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Reinvestigation of the Phenacyl Bromide Modification of α-Chymotrypsin†

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ABSTRACT: The modification of α-chymotrypsin with phenacyl bromide has been reinvestigated over a wide pH range. Evidence is presented that indicates that the nature of the phenacyl-modified enzymes prepared by this reaction is dependent upon the pH of the reaction medium. The phenacyl α-chymotrypsin produced at low pH is most probably the Met-192 phenacylsulfonium salt, as proposed earlier, since it readily undergoes dealkylation using 2-mercaptoethanol. However, the phenacyl-enzyme prepared at neutral pH possesses a much reduced enzymatic activity and does not react with 2-mercaptoethanol to regenerate native α-chymotrypsin.

In addition, incubation of the Met-192 phenacyl sulfonium enzyme at neutral pH causes a smooth irreversible change to the new phenacyl-enzyme as monitored by changes in enzymatic activity, susceptibility to dealkylation using 2-mercaptoethanol, and ultraviolet difference absorption spectral properties. The stoichiometries of both the low and neutral pH modification reactions have been determined, using [*carbonyl*-^{14}C]phenacyl bromide, to be 1 phenacyl group/enzyme molecule. In efforts to obtain information about the nature and mechanism of formation of the phenacyl α-chymotrypsin produced at neutral pH, alkylation reactions of modified α-chymotrypsins produced by His-57 functionalization with tosylphenylalanine chloromethyl ketone and by Met-192 oxidation to the sulfoxide have been investigated. The combined results of these studies have been initially interpreted in terms of a neutral pH, phenacyl bromide modification resulting in formation of a new modified enzyme via the Met-192 sulfonium salt.

Specific chemical modification of the methionine-192 amino acid residue of α-chymotrypsin using a variety of alkylating agents has been the subject of numerous investigations (Schramm and Lawson, 1963; Lawson and Schramm, 1965; Kezdy et al., 1967; Sigman et al., 1967, 1969; Gibian et al., 1969; Stevenson and Smillie, 1970; Jones and Hysert, 1972; Naider and Bohak, 1972; Alazard et al., 1973; Hille and Koshland, 1967). Schramm and Lawson (1963) were the first to provide evidence for the nature of the modification of α-chymotrypsin using hydrophobic alkyl halides at pH 5.1. The site of alkylation was deduced from amino acid analysis data that showed the absence of one of the two methionine residues of the native enzyme. Further evidence to support the postulate that modification of α-chymotrypsin with phenacyl bromide

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at pH 5.1 leads to the methionine-192 phenacyl sulfonium salt has been provided by Naider and Bohak (1972) who showed that regeneration of esterase activity can be accomplished by incubating the phenacyl-modified enzyme with 2-mercaptoethanol, a nucleophile expected to participate in displacement reactions that liberate native α-chymotrypsin. More recent investigations resulting from a continuation of earlier studies of phenacyl α-chymotrypsin (Glover et al., 1974), however, have led to results that indicate that the modification of this enzyme with phenacyl bromide is not as straightforward as originally expected. The results of our current studies demonstrate that 2-mercaptoethanol treatment of modified α-chymotrypsin, prepared at pH 7.0 or 4.0, followed by incubation at pH 7.0, does not lead to facile nucleophilic displacement at the phenacyl grouping and liberation of the native enzyme. We would like to report these results along with those that implicate a pH-dependent secondary modification of the initially formed methionine-192 modified enzyme that alters the nature and, perhaps, location of the phenacyl moiety.

Materials and Methods

Analytical Techniques. Bovine α-chymotrypsin (Worthington, three-times crystallized and lyophilized, lot CDI-2KD) was purified before use by gel filtration (Yapel et al., 1966). The number of equivalents of functional active sites in α-chymotrypsin and modified enzymes was determined to be greater than 90% of the theoretical amount in all cases using the titration method (Schonbaum et al., 1961) with *N-trans*-cinnamoylimidazole. Esterase rate assays used *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (Glover et al., 1974). All ultraviolet–visible spectrophotometric measurements were made using a Beckman Acta-III uv[1]–visible spectrophotometer. Radioactivity was determined in Aquasol solutions (New England Nuclear Corporation) using a Beckman LS-100 liquid scintillation system.

Chemicals. The sources of chemicals used are as follows: *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester, *N*-tosylphenylalanine chloromethyl ketone (Tos-PheCH_2Cl), and *N-trans*-cinnamoylimidazole (Sigma Chemical Co.), phenacyl bromide (Eastman Organic Chemicals), [Carbonyl-^{14}C]-benzoic acid (sp act. 14.9 mCi/mmol) (New England Nuclear), and Sephadex G-25 fine (Pharmacia Fine Chemicals). [Carbonyl-^{14}C]phenacyl bromide (sp act. 1.21×10^8 dpm/mmol) was prepared using the procedure described earlier (Glover et al., 1974). All other chemicals used were reagent grade or better.

Tos-PheCH_2Cl-Modified α-Chymotrypsin. Tos-PheCH_2Cl-inactivated α-chymotrypsin was prepared according to the procedure of Schoellmann and Shaw (1962).

