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Control of Conformation of α-Chymotrypsin through Chemical Modification†

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ABSTRACT: Oxidation of Met-192 of α-chymotrypsin enhances the stability of the active substate of this protein relative to the inactive substate and also reduces the autolysis rate making this derivative the currently most favorable one for studies of mechanism. A new reagent, trichloromethanesulfonyl chloride, oxidizes Met-192 to the sulfoxide form in a rapid stoichiometric reaction. In the usual pH range employed for chymotrypsin kinetics this derivatized enzyme exists entirely in the active substate until at least pH 9.1. In the course of this work we found that *N*-bromosuccinimide and *N*-chlorosuccinimide oxidize Met-192 at least as rapidly as they oxidize any indole groups. It is probable that this heretofore unrecognized behavior is common in studies with these reagents. The further oxidation by H_2O_2 in urea of Met-180 produces a dimethionine sulfoxide derivative which has a rapid autolysis rate especially at slightly higher temperature. Under conditions at which autolysis is minimized the derivative has relatively high amidase activity in contrast to previous reports.

There are a number of conformational substates which characterize α-chymotrypsin in solution (Hess, 1971; Lumry and Biltonen, 1969); any experimental procedures which can either limit the number of substates or explain their importance to the catalytic process will aid significantly in our understanding of the mechanism of action of this enzyme. We have used chemical modification to achieve these ends. It is not yet generally realized that the "inactive" species forms a significant fraction of the total enzyme in α-chymotrypsin solutions at all pH values usually chosen for investigation (Fersht and Requena, 1971; Fersht, 1972). The equilibrium between active and inactive forms is a sensitive function of substrate concentration, pH, and temperature, so that in effect a major fraction of previous quantitative studies of the enzyme with or without small specific molecules is in error. In this paper we show that a well-known chemical modification of the enzyme eliminates the inactive species over a wide pH range without qualitative change in catalytic behavior. Although additional substates exist (Lumry and Biltonen, 1969), the derivative provides considerable improvement in substate control leading to a respectable beginning of quantitative studies of this enzyme.

Previous work involving chemical modification of chymotrypsin is extensive (Dixon and Schacter, 1964; Hess, 1971; Kosman and Piette, 1972; Nakagawa and Bender, 1970; Schacter and Dixon, 1964). Reagents have been developed which are specific for either active-site residues or for the amino acids which are believed to contribute significantly to maintaining this enzyme's tertiary structure. We emphasize that it is crucial to all chemical modification experiments that such reactions be "clean" and well defined. Not only must it be clear which residues have been modified and how, in addition interpretation of resulting changes in the enzyme's activity in terms of "specificity," "structure," "conformation," etc., must be made with the utmost caution (Dixon and Schacter, 1964). Changes in enzymatic properties which are ascribed to chemical modifications of the active site can in fact be due to conformational changes which render the enzyme inactive (Dixon and Schacter, 1964).

In this paper we reexamine a number of well-known α-

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chymotrypsin derivatives. We use a new reagent (trichloromethanesulfonyl chloride, CCl_3SO_2Cl) to oxidize Met-192 (three residues from the active-site Ser-195) of α-chymotrypsin to the sulfoxide. We show through a variety of physical and chemical measurements that in marked contrast to the parent this derivative exists *entirely* in its active conformation from *ca.* pH 7 to at least pH 9.1.

Experimental Procedure

α-Chymotrypsin. Salt-free, three-times crystallized α-chymotrypsin was obtained from Worthington Biochemical Co. and further purified by previously published methods (Yapel *et al.*, 1966). Protein concentrations were determined on a Cary 15 recording spectrophotometer using the extinction coefficient of ϵ_{280} 2.00 l. g^{-1} cm^{-1} and assuming a mol wt of 25,000.

Activity Assays. $AcTyrNH_2$,[1] AcTrpOEt,[2] and AcTyrOEt were obtained from Cyclo Chemical Corp. and were used without further purification. Rates of hydrolysis of the esters by α-chymotrypsin and its derivatives were performed using previously published spectroscopic techniques (Kumar and Hein, 1969; Schwert and Takenaka, 1955; Zerner *et al.*, 1964). Hydrolysis of $AcTyrNH_2$ was followed using Nessler's reagent under the following conditions. Enzyme (5 μM in each case) and substrate (2.55 mM) were incubated in pH 7.8 0.05 M phosphate at either 37° or room temperature (24°). Periodically 500 μl of incubation solution was added to 3 ml of a diluted solution of Nessler's reagent (*ca.* one-fourth of the original concentration, Fisher Scientific), and the absorbance at 490 nm was measured. Duplicate samples were run and reproducibility was in general better than ±0.01 absorbance unit. A control sample consisting of the amide and buffer incubated under similar conditions showed no detectable hydrolysis (*i.e.*, no net absorption at 490 nm) over 43 hr.

Active-site titrations were performed using standard methods (Schonbaum *et al.*, 1961). Typical values were approximately 90–93%, before and after modification experiments.

