acetyl-imidazole,[13,14] it does suggest that the changes in AC-A which causes a decrease in absorbancy at 245 mμ are inhibited by protonation of possibly a single functional group of the enzyme. The apparent dissociation constant of this group (K_{app}) can be estimated from the pH dependence of the apparent first order rate constant (k'), if the assumption

(13) E. R. Stadtman, "Mechanism of Enzyme Action," W. D. McElroy and B. Glass, eds., Johns Hopkins Press, Baltimore, Md., 1954. p. 581.
(14) W. P. Jencks and J. Carriuolo. J. Biol. Chem., 234, 1272 (1959).

is made that the decay of absorbancy corresponds to the decomposition of a single absorbing species when the group in question is not protonated. Then $k' = k\left[\dfrac{K_{app}}{K_{app} + (H^+)}\right]$ where k is the limiting first order rate constant for the decay of the 245 mμ absorbing species. A plot of $1/k'$ versus (H^+) was found to be linear (Fig. 7) and pK_{app} calculated from the slope is 6.6 ± 0.2.

TABLE I

EFFECT OF pH ON THE RATE OF THE ABSORBANCY DECAY OF
AC-A AT 245 mμ

pH	$k' \times 10^3$ sec. $^{-1}$	Total decay ΔD
8.5	1.7	0.12
8.0	1.7	.11
7.6	1.4	.12
7.2	1.3	.12
6.4	0.8	.12
6.3	.7	.13
5.5	.3	.13

(AC-A) = 4.8 × 10^{-5} M, 0.033 M Tris-HCl buffer, 0.033 M
CaCl$_2$, T = 25 ± 0.5°

Discussion

The evidence presented[7] for the involvement of N-acetyl-imidazolyl in the deacylation of mono-acetyl-δ-chymotrypsin rests on two experimental observations: (1) the difference spectrum of this enzyme derivative at pH 8.9 versus pH 3.5, with a spectral peak near 245 mμ and (2) the spectral changes at 245 mμ accompanying the deacylation and in particular the rate of decrease in absorbancy at this wave length.

The Spectral Peak Near 245 mμ.—Not only the difference spectra of all preparations of mono-acetyl-α-chymotrypsin, pH 9.0 versus pH 3.5, show an absorption peak near 245 mμ, but also, this same peak is observed in the pH difference spectrum of α-chymotrypsin (Fig. 1). The absorption peak in the pH difference spectrum is, therefore, not a unique property of the monoacetyl-enzyme. The main component of this difference spectrum is most probably due to differences in the ionization state of tyrosyl residues of the enzyme molecules at two different pH values. The pH dependence of these difference spectra, as well as their similarity to the difference spectrum of N-acetyl-L-tyrosine ethyl ester, pH 9.0 versus pH 3.5, strongly suggest this (Fig. 1). While the pH difference spectrum of the enzyme molecules is not exactly the same as that of N-acetyl-L-tyrosine ethyl ester, it is well documented that incorporation of amino acid residues into proteins modifies their spectra.[15]

The Spectral Changes at 245 mμ.—The observations of Spencer and Sturtevant[4] that some chymotrypsin preparations give observable spectral changes at 245 mμ, even though considerably faster than those reported for monoacetyl-δ-chymotrypsin[7] and monoacetyl-α-chymotrypsin,[4,9] left some question as to whether these spectral changes are a unique property of AC-A, and the analogous species of monoacetyl-δ-chymotrypsin.

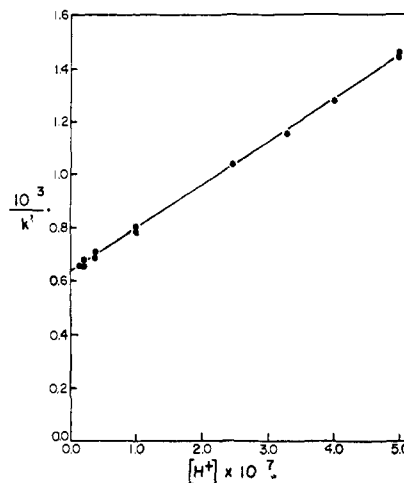(15) G. H. Beaven and E. R. Holiday, Advances Protein Chem., 7, 319 (1952).