α-Chymotrypsin Met-192 Sulfoxide. α-Chymotrypsin Met-192 sulfoxide was prepared using the method of Weiner et al., (1966).

Modification of α-Chymotrypsin at pH 4.0, 5.1, and 7.0 with Phenacyl Bromide and Reactivation with 2-Mercaptoethanol. Alkylation of purified α-chymotrypsin with phenacyl bromide was accomplished using a modification of the procedure reported by Schramm and Lawson (1963). Dealkylations of Met-192-modified phenacyl α-chymotrypsin with 2-mercaptoethanol followed the method of Naider and Bohak (1972).

Typical procedures were as follows: α-Chymotrypsin (50 mg, 2 μmol) was dissolved in deionized, argon-purged water (11.25 ml) and absolute ethanol (1.2 ml) was added slowly. The pH of the enzyme solution was then adjusted to either 4.0, 5.1, or 7.0 using appropriate volumes of 0.1 N potassium hydroxide. To this mixture was added a solution of phenacyl bromide (8 mg, 40 μmol) in absolute ethanol (0.125 ml). The reaction mixture was then stirred in the dark at 25 °C. Aliquots were removed at varying time intervals for measurement of the percent inactivation and reactivation by 2-mercaptoethanol, using the procedure and esterase activity assay described above. After the esterase activity decreased to a constant value (30, 150, and 150 min for the pH 4.0, 5.1, and 7.0 inactivations, respectively), the inactivation mixture was acidified to pH 3 (using 0.1 N hydrochloric acid), gel filtered (using 1.0 mM hydrochloric acid as eluent), lyophilized, and the resulting solid enzyme was stored in the dark at −5 °C. It should be noted that the pH of the inactivation mixtures did not change during the course of the alkylation reactions.

Reactivation reactions using the α-chymotrypsin inactivation mixtures were performed during the course of each experiment. Fixed aliquots of the inactivation mixtures were added to 1.0 ml of a 12 mM 2-mercaptoethanol solution in 0.05 M potassium phosphate (pH 7.5). Esterase activity assays were performed on these mixtures after 5 min of incubation to determine the percent reactivation compared to control mixtures not containing 2-mercaptoethanol.

The results of these experiments are recorded in Figure 1, which contains plots of the enzymatic activity of the phenacyl bromide–α-chymotrypsin modification reaction mixtures and of the reactivation mixtures after incubation with 2-mercaptoethanol vs. time. Enzymatic activities are recorded as percentages relative to the initial readings for solutions containing native α-chymotrypsin.

The Effect of pH on the Nature of Phenacyl Modified α-Chymotrypsin. The pH 4–7 Modified Enzyme. Phenacyl α-chymotrypsin, prepared by reaction of phenacyl bromide with α-chymotrypsin at pH 4.0 as described above, was used for this experiment. The pH of a gel-filtered solution of this material was rapidly raised to 7.0 with 0.1 N potassium hydroxide. The esterase activities of aliquots of this mixture before and after incubation with 2-mercaptoethanol were measured at various time intervals after the change to pH 7.0 and until the enzymatic activities had reached their minimum values (ca. 70 min). The results of this experiment are recorded in Figure 2, in which the enzymatic activities of the modification reaction mixture before and after incubation with 2-mercaptoethanol, during the pH 4.0 deactivation and after the increase in pH to 7.0, are plotted as a function of time. After the change in enzymatic activity was complete, the pH of the reaction mixture was lowered to 3.0.

An experiment similar to this was conducted using lyophilized phenacyl α-chymotrypsin, prepared at pH 4.0 according to the procedure described above. The pH of an aqueous solution of this enzyme was adjusted to 7.0 with 0.1 N potassium hydroxide. Enzymatic activity before and after incubation with 2-mercaptoethanol for 5 min at pH 7.5 was assayed using aliquots removed over a 90-min period. The results of this experiment are plotted in Figure 3. The resulting solution was gel filtered, lyophilized, and stored at −5 °C in the dark.

Results and Discussion

Dependence of Phenacyl Bromide Alkylation Reactions of α-Chymotrypsin on pH. α-Chymotrypsin was reacted with a 20 molar excess of phenacyl bromide in 10% aqueous ethanol solutions at pH 7.0. Unbuffered solutions were used in this and ensuing modifications, since we have noted that buffers sig-

[1] Abbreviations used are: uv, ultraviolet; Tos-PheCH_2Cl, *N*-tosylphenylalanine chloromethyl ketone.