Autolysis Studies. Autolysis of α-chymotrypsin and its derivatives was studied in the following manner. Samples were incubated in 0.05 M phosphate, pH 7.8, 37°, and periodically assayed for activity against AcTrpOEt. Control samples of the same concentration were held at 4°, pH 4–5, during the same period. The activity of the control samples changed less than 10% during this time. Glass vials (4 drams) were used for incubation. It was assumed that the activity toward AcTrpOEt of incubated samples was directly proportional to the concentration of unautolyzed enzyme. It is possible, of course, that initial autolysis could give rise to polypeptide chains which were still enzymatically active. However, the results of Kumar and Hein (1970), who followed autolysis of α-chymotrypsin by correlating the rate of peptide bond cleavage with the rate of activity loss (AcTyrOEt was the substrate), suggest that even partially autolyzed products are enzymatically inactive. Their strongest evidence to support this contention is the fact that as activity decreased, the Michaelis constant for AcTyrOEt hydrolysis remained constant. That is, activity changes were directly ascribable to a change in (k_{cat}[E]). We assumed that a similar situation obtains for autolysis studies using AcTrpOEt to monitor residual enzyme activity. Good second-order kinetics for α-chymotrypsin autolysis were obtained; the results were in satisfactory agreement with those reported experiments obtained under similar but not identical conditions (Kumar and Hein, 1970; Johnson and Whateley, 1972).

Proflavine Binding. Proflavine hemisulfate (Sigma) was recrystallized in the dark twice and stored over Drierite. Binding experiments using previously published difference-spectrum techniques were performed (Bernhard *et al.*, 1966; Brandt *et al.*, 1967). A computer program which processed binding data according to the analytical forms (Rossotti and Rossotti, 1961) of the equations relating binding and absorbance changes (no approximations such as the Benesi–Hildebrand method (1949) were used here) was used to determine proflavine–chymotrypsin dissociation constants. Results agreed well with most literature values (Bernhard *et al.*, 1966; Brandt *et al.*, 1967; Marini and Caplow, 1972).

Circular Dichroism. CD curves from 227 to 350 nm were measured on the CD attachment of the Cary Model 60 spectropolarimeter. Enzyme concentrations were *ca.* 2.5×10^{-5} M, and a cell of path length of 0.5 cm was used.

Stopped-Flow Experiments. Stopped-flow experiments were performed on a "home built" device with a "dead time" of *ca.* 2 msec. It will be discussed in a future publication.

Preparation of α-Chymotrypsin Derivatives. [2][Met(O)]chymotrypsin and $[Met(O)]_2$chymotrypsin were prepared by H_2O_2 oxidation (Dixon and Schacter, 1964; Schacter and Dixon, 1964; Weiner *et al.*, 1966) according to previously published methods. $[Met(O)]_2$chymotrypsin was never lyophilized but was used in solution after the urea was dialyzed away.

The preparation of [1][Met(O)]chymotrypsin *via* CCl_3SO_2Cl oxidation is as follows. To 500 mg of α-chymotrypsin dissolved in 50 ml of water (*ca.* 4×10^{-4} M) adjusted to pH 3.5[3] was added with stirring 1 ml of an acetone solution of CCl_3SO_2Cl (*ca.* 5×10^{-2} M). After 10 min of stirring the solution was exhaustively dialyzed against 10^{-3} N HCl and then lyophilized. No change in activity could be detected as a result of lyophilization.

Iodoacetic acid-*1*-^{14}C was obtained from New England Nuclear Corp. (lot no. 465-244, 6 mCi/mmol). It was diluted with a solution of 0.02 M recrystallized nonradioactive iodoacetic acid (in 8 M urea, pH 2.8) to give a final activity of 1 μCi/ml and 0.05 Ci/mol of iodoacetic acid. Exhaustive alkylations of the various α-chymotrypsin derivatives using this ^{14}C label were employed (Dixon and Schacter, 1964; Schacter and Dixon, 1964).

pH and Proton Release Experiments. The pH was measured on a radiometer type 4c pH meter. Release of protons during the reaction of α-chymotrypsin or methionine and trichloro-

[1] Abbreviations used are: [1][Met(O)]chymotrypsin, monomethionine sulfoxide (position 192) chymotrypsin prepared from trichloromethanesulfonyl chloride; [2][Met(O)]chymotrypsin, the same, but prepared from H_2O_2; $[Met(O)]_2$chymotrypsin, dimethionine sulfoxide (positions 180 and 192) chymotrypsin, prepared *via* H_2O_2 in 8 M urea; iPr_2P-chymotrypsin, chymotrypsin which had been modified by phosphorylation of Ser-195 by the diisopropylphosphoryl group; $AcTyrNH_2$, *N*-acetyl-L-tyrosinamide; AcTrpOEt, *N*-acetyl-L-tryptophan ethyl ester; AcTyrOEt, *N*-acetyl-L-tyrosine ethyl ester; k_{cat}, rate constant (turnover number) for an enzyme-catalyzed reaction following Michaelis–Menten kinetics; [E], concentration of unautolyzed enzyme monitored in autolysis assay; SucNBr, *N*-bromosuccinimide; X-chymotrypsin, chymotrypsin which had reacted with 4 mol of SucNBr at pH 4.0; SucNCl, *N*-chlorosuccinimide.

[2] Lot 027035 was used in this work. D. W. Bolen (personal communication) used thin layer electrophoresis and confirmed our observations that this lot was essentially 100% pure.

[3] pH control is not crucial here. The reaction appears to be stoichiometric at least between pH 3 and 5.

methanesulfonyl chloride was followed with a Radiometer TTT1 pH-Stat, kindly provided by Dr. Ben Halloway.