Fig. 7.—Effect of (H$^+$) on the rate of the absorbancy decay of AC-A solutions; 4.8 × 10^{-5} M AC-A, 0.033 M Tris-HCl buffer, 0.033 M CaCl$_2$, 25 ± 0.5°.

These observations by themselves do by no means exclude the N-acetyl imidazole hypothesis. It is well known that small peptides are tightly attached to some α-chymotrypsin preparations.[16] Proponents of the N-acetyl-imidazole hypothesis could suggest that these peptides are present in the form of an acyl-enzyme complex and that the spectral changes observed with some chymotrypsin preparations are due to the decomposition of this complex.

The experimental observations reported here do, in fact, suggest that the spectral changes at 245 mμ first observed by Dixon and Neurath[7] are a unique property of AC-A type molecules. The rapid absorbancy changes ($t_{1/2} \approx 30$ seconds) are ubiquitous with lyophilized chymotrypsin and monoacetyl-chymotrypsin preparations, providing the solutions are freshly prepared and do not contain calcium ions. In the presence of calcium ions the only changes observed at 245 mμ are those reported for AC-A. These changes are an increase followed by a slow decrease ($t_{1/2} = 400$ seconds) when solutions of AC-A are brought to pH 8.0–9.0.

The experiments presented suggest that the absorbancy changes at 245 mμ observed with AC-A in the presence of calcium ions are due to molecular aggregation, followed by disaggregation, which accompanies the deacylation of AC-A. If deacylation of monoacetyl-chymotrypsin produces a true absorption peak at 245 mμ, as reported, this peak should be observable in the experiment illustrated in Fig. 4. In this experiment the difference spectrum of monoacetyl-α-chymotrypsin versus its deacylated control was obtained at the same pH, ionic strength, enzyme concentration and temperature. One cannot observe an absorption peak at 245 mμ, but a generalized increase in the absorbancy is seen. This absorbancy increase, observed only with AC-A, extends into the visible wave length region. The log-log plot of ΔD versus λ is linear, and the rate of decrease in absorbancy, observed during deacylation of AC-A, is the same at 245

(16) P. Desnuelle, M. Rovery and C. Fabre, Biochem. et Biophys. Acta, 9, 109 (1952).

mμ as in the whole wave length region examined, up to 500 mμ.

It was observed[17,18] that apparent absorbancy in difference spectra, at wave length regions where the protein does not absorb, can result from Raleigh light scattering due to differences in molecular aggregation. If the absorbancy differences are due to light scattering, a log–log plot ΔD versus λ should be linear in the visible region and extrapolation of this linear portion into the region of absorption allows correction of the scattering contributions.[18] The linearity of the log–log plot ΔD versus λ, as well as the identical rate of decrease of absorbancy at 245 mμ and in the visible region (Fig. 4) establish a relationship between the events observed at 245 mμ and in the visible region. This constitutes strong evidence that the absorbancy results from Raleigh light scattering due to differences in molecular aggregation between solutions of AC-A and the deacylated control. Further evidence for the origin of the absorbancy at 245 mμ is the observation that ΔD at 245 mμ is not proportional to enzyme concentration (Fig. 5), as it should be if the absorbancy were due to a new chromophore. On the other hand, if the apparent increase in optical density is due to light scattering, the observed deviation from Beer's law is to be expected.[18] The effect of enzyme concentration on the rate of increase in absorbancy is consistent with the intermolecular reaction involved in the aggregation of the monoacetyl-enzyme. The rate of the intramolecular formation of an N-acetyl-imidazolyl derivative of α-chymotrypsin, however, should be independent of enzyme concentration. Similarly, the instantaneous decrease in absorbancy at 245 mμ observed in 8 M urea is consistent with disaggregation due to the effect of urea on the secondary valence bonds known to participate in the aggregation of chymotrypsin.[19] The instantaneous disappearance of an N-acetylimidazolyl derivative in 8 M urea could be explained only with difficulty, if at all. Finally, the observation[4] that the rate of decrease in absorbancy at 245 mμ is independent of temperature, between 10 and 25°, is consistent with the known temperature independence of the aggregation-disaggregation of chymotrypsin in this temperature region.[19]

The weight of experimental evidence strongly supports the view that the absorbancy changes at 245 mμ are a result of light scattering due to difference in molecular aggregation between solutions of AC-A and the deacylated controls. Certainly, these experiments cannot be reconciled with the suggestion that the changes in absorbancy at 245 mμ are due to the formation and decomposition of an N-acetyl-imidazolyl derivative of α-chymotrypsin.