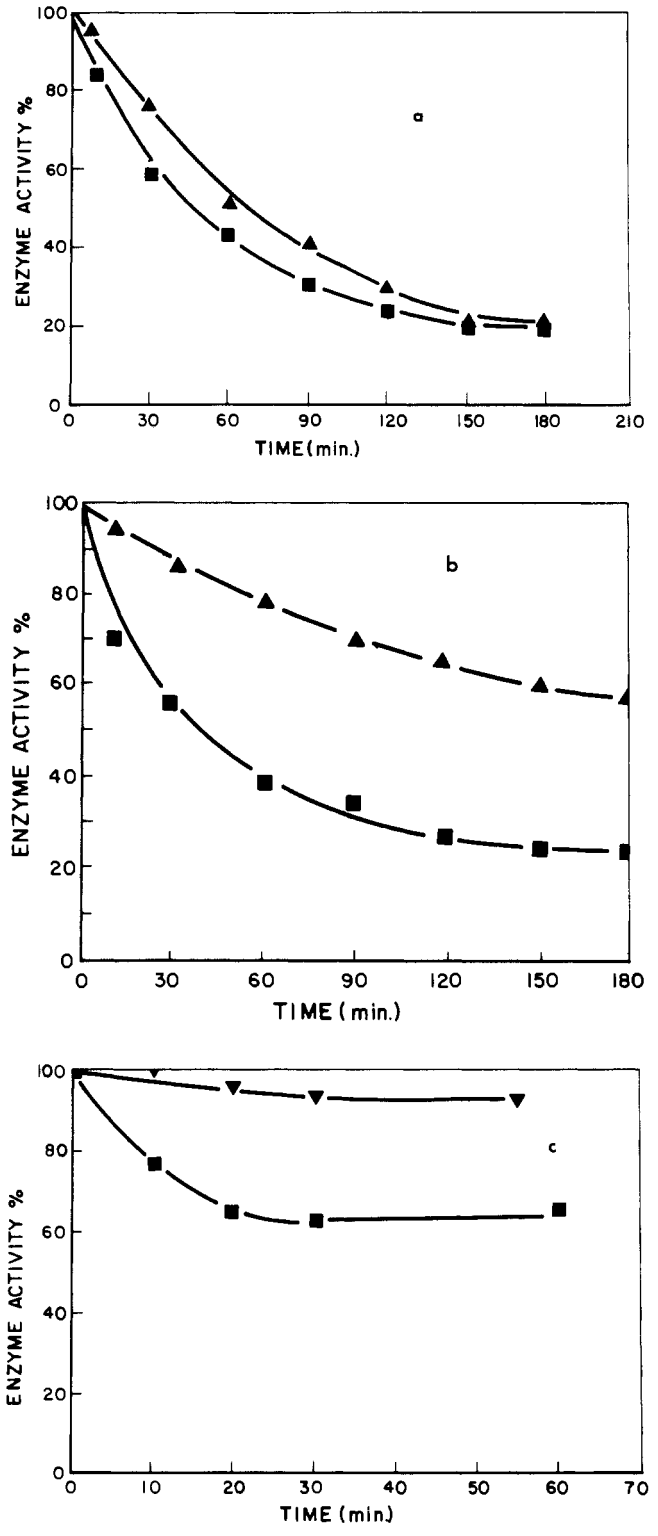


FIGURE 1: Enzymatic activities, recorded as percentages relative to native α-chymotrypsin solutions present at zero time, of aliquots from phenacyl bromide-α-chymotrypsin modification reaction mixtures (■), at (a) pH 7.0, (b) pH 5.1, and (c) pH 4.0, and of aliquots from modification reaction mixtures after incubation for 5 min at pH 7.5 with 2-mercaptoethanol (▲).

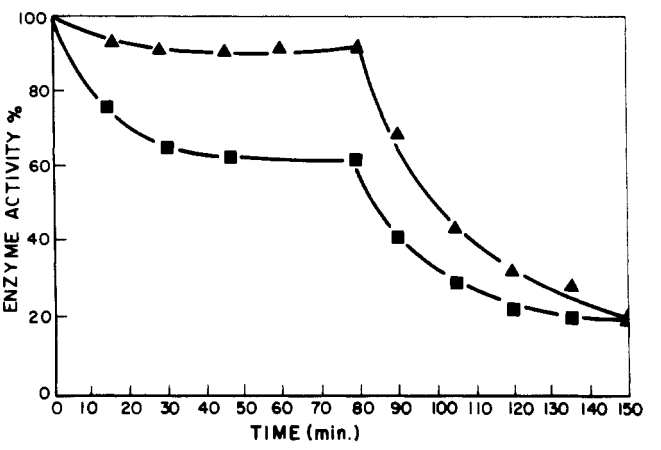


FIGURE 2: Enzymatic activities, recorded as percentages relative to native α-chymotrypsin solutions present at zero time, of aliquots from phenacyl bromide-α-chymotrypsin modification reaction mixtures (■), at pH 4.0 (0–80 min) and after adjustment of the pH to 7.0 (80–150 min), and of aliquots from modification reaction mixtures after reactions for 5 min at pH 7.5 with 2-mercaptoethanol (▲).