Other Chemicals. SucNBr (Arapahoe Chemical Co.) and SucNCl (Aldrich Chemical Co.) were twice recrystallized from water. Trichloromethanesulfonyl chloride (Eastman) was recrystallized from ethanol and stored over Drierite. Buffers, salts, acetone, and H_2O_2 were all of reagent grade or better. Urea was deionized on an Amberlite MB 3 column at 40° before use.

Oxidation of Methionine to the Sulfoxide. Oxidation of the thioether linkage of methionine to the sulfoxide was performed using H_2O_2 according to published procedures (Toennies and Callan, 1939). The product was isolated by precipitating it from aqueous solution through the addition of excess acetone. Oxidation using CCl_3SO_2Cl was performed as follows. To 1.6 g of methionine dissolved in 100 ml of water (*ca.* 0.11 M) and adjusted to pH 3.2 with HCl was added with stirring 10 ml of an acetone solution of CCl_3SO_2Cl (*ca.* 1.1 M). At first much of the CCl_3SO_2Cl precipitated. After about 40 min of stirring it had all dissolved and the pH was about 0.8. The pH was readjusted to 3.3 with KOH. Excess acetone was added and the product was obtained by stirring the cloudy solution in a Dry Ice–acetone bath. Yields greater than 70% of the sulfoxide product were obtained.

Nuclear Magnetic Resonance Experiments. Experiments were performed on a Varian T-60 or A-60-A nmr spectrometer with probe temperatures of about 35 and 40°, respectively. The proton magnetic resonance spectrum of methionine dissolved in D_2O was essentially identical with the published spectra (Roberts and Jardetzky, 1970). The methionine sulfoxide derivative can be easily identified because of the significant downfield shift (*ca.* 0.7 ppm) of the sharp SCH_3 resonance upon oxidation to the sulfoxide. The other protons exhibit smaller changes in chemical shifts upon oxidation.

Results

Reaction of α-Chymotrypsin with CCl_3SO_2Cl. At moderately low pH α-chymotrypsin and CCl_3SO_2Cl react stoichiometrically (1:1) to generate a derivative which has enhanced activity toward AcTrpOEt and decreased activity toward AcTyrOEt (Figure 1).[4] Based on the stoichiometry of this reaction a "clean" derivative, in which only Met-192 has been oxidized, is produced. The following evidence supports this conclusion.

(1) Our independent experiments indicate that DL-methionine is oxidized by CCl_3SO_2Cl to the sulfoxide; the proton magnetic resonance spectrum of methionine which had been oxidized with H_2O_2 was identical with that of the product which was isolated from the reaction of methionine with CCl_3SO_2Cl.

(2) ^{14}C labeling experiments with iodoacetate in 8 M urea indicate that only half of the available methionines could be labeled after treatment of α-chymotrypsin with CCl_3SO_2Cl (Table I). This is consistent with the value expected if only Met-192 had been oxidized to the sulfoxide (Dixon and Schacter, 1964; Schacter and Dixon, 1964).

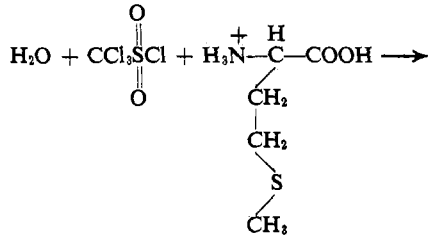
TABLE I: Radioactive Labeling of Methionines of α-Chymotrypsin and Its Derivatives.

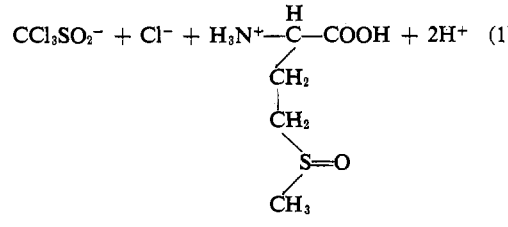
$$I^{14}CH_2COO^- + \overset{|}{S} \longrightarrow I^- + {}^{+}\overset{|}{S}-{}^{14}CH_2COO^-$$

	% Label Incorp.[d]
α-Chymotrypsin	100 ± 3[a]
[Met(O)]chymotrypsin	48 ± 1[b]
X-Chymotrypsin[c]	40 ± 2[b]

[a] Based on four separate determinations. [b] Based on two separate determinations. [c] In this derivative 4 mol of SucNBr was allowed to react with 1 mol of α-chymotrypsin at pH 4.0. After exhaustive dialysis and lyophilization, the resulting derivative was treated by the same labeling procedure. [d] Based on two methionines.

(3) Titration experiments indicate that in both the model reaction (methionine with CCl_3SO_2Cl), and the reaction of α-chymotrypsin with CCl_3SO_2Cl, 2 mol of proton/mol of methionine oxidized (or per mol of enzyme) is liberated. The probable mechanism is that of eq 1.





(1)

There is some precedent for such an oxidation reaction. CCl_3SO_2Cl can be thought of as a potential source of "positive" halogen, similar to reagents such as *tert*-butyl hypochlorite or SucNCl or SucNBr. These latter reagents are all potential oxidizing agents and have in fact been used for the oxidation of disulfides to the corresponding sulfoxides (Harville and Reed, 1968; Walling and Mintz, 1967). CCl_3SO_2Cl may act as an oxidizing agent in a related manner.