A reasonable explanation of previous observations is now possible. The reported spectral peak and the absorbancy changes at 245 mμ are independent phenomena. The spectral peak is a component of the pH difference spectrum. This peak

is therefore observed with the two monoacetyl-enzymes, AC-I and AC-A and with α-chymotrypsin. In presence of calcium ions, the absorbancy changes at 245 mμ are only observed with AC-A. These are light scattering changes caused by pH dependent molecular aggregation and by disaggregation which accompanies the deacylation of AC-A. The light scattering superimposed on the pH difference spectrum explains why the difference spectrum of AC-A, pH 9.0 versus 3.5, has an absorbancy near 245 mμ greater than observed with AC-I, α-chymotrypsin, or T-CT (Fig. 1).

The Relation Between AC-I and AC-A.—The molecular aggregation-disaggregation phenomenon as evidenced by absorbancy changes at 245 mμ appears to be intimately associated with the acetyl-group at or near the active center of α-chymotrypsin. When α-chymotrypsin and AC-I are treated in an identical manner to bring about the AC-I →AC-A transformation, T-CT cannot be differentiated, spectroscopically or kinetically, from 3 × recrystallized α-chymotrypsin. This suggests that the enzymatic acylation of α-chymotrypsin is accompanied by changes in the structure of the enzyme. Experimental evidence for structural changes accompanying the phosphorylation of α-chymotrypsin by diisopropylphosphorofluoridate and the formation of monoacetyl-α-chymotrypsin have been presented.[11,12,20] These structural changes which occur in the formation of monoacetylchymotrypsin appear to be important for further structural changes which bring about the AC-I→AC-A transformation. Previous experiments[9] have suggested that the equilibrium of this transformation is in favor of AC-A, the rearranged form of AC-I.

The events at a pH where AC-A deacylates can be correlated with the experiments presented here and with those previously reported.[4,7,9] At a pH greater than 5.0, AC-A aggregates as evidenced by an increase in absorbancy at 245 mμ (Fig. 6. Curve a). This aggregate is large enough to cause an absorbancy increase due to light scattering which is observable at all wave lengths between 240 and 500 mμ (Figs. 2 and 4).

As AC-A deacylates, disaggregation occurs as evidenced by a decrease of absorbancy. Both the rate of aggregation and disaggregation are pH dependent (Table I). The rate of disaggregation, measured by the decrease in absorbancy at 245 mμ, can be correlated with the rate of deacylation of AC-A. The rates of deacylation of AC-I + AC-A have been obtained previously[9] by measuring the rate of liberation of p-nitrophenol from p-nitrophenyl acetate on the addition of the monoacetyl enzymes to solutions of p-nitrophenyl acetate at the appropriate temperature and pH, and more directly,[9,21,22] by measuring the rate of recovery of the catalytic properties of these monoacetyl enzymes toward the hydrolysis of N-acetyl-L-tyrosine ethyl ester. The rate of deacylation of AC-I was characterized by a single first order rate constant which is identical to the rate limiting

(17) M. Laskowski, Jr., S. J. Leach and H. A. Scheraga, *J. Am. Chem. Soc.*, **82**, 571 (1960).

(18) S. J. Leach and H. A. Scheraga, *ibid.*, **82**, 4790 (1960).

(19) R. F. Steiner, *Arch. Biochem. Biophys.*, **53**, 457 (1954).

(20) H. Neurath, J. A. Rupley and W. J. Dreyer, *ibid.*, **65**, 243 (1956).

(21) M. A. Marini and G. P. Hess, "Abstracts of the 136th ACS meeting," Atlantic City, September, 1959, p. 58c.