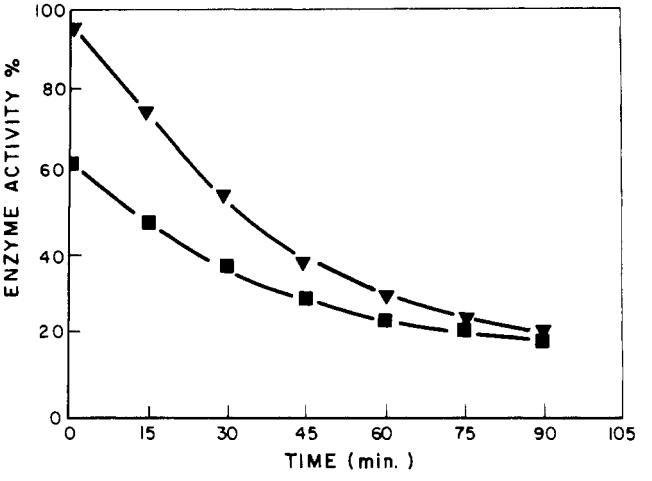


FIGURE 3: Enzymatic activities, recorded as percentages relative to native α-chymotrypsin, of aliquots from pH 4.0-prepared phenacyl α-chymotrypsin, gel filtered, lyophilized, and incubated at pH 7.0 (■) and after incubation at pH 7.5 for 5 min with 2-mercaptoethanol (▲).

nificantly retard the rates of these processes. The enzymatic activity of the modification-reaction solution decreased with time to a constant value 15–20% of the original after 150 min (Figure 1a). Throughout the course of the alkylation reaction, the ability of 2-mercaptoethanol to regenerate enzymatic activity was monitored. Aliquots were removed at the time periods indicated in Figure 1a and subjected to conditions identical to those used by Naider and Bohak (1972) to regenerate α-chymotrypsin from the phenacyl enzyme prepared at pH 5.1. Interestingly, the amount of free α-chymotrypsin resulting from treatment of the reaction medium with 2-mercaptoethanol, as judged by the percent increase in esterase activity relative to α-chymotrypsin solutions at zero time, decreased smoothly from 97% after an alkylation reaction time of 10 min to essentially 0% after 150 min.

The clear contrast between these results and those presented by Naider and Bohak for the modified enzyme prepared at pH 5.1 indicated that the nature of the α-chymotrypsin alkylation reaction with phenacyl bromide is dependent on the pH of the reaction medium. In order to confirm this hypothesis, modification reactions were conducted at pH 5.1 and 4.0. Both the decrease in esterase activity and ability to regenerate free α-chymotrypsin with 2-mercaptoethanol were monitored as a function of time (Figure 1b,c). The esterase activity of the pH 5.1 alkylation reaction mixture decreased to a constant value 20–25% of the original after a 150-min reaction time. In contrast to the results from the pH 7.0 modification reaction, re-

TABLE I: Stoichiometries of α-Chymotrypsin Modification Reactions Using [*carbonyl*-^{14}C]Phenacyl Bromide.

Entry	Enzyme Reactant (pH of Reaction Medium)	dpm/mmol of Phenacylated Enzyme[a]	Reaction Stoichiometry as Number of Phenacyl Groups/Enzyme Molecule: Based on [*carbonyl*-^{14}C] Phenacyl Bromide[b]	Reaction Stoichiometry as Number of Phenacyl Groups/Enzyme Molecule: Relative to pH 4 Enzyme
1	α-CT (4.0)	9.72×10^7	0.80	1.00
2	α-CT (4.0–7.0)	1.00×10^8	0.83	1.04
3	α-CT (7.0)	1.44×10^8	1.19	1.48
4	Tos-PheCH$_2$Cl-α-CT (4.0)[c]	5.72×10^6	0.05	0.06
5	Tos-PheCH$_2$Cl-α-CT (7.0)[d]	4.40×10^7	0.36	0.45
6	Met-192-sulfoxide-α-CT (4.0)[e]	1.26×10^7	0.10	0.13
7	Met-192-sulfoxide-α-CT (7.0)[f]	3.46×10^7	0.29	0.36

[a] Radioactivity determined on enzyme purified by gel filtration at pH 3.0; enzyme concentrations were by weight under identical conditions after gel filtration and lyophilization. [b] Specific activity of phenacyl bromide used, determined on the dimethyl phenacyl sulfonium salt derivative, was 1.21×10^8 dpm/mmol. [c] Tos-PheCH$_2$Cl-α-chymotrypsin was incubated with [*carbonyl*-^{14}C]phenacyl bromide at pH 4.0 for 45 min. [d] Tos-PheCH$_2$Cl-α-chymotrypsin was incubated with radioactive phenacyl bromide at pH 7.0 for 150 min. [e] Met-192-sulfoxide derivative of α-chymotrypsin was incubated with radioactive phenacyl bromide for 45 min at pH 4.0. [f] Met-192-sulfoxide derivative of α-chymotrypsin was incubated with radioactive phenacyl bromide for 150 min at pH 7.0.

generation of free α-chymotrypsin using 2-mercaptoethanol still occurred after 180 min at which time a value of 55% regenerated activity was obtained. A confirmation of this trend was obtained by monitoring the pH 4.0 modification reaction. The esterase activity in this case reached a constant value 60–65% of the original after short reaction times. In addition, the activity regenerated by treatment with 2-mercaptoethanol remained at the high value of 90–95% throughout the entire reaction.