(4) The K_m for hydrolysis of AcTyrOEt by [Met(O)]-chymotrypsin[5] increases by a factor of *ca.* 3–4 in agreement with the changes observed in [Met(O)]chymotrypsin (Dixon and Schacter, 1964; Knowles, 1965; Kumar and Hein, 1969; Schacter and Dixon, 1964; Weiner *et al.*, 1966).

(5) The CD curves for α-chymotrypsin and the derivatives we examined are similar in the wavelength region under investigation (*ca.* 350–227 nm). However, the *magnitude* of the strongly negative band ($[\theta] \simeq -2 \times 10^3$ to -5×10^3 deg

[4] These activity changes exactly parallel the changes in activity which are observed for [Met(O)]chymotrypsin. However, we noted that in all cases the derivative prepared *via* H_2O_2 had slightly lower activity than [Met(O)]chymotrypsin. We believe these derivatives are nearly the same but that the H_2O_2 oxidation method must produce a side reaction which results in a derivative of slightly lower net activity. Active-site titrations, however, gave approximately the same results for both species (*ca.* 90–93%). There is some evidence (Dixon and Schacter, 1964) that side reactions do indeed occur under the conditions used for oxidation of Met-192 by H_2O_2.

[5] R. P. Taylor, unpublished observations.

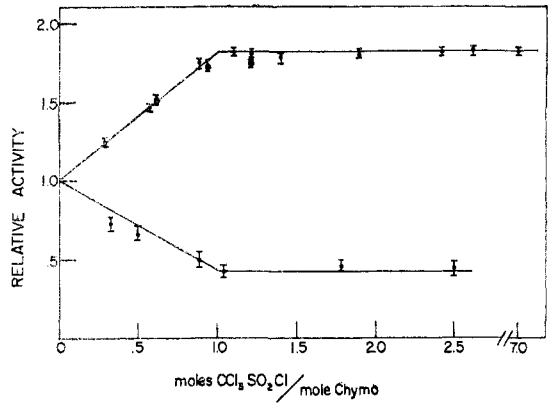


FIGURE 1: Stoichiometry of reaction between CCl_3SO_2Cl and α-chymotrypsin. Small aliquots of acetone solutions of CCl_3SO_2Cl (*ca.* 5×10^{-3} M) were added to aqueous solutions of α-chymotrypsin (*ca.* 2×10^{-5} M). Conditions for assays: (1) AcTrpOEt, 1.5 mM substrate, 0.2 M KCl, 0.05 M phosphate, pH 7.50, *ca.* 0.2 μM enzyme, 25°; under these conditions a turnover number of 33 sec^{-1} for untreated α-chymotrypsin is observed (not corrected for CI titration); (2) AcTyrOEt, 1.0 mM substrate, 0.10 M phosphate, pH 7.0, 25°; upper curve, AcTrpOEt; lower curve, AcTyrOEt. Chymo, chymotrypsin.

cm^2 $dmol^{-1}$) centered at about 231 nm has been shown to be quite sensitive to this protein's conformation (Cuppett *et al.*, 1971; Fasman *et al.*, 1966; Hess, 1971). The effects of chemical modification, temperature, salt, and pH on chymotrypsin conformation have been examined by monitoring their influence on this band. Our results indicate that in the very pH-sensitive region between 7 and 9, [1][Met(O)]chymotrypsin and [2][Met(O)]chymotrypsin are identical within small experimental error and are significantly different from α-chymotrypsin (Figure 2).

(6) Proflavine binding experiments indicate that [2][Met(O)]-chymotrypsin and [1][Met(O)]chymotrypsin have the same dissociation constants for proflavine binding (Figure 3) at pH 8.3 where α-chymotrypsin itself gives a significantly higher dissociation constant.

(7) There is no detectable change in activity when H_2O_2 is added to a solution of [1][Met(O)]chymotrypsin or CCl_3SO_2Cl is added to a solution of [2][Met(O)]chymotrypsin. Addition of either of these reagents to α-chymotrypsin solution does cause activity changes due to oxidation of Met-192 (Dixon and Schacter, 1964; Schacter and Dixon, 1964; Figure 1).

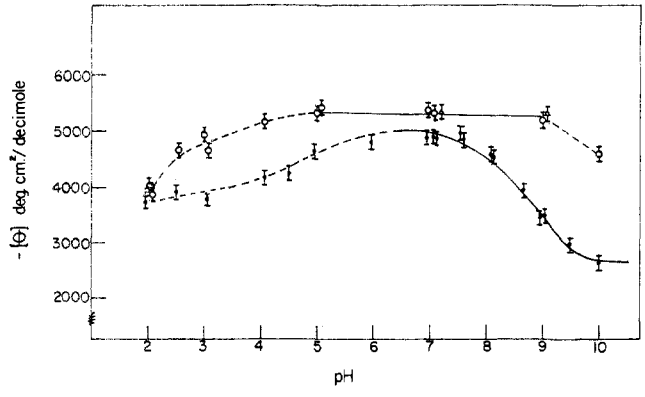


FIGURE 2: pH dependence of circular dichroism of α-chymotrypsin and [1][Met(O)]chymotrypsin: (○) [1][Met(O)]chymotrypsin; (△) [2][Met(O)]chymotrypsin; (●) α-chymotrypsin. The solid line for α-chymotrypsin is based on a computer fit to the data which gives a titration curve of $pK_a = 8.73$; λ 231 nm.