(22) G. H. Dixon and H. Neurath, *J. Biol. Chem.*, **225**, 1049 (1957).

constant of the α-chymotrypsin catalyzed hydrolysis of p-nitrophenyl acetate. ($k' = 2.0 \times 10^{-2}$ sec.$^{-1}$, pH 8.0, 20°.) The kinetics of deacylation of AC-A can be expressed in terms of two simultaneous first order processes with rate constants of 1.9×10^{-2} sec.$^{-1}$ and 0.16×10^{-2} sec.$^{-1}$ at pH 8.0 and 20°.[9] It should be noted that the first rate constant corresponds to the rate of deacylation of AC-I, while the second corresponds to the rate of decrease in absorbancy at 245 mμ at pH 8.0 and 20°. It appears therefore, that the disaggregation and deacylation of AC-A are intimately related events. One explanation of these events is that at a pH where AC-A deacylates a mixture is formed of AC-A and an aggregate of AC-A, [(AC-A)$_n$]. (AC-A)$_n$ deacylates at a slower rate than AC-A for one of two reasons. Either the deacylation takes place in the aggregate at a slow rate and the deacylated chymotrypsin dissociates, or there is at any pH an AC-A, (AC-A)$_n$ equilibrium, the deaggregation of (AC-A)$_n$ is rate limiting, and only AC-A is deacylated. The experimental evidence favors the latter possibility. As mentioned previously, the rate of decrease in absorbancy at 245 mμ as well as aggregation–disaggregation of α-chymotrypsin are temperature independent in the region examined. The deacylation of normal chymotrypsin intermediates, however, is quite temperature dependent. The Arrhenius activation energy, ΔE, was reported to be 18,500 cal per mole for the deacylation reaction.[22] Therefore, if (AC-A)$_n$ is deacylated at all, it presumably occurs at an insignificant rate and disaggregation must precede deacylation and be rate determining. This conclusion agrees with the previous findings of Martin and Niemann.[23] The kinetic experiments of these authors suggested that only the monomeric form of α-chymotrypsin-substrate complexes can decompose and that, perhaps, a common part of the active center is involved in the deacylation reaction and in the aggregation of the chymotrypsin-substrate complex. In this regard, it is interesting to note that the apparent pK of the group, which apparently controls the rate of decay of absorbancy at 245 mμ (Fig. 7), is similar to the apparent $pK(s)$ of the group(s) controlling both the rates of acylation and deacylation of α-chymotrypsin.[2,22]

Conclusion

The events which lead to the absorbancy changes at 245 mμ during the deacylation of AC-A are proposed in Fig. 8.

$$CT + NPA \xrightarrow{\ pH\ 5.0\ }$$

$$NP + AC\text{-}I \xrightarrow{\ pH \geq 5.5\ } CT + Acetate$$

$$pH\ 5.0 \downarrow Slow \qquad\qquad \uparrow$$

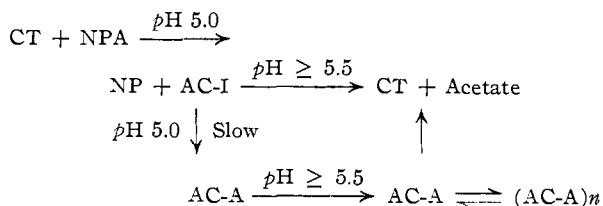$$AC\text{-}A \xrightarrow{\ pH \geq 5.5\ } AC\text{-}A \rightleftharpoons (AC\text{-}A)n$$

Fig. 8.—Proposed scheme for the formation and deacylation of monoacetyl-α-chymotrypsins. CT = α-chymotrypsin; NPA = p-nitrophenyl acetate; NP = p-nitrophenol.

(23) R. B. Martin and C. Niemann, J. Am. Chem. Soc., 80, 1473 (1958).

Monoacetyl-α-chymotrypsin (AC-I) formed in the reaction of NPA with α-chymotrypsin and isolated according to the procedure of Marini and Hess[9] is kinetically indistinguishable from the monoacetyl-enzyme formed in the α-chymotrypsin catalyzed hydrolysis of NPA at a higher pH. The enzymatic acylation of α-chymotrypsin leads to structural changes in the enzyme. Prolonged standing of AC-I at pH 5.0 or below brings about further structural changes and the formation of a new species of monoacetyl-chymotrypsin (AC-A). At a pH where the monoacetyl-enzymes deacylate, AC-A, unlike AC-I, aggregates and forms an AC-A, (AC-A)$_n$ equilibrium. (AC-A)$_n$ is not deacylated directly. The deacylation proceeds only through AC-A and the reestablishment of the (AC-A)$_n$, AC-A equilibrium. Since disaggregation of (AC-A)$_n$ is considerably slower than AC-A hydrolysis, deviation from first order kinetics is observed in the deacylation of this monoacetyl-enzyme.[9]

The formation and disappearance of (AC-A)$_n$ cause the observed increase and subsequent decrease of absorbancy at 245 mμ due to light scattering. The reported spectral peak near 245 mμ is a phenomenon independent of the absorbancy changes. This spectral peak is a component of the pH difference spectrum of the enzyme and is, therefore, observable not only with both forms of monoacetyl-chymotrypsin but with α-chymotrypsin itself.