Secondary Modification Reaction of Phenacyl α-Chymotrypsin. The pH dependence of the nature of phenacyl-modified α-chymotrypsin, as judged by final esterase activity and the ability to regenerate free α-chymotrypsin using 2-mercaptoethanol, indicates that, perhaps, a competing or ensuing modification reaction is occurring in addition to that resulting in alkylation of Met-192 and production of a sulfonium salt. Importantly, this secondary modification would need to meet the criterion of being insensitive to dealkylation by 2-mercaptoethanol. In order to determine the origin of a possible secondarily-modified α-chymotrypsin, i.e., if it is forming directly from phenacyl bromide and α-chymotrypsin at high pH or indirectly from the initially formed Met-192 phenacylsulfonium salt, modified α-chymotrypsin prepared at pH 4.0 was incubated at pH 7.0. The enzymatic activity of the reaction mixture and the amount of α-chymotrypsin regenerated by treatment with 2-mercaptoethanol displayed parallel decreases after the pH was increased (Figure 2). The esterase activity reached a constant value of ca. 20% based upon pure α-chymotrypsin. The coincidence of this value with that obtained by the direct alkylation at pH 7.0 indicates that the same modified enzyme is being produced from both reactions. Also, the inability of 2-mercaptoethanol to dealkylate the phenacyl-enzyme prepared in this indirect fashion again parallels the behavior of the enzyme prepared at pH 7.0. Modified enzyme, prepared at pH 4.0, separated from excess phenacyl bromide by gel-filtration at pH 3.0 and incubated at pH 7.0, showed similar changes in esterase activity and sensitivity to 2-mercaptoethanol (Figure 3). This observation allows elimination of the trivial explanation of the initial result that attributes loss of activity and reactivation to the presence of excess phenacyl bromide in the reaction mixture and an ensuing, rapid and direct pH 7.0 alkylation.

Stoichiometry of the Modification Reaction. The stoichiometries of the phenacyl bromide alkylation and 2-mercaptoethanol reactivation reactions were determined using ^{14}C-labeled phenacyl bromide. The radioactive enzymes were prepared from α-chymotrypsin and [carbonyl-^{14}C]phenacyl bromide at pH 4.0 and 7.0, and by incubation of the enzyme prepared at pH 4.0 and incubated at 7.0 (the pH 4.0, 7.0, and 4.0–7.0 enzymes, respectively). Each of the enzymes was separated from unreacted phenacyl bromide by gel filtration at pH 3.0 and subjected to liquid scintillation counting. The ^{14}C content of the pH 4.0 and 4.0–7.0 enzymes indicated the presence of approximately one phenacyl group per α-chymotrypsin molecule (entries 1 and 2 in Table I). The pH 7.0 enzyme, on the other hand, contained 1.19 equivalents of phenacyl moieties (entry 3 in Table I). Significantly, the excess radioactivity in the pH 7.0 enzyme could be removed by treatment with hydroxylamine (entry 5 in Table II), a reagent known to participate in dealkylation reactions which displace modifying groups covalently bound through ester linkages (Wilcox, 1967). Treatment of the pH 4.0–7.0 enzyme with hydroxylamine caused no measurable change in radioactive content (entry 4 in Table II).

From these experiments, it can be concluded that the site of final modification in the pH 7.0 and 4.0–7.0 enzymes is not a carboxylate grouping. In addition, the excess over the stoichiometric amount of radioactivity incorporated during the pH 7.0 modification reaction appears to be primarily due to alkylation of carboxylate groups. As expected, at pH 4.0 where most of these groups are unionized and, thus, less nucleophilic, no excess alkylation is detected (entry 1 in Table I).

Treatment of the radioactively labeled pH 4.0 enzyme with 2-mercaptoethanol resulted in removal of 94% of the radioactive label (entry 1 in Table II). On the other hand, no significant loss of radioactivity was detected when the pH 7.0 or the 4.0–7.0 modified enzyme was subjected to identical reaction conditions with this thiol reagent (entries 2 and 3 in Table II). These results parallel closely those obtained using enzymatic activity as a monitor of dealkylation or α-chymotrypsin regeneration and confirm the conclusion that the pH 7.0 and 4.0–7.0 modified enzymes do not contain the simple Met-192 sulfonium salt grouping that is formed from the phenacylation reaction at pH 4.0.

Active Site and Met-192 Blocked Enzymes. Further experiments were designed and conducted in order to obtain additional information about the nature of the pH 7.0 and 4.0–7.0 phenacyl enzymes and the mechanism of formation. If alkylation of Met-192 of α-chymotrypsin by phenacyl bro-

TABLE II: Dealkylation Reactions of Phenacyl-Modified Enzyme Monitored by Loss of Radioactive Label.