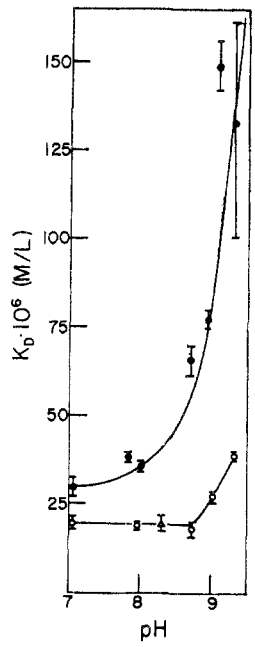


FIGURE 3: pH dependence of binding of proflavine to α-chymotrypsin and [1][Met(O)]chymotrypsin, 24°; same key as in Figure 2. The solid line for α-chymotrypsin is drawn for a titration curve of $pK = 8.75$.

Reaction of α-Chymotrypsin with N-Bromosuccinimide. Our results indicate that "positive" halogen sources such as CCl_3SO_2Cl are capable of oxidizing methionines. We have already noted that other positive halogen sources such as SucNBr can oxidize disulfide groups to sulfoxides. There is evidence which suggests that SucNBr can oxidize methionines (Lokshina and Orekhovich, 1964; Schmir and Cohen, 1961) and a recent report (Myer, 1972) indicates that SucNBr does oxidize the methionines in horse heart cytochrome *c*. Considering the fact that traditionally SucNBr has been used under mild conditions (pH 4, room temperature) to oxidize tryptophans in α-chymotrypsin (Spande *et al.*, 1966; Spande and Witkop, 1967; Viswanatha and Lawson, 1961) and in view of the apparent great susceptibility of Met-192 of α-chymotrypsin to oxidation, we decided to reinvestigate the reaction between α-chymotrypsin and SucNBr to determine if methionine oxidation occurs.

Oxidation of the indole ring of tryptophan to an oxindole results in a decrease in molar extinction coefficient for this chromophore of about 4.0×10^3 at 280 nm (Spande and Witkop, 1967). Thus, tryptophan oxidation in proteins is most easily studied by monitoring the effect of the potential oxidizing agent on the absorbance of the protein solution at 280 nm. These results can be analyzed readily to determine the number of moles of tryptophan oxidized per mole of reagent consumed (Spande and Witkop, 1967). Our experiments show that approximately 1 mol of SucNBr is involved in a secondary reaction with chymotrypsin which does not cause tryptophan oxidation (see extrapolated dotted line, Figure 4).[6] It is reasonable that this initial quantity of SucNBr is consumed in the oxidation of Met-192. The following evidence supports this hypothesis.

(1) We have found that the product isolated from the reaction of 1 mol of SucNBr with 1 mol of methionine is the sulf-

[6] Similar results have been obtained for SucNCl.

oxide (based on nmr evidence). The experimental procedure for oxidizing methionine with SucNBr is essentially identical with the method used with CCl_3SO_2Cl as the oxidizing agent.

(2) ^{14}C labeling experiments confirmed that treatment of α-chymotrypsin with SucNBr reduces the number of methionines available for alkylation (Table I). The fact that only 40% of the theoretically maximum possible label was incorporated suggests that some Met-180 oxidation occurs as more and more tryptophans are oxidized, probably concurrent with unfolding of the protein.

(3) There is no side reaction between SucNBr and either ^{2}[Met(O)]chymotrypsin or ^{1}[Met(O)]chymotrypsin (Figure 4). In these two species Met-192 has already been oxidized and addition of SucNBr apparently only causes tryptophan oxidation. The results extrapolate to the origin for these derivatives. In fact, it should be noted that the slopes of *all three lines* are approximately the same after the proposed initial oxidation of Met-192 is completed for α-chymotrypsin (Figure 4).[7]

In summary, we note that there is a 1:1 stoichiometry in the reaction between α-chymotrypsin and CCl_3SO_2Cl. Model experiments with methionine show that it is oxidized by CCl_3SO_2Cl to the sulfoxide with the liberation of two protons. Experiments with α-chymotrypsin itself indicate that this stoichiometric reaction also liberates two protons and, in fact, radioactive labeling experiments with ^{1}[Met(O)]chymotrypsin confirm that one methionine cannot be alkylated. The properties of this derivative are identical with ^{2}[Met(O)]chymotrypsin except for small differences in activity which are probably due to side reactions with H_2O_2. Our SucNBr experiments provide additional evidence that Met-192 is oxidized in ^{1}[Met(O)]chymotrypsin.

Properties of ^{1}[Met(O)]chymotrypsin,[8] A Comparison with α-Chymotrypsin. CIRCULAR DICHROISM. CD studies demonstrate that the magnitude of the negative ellipticity band at 231 nm is larger in ^{1}[Met(O)]chymotrypsin and less sensitive to pH than in α-chymotrypsin (Figure 2). The solid line drawn through the high pH experimental points for α-chymotrypsin is based on an apparent pK_a of 8.73 (see below).