The reported observations cannot be reconciled with the N-acetylimidazole hypothesis of Dixon and Neurath,[7] but they are compatible with the kinetic data of Gutfreund and Sturtevant[2] and the spectroscopic data of Bender, et al.,[24] which suggest the direct deacylation of the acyl group from the β-hydroxy group of a serine residue of α-chymotrypsin.

Experimental

Materials. Enzyme.—Salt-free, 3 times recrystallized α-chymotrypsin, obtained from Worthington Biochemical Corporation, was used without further purification.

Buffers.—Unless otherwise indicated, all experiments were performed in 0.033 M tris-(hydroxymethyl)-aminomethane (Tris) (Sigma 7–9), 0.033 M calcium chloride (Mallinckrodt Analytical Reagent Grade), the pH being adjusted with hydrochloric acid (Baker Analyzed Reagent). pH adjustments were made with reference to Beckman pH 4.0 and pH 7.0 standard buffers, using a glass electrode (Beckman #40498) and Beckman Model G pH meter.

p-Nitrophenyl Acetate.—Prepared from p-nitrophenol (recrystallized from 0.001 M HCl, m.p. 114–116°) and acetic anhydride and recrystallized from hexane to give light yellow crystals m.p. 80°.[25]

(24) G. R. Schonbaum, K. Nakamura and M. L. Bender, ibid., 81, 4746 (1959).

(25) F. D. Chattaway, J. Chem. Soc., 2495 (1931).

Urea.—Mallinckrodt Analytical Reagent Grade crystalline urea was used without further purification.

Monoacetyl α-Chymotrypsin (AC-A).—The isolation procedure of Balls and Wood[3] was used with certain modifications. The adsorption step employing acid washed charcoal was eliminated. With certain lots of α-chymotrypsin, the unique spectral properties of this intermediate did not appear after 45 minutes incubation at room temperature. In those cases, the incubation times were extended until aliquots of the reaction mixture, after extraction of excess reagent and p-nitrophenol, showed the increase in absorbancy at 245 mμ upon raising the pH.

Monoacetyl-α-chymotrypsin (AC-I).—The method of preparation of this intermediate was identical to the method above, except that the time of incubation of chymotrypsin with p-nitrophenyl acetate was reduced to ten minutes at room temperature. An increase in absorbancy at 245 mμ was not observed when solutions of AC-I were adjusted to pH 8.0.

Determination of Extent of Acylation.—Complete acetylation of the monoacetyl α-chymotrypsin preparations used was ascertained both by spectrophotometric methods[22] and by reaction with hydroxylamine.

Treated α-Chymotrypsin (T-CT).—Solutions of α-chymotrypsin were treated in a manner identical to the preparation of AC-A, except for the exclusion of p-nitrophenyl acetate.

Methods. Instrument.—A Cary Model 14 self-recording spectrophotometer was employed for all kinetic and spectral studies. Temperature control (±0.5°) was effected by the use of a water jacketed cell holder.

Kinetic Studies.—Unless otherwise indicated, water solutions of 6 mg. per ml. of enzyme by weight were filtered through Whatman 42 filter paper and kept on ice until used. Before use, aliquots of these solutions were preincubated at the reaction temperature, usually 25 ± 0.5°. A 1.0 ml. aliquot of enzyme solution was introduced into the sample cuvette, and, at zero time, 2.0 ml. of 0.05 M Tris-HCl buffer, which was also 0.05 M with respect to calcium chloride, was added with a syringe type mixing device. Good mixing was obtained and readings of absorbancy at 245 mμ were begun within a maximum time of five seconds. Water was used as the reference solution. Apparent first order rate constants for the decay of absorbancy at 245 mμ were estimated from the slopes of plots of −ln (ΔD) versus time in seconds, where ΔD is the difference between absorbancy at time t and the final absorbancy. Apparent first order rate constants for the increase in absorbancy were estimated from plots of −ln (−ΔD) versus time in seconds, where ΔD was the difference between absorbancy at time t and the maximum absorbancy.