Entry	Nature of Phenacyl Modified Enzyme[a]	Dealkylation Reagent	dpm/mmol of Recovered Enzyme[b]	Number of Phenacyl Groups Remaining/Enzyme Molecule[c]	Extent of Dealkylation; Number of Phenacyl Groups Lost/Enzyme Molecule[c]
1	pH 4.0 α-CT	2-Mercapto-ethanol[d]	6.10×10^6	0.06	0.94
2	pH 4.0–7.0 α-CT	2-Mercapto-ethanol[d]	1.07×10^8	1.10	~0
3	pH 7.0 α-CT	2-Mercapto-ethanol[d]	1.33×10^8	1.37	0.11
4	pH 4.0–7.0 α-CT	Hydroxyl-amine[e]	1.02×10^8	1.05	~0
5	pH 7.0 α-CT	Hydroxyl-amine[e]	1.09×10^8	1.12	0.36
6	pH 4.0–7.0 α-CT	2-Mercapto-ethanol[d,f]	1.02×10^8	1.05	0.01
7	pH 7.0 Tos-PheCH$_2$Cl-α-CT	Hydroxyl-amine[e]	1.30×10^7	0.13	0.32
8	pH 7.0 Met-192-sulfoxide-α-CT	Hydroxyl-amine[e]	1.60×10^7	0.16	0.20

[a] Modified enzymes used are those described having the specific activities given in Table I. [b] Radioactivity determined on enzyme purified by gel filtration at pH 3.0. [c] Based upon dpm/mmol of pH 4.0 modified α-CT given in Table I, entry 1. [d] Modified enzymes incubated with 0.012 M 2-mercaptoethanol, 0.05 M potassium phosphate, 0.1 M potassium chloride at pH 7.5 for 5 min. [e] Modified enzymes incubated with 1.0 M hydroxylamine at pH 6.0 for 300 min, then gel filtered and lyophilized. [f] Modified enzyme was incubated at pH 3.0 for 5 days prior to treatment with 2-mercaptoethanol.

mide is a necessary step in a sequential process for generation of the pH 7.0 enzyme, as the data obtained from monitoring the pH 4.0–7.0 reaction appear to indicate, the presence of covalently bound, sterically bulky groups in the active-site region should act to decrease the rate of phenacylation of the Met-192 residue at all pH values and, importantly, at pH 7.0. This expectation is based upon preliminary data (Mariano et al., 1974) that indicates that alkylation of Met-192 of α-chymotrypsin by phenacyl bromide is inhibited by the close structural analogue, acetophenone, and, therefore, that the reaction follows an affinity-controlled pathway requiring preliminary reversible binding of the reagent to the hydrophobic, active center of the enzyme. The Tos-PheCH$_2$Cl (Schoellman and Shaw, 1962) derivative of α-chymotrypsin appeared useful in this regard, since it has been shown that Tos-PheCH$_2$Cl alkylates the active site His-57 giving an enzyme having the bulky tosylphenylalanyl ketonic grouping in close proximity to the Met-192 residue (Sigler, 1968). Incubation of this derivatized enzyme with radioactive phenacyl bromide at pH 4.0 for 45 min led to incorporation of radioactive label that corresponds to only 0.06 equiv of phenacyl groups/enzyme molecule when compared with phenacyl uptake under similar reaction conditions using the native enzyme (entry 4 in Table I). Similarly, when the alkylation reaction was conducted at pH 7.0 using Tos-PheCH$_2$Cl α-chymotrypsin, only 0.45 equiv of radioactivity were incorporated in comparison to 1.48 equiv when the native enzyme was used (entry 5 in Table I). The excess phenacyl incorporation during the pH 7.0 reaction using the Tos-PheCH$_2$Cl enzyme was shown to be the result of carboxylate modifications, since hydroxylamine treatment serves to remove nearly all radioactive content (entry 7 in Table II).

The question of whether or not alkylation by phenacyl bromide at Met-192 is an obligatory first step in production of the new pH 7.0 phenacyl enzyme was pursued further using the Met-192-sulfoxide derivative (Weiner et al., 1966) of α-chymotrypsin, in which the Met-192 thioether functional group is replaced by the much less nucleophilic sulfoxide. Treatment of this oxidized α-chymotrypsin with [^{14}C]phenacyl bromide at pH 4.0, the same reaction conditions used previously, led to the incorporation of only 0.13 equiv of radioactivity (entry 6 in Table I). Similarly, alkylations with [*carbonyl*-^{14}C]-phenacyl bromide at pH 7.0 gave recovered enzyme containing 0.36 equiv of phenacyl groups (entry 7 in Table I). Here as in the case above, a portion of these groups could be removed by incubation with hydroxylamine (entry 8 in Table II).

Spectrophotometric Analysis. Spectrophotometric methods were employed in order to gain additional information about the nature of the pH 7.0 enzyme and the mechanism for the changes occurring upon incubation of the pH 4.0 enzyme at pH 7.0. The difference uv spectra of purified pH 4.0 phenacyl α-chymotrypsin, vs. α-chymotrypsin, both in pH 7.0 solution, were recorded at various times after initial mixing (Figure 4). The continuous changes occurring during the time course of this reaction after the initial 10-min period were characterized by the presence of an isosbestic point at 268 nm, an increase in the absorbance at the low energy maximum (300 nm), and a decrease in absorbance and shift in the high energy maximum. The changes noted appear consistent with a pH 7.0-induced slow chemical change of the pH 4.0 enzyme leading to a new modified α-chymotrypsin. The initial rapid process occurring within the first 10-min period is most probably a conformational change (Vandlen and Tulinsky, 1973).