PROFLAVINE BINDING STUDIES. At a given temperature and pH ^{1}[Met(O)]chymotrypsin binds proflavine more effectively than does α-chymotrypsin (Figure 3). The pH dependence of this interaction is much less pronounced for ^{1}[Met(O)]chymotrypsin than for α-chymotrypsin itself (Figure 3). The results for α-chymotrypsin are consistent with a transition of $pK_a \simeq 8.75$.

STOPPED-FLOW EXPERIMENTS. Fersht and Requena have recently used stopped-flow techniques to monitor the slow transient which occurs on mixing α-chymotrypsin and proflavine (Fersht and Requena, 1971; Fersht, 1972). We have duplicated their work and find similar results for α-chymotrypsin (Table II). We find no evidence for any slow transients for ^{1}[Met(O)]-chymotrypsin or for ^{2}[Met(O)]chymotrypsin. At much higher pH such transients appear (Table II) and they are faster than those observed for α-chymotrypsin at any pH we have used.

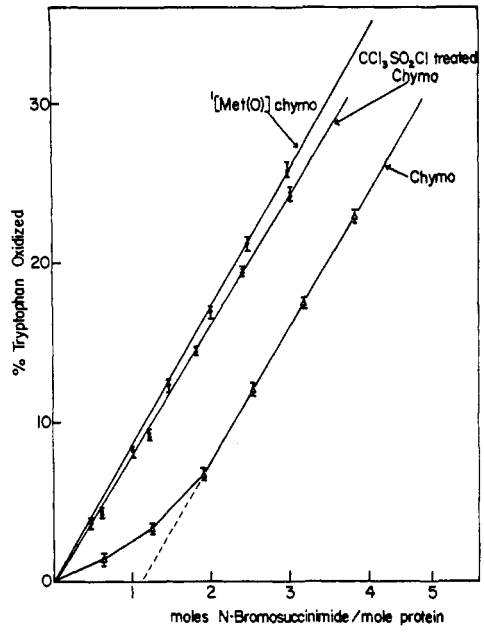


FIGURE 4: Oxidation of tryptophan by *N*-bromosuccinimide in α-chymotrypsin and its derivatives; small aliquots of aqueous solutions of SucNBr were added to enzyme solutions (24°, pH 4.0, *ca.* 2.6×10^{-5} M) and the resulting decrease in net absorbance at 280 nm was monitored. The absorbance changes were converted to moles of oxidized tryptophan using a difference in molar extinction coefficient between oxidized and unoxidized indoles of 4000 (Spande and Witkop, 1967). Chymo, chymotrypsin.

AUTOLYSIS EXPERIMENTS. ^{1}[Met(O)]chymotrypsin is more stable toward autolysis than α-chymotrypsin (Figure 5).

Properties of [Met(O)]$_2$chymotrypsin. One of the most interesting properties of this derivative involves its reported apparent lack of reactivity toward $AcTyrNH_2$ (Weiner *et al.*, 1966). We have repeated assays of the activity of this deriva-

TABLE II: pH Dependence of the First-Order Rate Constants Observed upon Mixing α-Chymotrypsin or ^{1}[Met(O)]chymotrypsin with Proflavine.[a]

	α-Chymotrypsin		^{1}[Met(O)]chymotrypsin	
pH	k_{obsd} (sec^{-1})	ΔA[b]	k_{obsd} (sec^{-1})	ΔA[b]
6.84[c]	2.52	0.011	*d*	
7.84[c]	1.30	0.018		
8.62[c]	0.67	0.035		
8.64[e]	0.44	0.037		
9.06[e]	0.52	0.049		
9.56[e]	0.88	0.035	20.9	0.013
10.00[e]	2.12	0.018	21.7	0.041

[a] 25.00 ± 0.02°, 1.90×10^{-4} M proflavine mixed with 4×10^{-5} M enzyme, 0.1 M KCl, 0.05 M buffer, λ 470 nm. Errors in k_{obsd} are 5% or less; errors in ΔA are 10% or less. Each result is based on at least three separate determinations, each of which consisted of at least three–four photographs for a given run. The rates were significantly slower at 2° ($k_{obsd} \sim 0.02$ sec^{-1} at pH 8.0). Even at 2° no transients were observed for ^{1}[Met(O)]chymotrypsin at pH 8.0. [b] Increase in absorbance of signal due to observed transient. [c] Phosphate. [d] No detectable transient until pH 9.56. [e] Borate.

[7] Loss of methionine in α-chymotrypsin due to SucNBr oxidation was not seen by the original investigators (Viswanatha and Lawson, 1961) because under the conditions of their amino acid analyses (acid hydrolysis) the sulfoxide reverts to the sulfide (Ray and Koshland, 1962).

[8] As mentioned earlier, aside from a slightly lower activity for ^{2}[Met(O)]chymotrypsin both "[Met(O)]chymotrypsin" derivatives have essentially the same properties. Unless otherwise noted, any observations on ^{1}[Met(O)]chymotrypsin have been confirmed on ^{2}[Met(O)]chymotrypsin as well.