Effect of Urea on Absorbancy.—Water solutions of 60 mg./ml. of enzyme by weight were prepared as needed. Aliquots of 0.10 ml. were pipetted into each of two cuvettes. 0.20 ml. of 0.05 M Tris was added to adjust the pH to 8.0.

To one cuvette 2.7 ml. of 8.9 M urea in 0.05 M Tris-HCl at pH 8.0, were added after a suitable interval to allow for increase in absorbancy. The other sample was allowed to deacylate for 1 hr., and than an equal amount of buffered urea was introduced. The absorbancy of these solutions at 245 mμ was compared to a reference solution of 8 M urea at various time intervals. Control solutions were compared in which the urea solution was replaced with aqueous buffer to insure that, at the initial high enzyme concentration, the increase and subsequent decay in absorbancy occurred normally in the absence of urea.

Difference Spectra.—Water solutions usually containing 3 mg. per ml. of enzyme by weight were prepared, filtered through Whatman #42 filter paper and kept on ice until needed. A 1.0 ml. aliquot of this solution was pipetted into the reference cuvette and 2.0 ml. of 0.05 M Tris-HCl, 0.05 M calcium chloride buffer at the desired pH were added and the sample allowed to deacylate. Then sufficient dilute HCl was added with a micropipet to bring the solution to the desired final pH and the solution was mixed. This constituted the reference solution. Unless otherwise indicated, for the sample solution 2.0 ml. of buffer and the desired amount of acid were placed in the cuvette. The enzyme solution was added and the solution mixed. For each pair of samples, the same pipette was used to add each solution, and extreme care was exercised to prevent concentration discrepancies. Gravimetric trial experiments indicated that total pipetting errors could be kept within limits such that, at an optical density of 2.0 at 280 mμ, the two solutions would not differ by more than an optical density of 0.003. Silicone coated pipets were used for addition of buffer. For introduction of enzyme solutions, however, acid cleaned transfer pipets were found to be far superior in reproducibility.

The difference spectra were scanned from 320 to 230 mμ at a scanning speed of 2.5 ångströms per second. At pH's at which absorbancy was changing, the scanning speed was increased to 10 Angstroms per second, and smaller wave length regions of particular interest were covered.

Below 240 mμ the slit width was extremely wide (1 to 3 mm.) and changing rapidly. Therefore, at the enzyme concentrations used, any spectral findings in this region must be viewed with skepticism. The maximum slit width observed in the 245–320 region was 0.2 mm.

Determination of Protein Concentration.—Protein concentrations were determined spectrophotometrically at 280 mμ by using $E_{1\%}^{280} = 20.0$ to relate extinction to protein concentration.[22] The molecular weight of α-chymotrypsin was taken as 25,000.

Determination of Extent of Acylation.—The extent of acylation of the enzyme was ascertained by reaction with hydroxylamine. A slight modification[9] of the procedure of Hestrin was used.[26]

(26) S. Hestrin, J. Biol. Chem., 180, 249 (1949).

Rauwolfia Alkaloids. XXXIX. Methyl Neoreserpate, an Isomer of Methyl Reserpate. Part 2. Mechanism of Formation

BY WILLIAM E. ROSEN AND HERBERT SHEPPARD

The equilibrium ratio of methyl reserpate (I) to methyl neoreserpate (II) has been found to be approximately 1:5. The mechanism of the conversion of I to II has been studied using methanol-C[14], and the results support the methanol elimination, methanol addition scheme previously proposed.

In our previous paper,[1] the formation of methyl neoreserpate from methyl reserpate by treatment with refluxing methanolic sodium methoxide was reported. Structure II was assigned to methyl neoreserpate on the basis of chemical conversions. In the present paper, the equilibrium between the two isomers is examined, and experiments using

methanol-C[14] are described which support the mechanism previously proposed.[1]

The ratio of methyl reserpate (I) to methyl neoreserpate (II) at equilibrium was found to be approximately 1:5. This ratio represents a free energy difference of 1–1.5 kcal./mole in favor of II.[2] Table I lists the ratios of I:II found after re-

(1) W. E. Rosen and J. M. O Connor. J. Org. Chem., 26, 3051 (1961).

(2) A discussion of stabilities based on conformational considerations was presented in Part 1.[1]