Importantly, the velocity of the process detected by the uv difference spectral changes during pH 7.0 incubation of the phenacyl enzyme, prepared at pH 4.0, is qualitatively the same as that measured using enzymatic activity as the monitoring method. This demonstrated that the decrease in enzymatic activity, the decrease in the ability of 2-mercaptoethanol to regenerate α-chymotrypsin, and the changes in uv difference spectra are all the result of one secondary process that is taking place to transform the modified α-chymotrypsin containing the Met-192 phenacyl sulfonium salt to the new pH 7.0 enzyme.

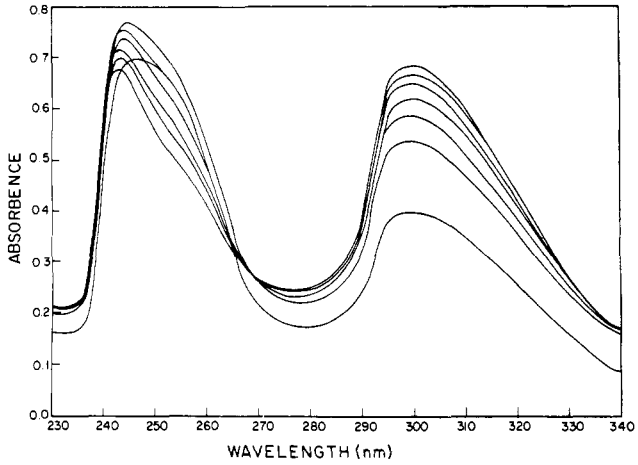
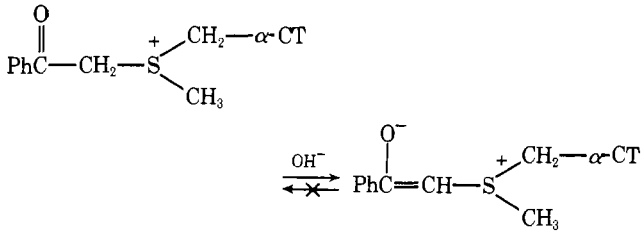


FIGURE 4: Difference spectra of pH 4.0 phenacyl α-chymotrypsin (35 μM) vs. α-chymotrypsin (35 μM) in 0.05 M potassium phosphate containing 0.1 M potassium chloride at pH 7.0. The spectra in order of increasing absorbance at 300 nm were recorded at 0-, 10-, 20-, 30-, 45-, 60-, and 90-min intervals after initial mixing.

Sulfonium Salt–Ylide Equilibrium. A simple explanation of the changes occurring in phenacyl α-chymotrypsin at high pH implicates the Met-192 sulfonium ylide. It is possible that the sulfonium salt function is undergoing an irreversible deprotonation to generate the ylide grouping (Sigman et al., 1969) as shown schematically below. The sulfonium ylide of

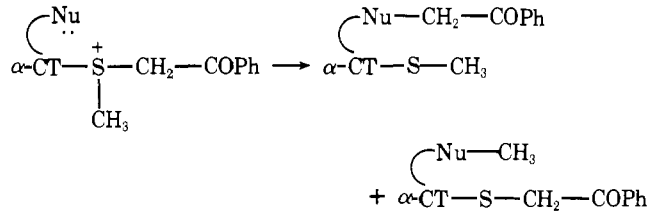


phenacyl α-chymotrypsin would of course be resistant to dealkylation under 2-mercaptoethanol reaction conditions. In addition, the uv difference spectral changes associated with this acid–base reaction induced by changes in the pH of the medium from 4.0 to 7.0 are expected to be similar to those we have noted. The increased absorbance of the long wavelength maximum would be a result of the increased conjugation in the ylide chromophore. However, several observations allow assignment of a low probability to this rationale.

First, the radioactive pH 4.0–7.0 enzyme was incubated in pH 3.0 solution for 5 days and subjected to 2-mercaptoethanol dealkylation reaction conditions. The radioactive content of the recovered enzyme was nearly equal to that found initially indicating no loss of phenacyl groups (entry 6 in Table II). Second, reversible heat denaturation at pH 2.1 (Havsteen et al., 1963) of both the pH 7.0 and 4.0–7.0 phenacyl enzymes gave recovered enzymes with 50% of the initial activities of the modified enzymes. Treatment of each of these with 2-mercaptoethanol under the typical conditions led to no observable increase in the enzymatic activity. Thus, if an irreversible sulfonium salt–ylide interconversion were responsible for the observed behavior of the pH 7.0 or 4.0–7.0 enzyme, a rationalization for the diminishingly slow reprotonation back to the sulfonium salt form, even under denaturating conditions, would need to be available. Therefore, we can conclude that the pH-dependent changes noted for phenacyl α-chymotrypsin are most probably not due to a simple irreversible acid–base process.