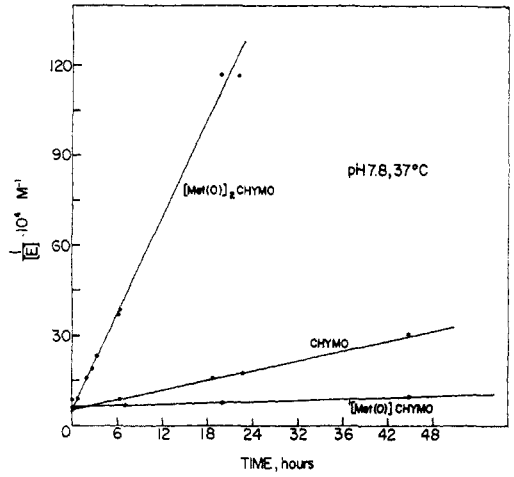


FIGURE 5: Autolysis of α-chymotrypsin and its derivatives. Samples were incubated in 0.05 M phosphate, pH 7.8, 37°, and periodically assayed for AcTrpOEt activity. It was assumed that the remaining activity toward AcTrpOEt was directly proportional to the concentration of unautolyzed enzyme. The reciprocal of unautolyzed enzyme concentration plotted *vs.* time gives straight lines indicative of good second-order kinetics. Initial enzyme concentrations were about 2×10^{-5} M. Approximate second-order rate constants were: [Met(O)]$_2$chymotrypsin, 4.8×10^4 M^{-1} sec^{-1}; α-chymotrypsin, 5.4×10^3 M^{-1} sec^{-1}; ^{1}[Met(O)]chymotryspin, 1.2×10^3 M^{-1} sec^{-1}. Chymo, chymotrypsin.

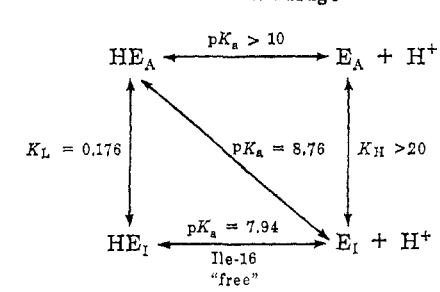
tive toward AcTyrNH$_2$ and find that the nonexistent activity can be explained by a much faster autolysis rate for [Met(O)]$_2$-chymotrypsin under conditions of the assay (37°; pH 7.8) (Figure 5). At lower temperatures, significant amidase activity is present (Table III). The marked instability of [Met-(O)]$_2$chymotrypsin toward autolysis is consistent with earlier observations which show that it is less stable relative to the unfolded forms than α-chymotrypsin or its monosulfoxide derivative (Biltonen and Lumry, 1971).

TABLE III: Hydrolysis of AcTyrNH$_2$ by α-Chymotrypsin and Its Derivatives.

		% Theoretically Possible Hydrolysis[a]		
T (°C)	Enzyme	6 hr	22 hr	44 hr
24	α-Chymotrypsin	45	93	100
24	^{1}[Met(O)]chymotrypsin	14	40	65
24	[Met(O)]$_2$chymotrypsin	4.0	16	20
37	α-Chymotrypsin	60	78	98
37	^{1}[Met(O)]chymotrypsin	31	61	78
37	[Met(O)]$_2$chymotrypsin	1.8	2.3	2.5

[a] In each experiment AcTyrNH$_2$ at a concentration of 2.5×10^{-3} M was incubated with enzyme at a concentration of 5×10^{-6} M in 0.05 M phosphate, pH 7.8. Aliquots (500 μl) of the incubation solution were added to 3 ml of a stock solution of Nessler's reagent and the absorbance at 490 nm was determined. Complete hydrolysis corresponded to liberation of enough NH_3 to give a final concentration of 2.5×10^{-3} M, and a net absorbance of 0.40, under the conditions of the experiment. This was confirmed using prepared stock solutions of $(NH_4)_2SO_4$. Errors are $\pm 2.5\%$ or less.

Scheme I: Equilibria for α-Chymotrypsin.



Discussion

The enhanced ability of ^{1}[Met(O)]chymotrypsin to bind proflavine, the increase in magnitude of the molar ellipticity at 231 nm, and the lack of a transient in the stopped-flow experiment can all be explained in terms of an enhanced *stabilization* of the active form of α-chymotrypsin at neutral pH. We briefly review now the relevant points of Fersht's analysis which are necessary for the development of this conclusion (Fersht and Requena, 1971; Fersht, 1972; Hess *et al.*, 1970).

Fersht's Analysis (*See Scheme I*). At neutral pH, α-chymotrypsin exists primarily in the "active" form (HE_A). At high pH the ion pair between Ile-16 and Asp-194 is deprotonated and the enzyme partially unfolds to an inactive conformation (E_I). The equilibrium between active and inactive chymotrypsin is apparently slow and is complete within the time range of the stopped-flow experiment. Thus, when α-chymotrypsin at moderately high pH is mixed with proflavine active α-chymotrypsin should bind at nearly diffusion-controlled rates (Havsteen, 1967). Then, due to mass action, some inactive α-chymotrypsin absorbs a proton and changes to active α-chymotrypsin in a slow first-order process. It is the second process ($E_I + H^+ \rightleftarrows HE_I \rightleftarrows HE_A$) which is presumed to give rise to the observed transients when the reaction is monitored at approximately 465–470 nm, the wavelength maximum for the α-chymotrypsin–proflavine complex.

The fact that transients are observed even at relatively low pH (6.9), far below the apparent pK of the ion pair (*ca.* 8.8), suggests that even when all of Ile-16 is protonated, a significant fraction of the enzyme exists in the inactive conformation, HE_I. The stopped-flow results show that the conversion of HE_I to HE_A must be slow, even at pH 6.9.