Conclusions

From these studies, it has become apparent that the chemistry of phenacyl methionine sulfonium salts of proteins can perhaps be complicated by secondary intraprotein processes that result from the chemical lability of the initial modification. The results found from studies of the phenacyl bromide–α-chymotrypsin reaction appear to provide evidence for an initial alkylation of Met-192, located near the active site of the enzyme, and a secondary alkylation reaction at higher pH values in which either the phenacyl or methyl group of the sulfonium salt is transferred to a nucleophilic side chain of a proximal amino acid residue. This conversion of the sulfonium



salt form of the modified enzyme to a new phenacyl α-chymotrypsin would represent another example of a secondary, selective chemical modification reaction of proteins that should lead to chemical information concerning the proximity of nucleophilic side chains to the site of initial labeling. Further studies are currently underway to define more precisely the nature of the new modified α-chymotrypsin produced at high pH by reaction with phenacyl bromide and the mechanism for its production.

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Pyrrolo[1,4]benzodiazepine Antibiotics. Biosynthesis of the Antitumor Antibiotic 11-Demethyltomaymycin and Its Biologically Inactive Metabolite Oxotomaymycin by *Streptomyces achromogenes*†

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ABSTRACT: 11-Demethyltomaymycin, an antitumor antibiotic produced by *Streptomyces achromogenes*, and its biologically inactive metabolite oxotomaymycin are biosynthesized from L-tyrosine, DL-tryptophan, and L-methionine. The anthranilate part of 11-demethyltomaymycin is derived from tryptophan probably via the kynurenine pathway. The predominant loss of tritium from DL-[5-^{3}H]tryptophan, during its conversion to 11-demethyltomaymycin and oxotomaymycin is interpreted to mean by NIH shift rules, that the main pathway to the 5-methoxy-4-hydroxy anthranilate moiety is through hydroxylation at C-8 prior to hydroxylation at C-7. The methoxy carbon is derived from the *S*-methyl group of methionine by transfer of an intact methyl group. The ethylideneproline moiety of 11-demethyltomaymycin is biosynthesized from tyrosine, without a 1-carbon unit from methionine. The results of biosynthetic feeding experiments with L-[1-^{14}C, 3- or 5-^{3}H]tyrosine are consistent with a "meta" or extradiol cleavage of 6,7-dihydroxycyclodopa as has also been demonstrated previously for anthramycin and lincomycin A. An experiment in which L-[1-^{14}C, Ala-2,3-^{3}H]tyrosine was fed showed that both of the β hydrogens of this amino acid are retained in 11-demethyltomaymycin. It has been demonstrated in cultures and washed cell preparations that 11-demethyltomaymycin is enzymatically converted to oxotomaymycin by an intracellular constitutive enzyme. Conversion of oxotomaymycin to 11-demethyltomaymycin by these same preparations could not be demonstrated. The enzymatic activity associated with the conversion of 11-demethyltomaymycin to oxotomaymycin is not limited to the 11-demethyltomaymycin production phase, since trophophase cells and even cells from 11-demethyltomaymycin nonproducing cultures of *S. achromogenes* were equally active in converting 11-demethyltomaymycin to oxotomaymycin.

11-Demethyltomaymycin (DMT)[1] is one of three pyrrolo[1,4]benzodiazepine antitumor antibiotics isolated from culture filtrates of actinomycetes. The other antibiotics within this class are anthramycin and sibiromycin. The structures of these compounds and the DMT related compound oxotomaymycin (OT) are shown in Scheme I. DMT was originally isolated from the culture filtrates of *Streptomyces achromogenes* by Professor K. Arima and co-workers at the University of Tokyo and the Fujisawa Pharmaceutical Company (Arima et al., 1972). The structures of DMT and the closely related compound, OT, which is also produced by cultures of *S. achromogenes*, were established by Kariyone et al. (1971). DMT and its methylated product tomaymycin have been shown to possess antiviral activity against *Escherichia coli* T_1 and T_3 phages, antibacterial activity against gram-positive bacteria (Arima et al., 1972), and cytotoxic activity towards Leukemia L1210 cells (Nishioka et al., 1972). All of the biological properties of these pyrrolo[1,4]benzodiazepine antibiotics can be attributed to the effects of these compounds on nucleic acid biosynthesis. DMT and tomaymycin appear to act by virtue of their ability to bind to DNA and thereby interfere with the function of DNA (Nishioka et al., 1972).

As part of a continuing effort to discover more about the biosynthesis of these pyrrolo[1,4]benzodiazepine antibiotics, we are currently studying in our laboratory the biosynthesis of anthramycin, DMT, OT, and sibiromycin. Our work on anthramycin (Hurley et al., 1975b) and preliminary data on DMT (Hurley et al., 1975a) have already appeared. For both of these antibiotics and sibiromycin (Hurley et al., unpublished data) we have shown that the anthranilate moiety of these antibiotics is derived from tryptophan probably via the kynurenine pathway. Our studies on anthramycin (Hurley et al., 1975b) show conclusively that the acrylamide proline moiety of this antibiotic is derived from tyrosine (7 carbons) and methionine (1 carbon) to give rise to the demonstrated labeling pattern shown in Scheme II. Dopa, or more likely its condensation product with the anthranilate part of the molecule, is considered to be the intermediate that undergoes "meta" or extradiol cleavage.

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[1] Abbreviations used are: DMT, 11-demethyltomaymycin; OT, oxotomaymycin; DMAA, methyl-4,5-dimethoxyanthranilate; TLC, thin-layer chromatography; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.