Fersht used the amplitudes of the transients observed on mixing α-chymotrypsin and proflavine to estimate the fraction of active α-chymotrypsin as a function of pH. His results indicate that the fraction of active α-chymotrypsin depends on a basic group of $pK_a = 8.7(6)$ at 25°, suggested to be Ile-16, which must be protonated. We detect a similar transition for α-chymotrypsin using CD or proflavine binding, the pK_a for the process being 8.7(3) (Figure 2) or 8.7(5) (Figure 3), respectively. Our results and those of Fersht are in good agreement with those of other workers who have also determined that deprotonation of a basic group of approximately $pK_a = 8.8$ renders α-chymotrypsin inactive (Hess *et al.*, 1970). We emphasize that the important point of Fersht's analysis is that even at low pH a significant portion (*ca.* 15%) of *inactive* α-chymotrypsin (HE_I) exists even though Ile-16 is protonated.

Stability of ^{1}*[Met(O)]chymotrypsin.* For this derivative we could detect no slow transient of the type observed by Fersht (Table II). It has full catalytic activity and in addition binds proflavine even more effectively than does α-chymotrypsin

(Figure 3). The results suggest that at neutral pH all [Met(O)]-chymotrypsin is in the active conformation (HE_A). Elimination of the HE_I state (*i.e.*, $K_L = 0$) should result in an enzyme which is fully in the active form over a wider pH range. It has been estimated that the pK_a of the α-chymotrypsin ion pair itself is probably greater than 10 (Fersht and Requena, 1971; Hess, 1971). If this estimate is correct, the apparent pK_a for inactivation of [Met(O)]chymotrypsin should be at least 10 (Scheme I). No transients in the stopped-flow experiment should be observable until at least pH 9, and the CD spectrum should be flat and independent of pH over a long range as well.[9] Our results are consistent with these predictions (Table II, Figure 2).

It is possible that the high pH results for [Met(O)]chymotrypsin (observed stopped-flow transient, CD changes) are related to the deprotonation of the buried ion pair. However, at high pH other groups (*e.g.*, tyrosine) can be deprotonated and destabilize the enzyme as well.[10] Thus, we hesitate at this time to assign any particular mechanism to the high pH results for [Met(O)]chymotrypsin.

Importance of Met-192. ZYMOGEN ACTIVATION (KRAUT, 1971). It has been documented that the position of Met-192 depends critically upon the state of the ion pair in α-chymotrypsin. X-Ray studies suggest that when chymotrypsinogen A is converted to the active form, the movements of Ile-16, Asp-194, and Met-192 are all interrelated. Formation of the Ile-16–Asp-194 ion pair causes Met-192 to move from a "buried" to a surface position on the "specificity cavity" of the enzyme. "The conformational changes involving Ile-16, Met-192, Gly-193 and Asp-194 and formation of the specificity cavity during zymogen activation can be viewed as a single unified event" (Kraut, 1971).

DEPROTONATION OF ILE-16. The elegant electron spin resonance (esr) experiments of Piette and Kosman in which a spin label group was attached to Met-192 show that titration of the ion pair ($pK_a \simeq 8.8$) causes a significant increase in the rotational freedom of the label (Kosman and Piette, 1972; Kosman, 1972). Thus, it is highly likely that in the inactive form of α-chymotrypsin (protonated or not) Met-192 occupies a very different position than in the active form.

Whether the high pH form of α-chymotrypsin is "zymogen-like," in which case Met-192 would be "reburied," or less well folded remains to be established. The esr experiments favor the latter view, but the large probe used in those experiments could make analysis of these esr data complicated. There are arguments in the literature to support both points of view (Hess *et al.*, 1970; Kosman, 1972; Wedler and Bender, 1969).

The important point is that unfolding of α-chymotrypsin to the inactive form is associated with a significant movement of Met-192 and that this movement is blocked in the pH region from *ca.* 9 to 7 for the sulfoxide derivative. Whether the effect is thermodynamic (*e.g.*, perhaps Met-192 must be "reburied," and this is energetically unfavorable for a hydrophilic sulfoxide) or there is a kinetic barrier (*e.g.*, the sulfoxide is too bulky), the results indicate the sulfoxide group locks the protein in the "active" conformation. A precedent for such a situation is found in the CD spectrum of iPr_2P-chymotrypsin. The spectrum is independent of pH, and it has been suggested that the bulky iPr_2P group "locks" the ion pair in place (Hess *et al.*, 1970; Parker and Lumry, 1963). Stabilization of the enzyme's conformation is not quite so complete in the case of the monosulfoxide since at moderately high pH (>9.1) conformational changes do occur as judged by CD and stopped-flow work.

We are currently reinvestigating other properties of [Met-(O)]chymotrypsin. It will be most interesting to see how the $A_b \rightleftarrows A_f$ transition (Kim and Lumry, 1971) is affected by oxidation of Met-192. This derivative is particularly attractive for future studies of conformational and catalytic behavior of the chymotrypsins since it is more stable toward autolysis, can be prepared cleanly, and maintains an *entirely* active conformation at all but extreme pH values. It does much to simplify the complexities of the substate problem for this protein but does not by any means solve them.

[9] At neutral pH the absolute magnitude of the molar ellipticity at 231 nm of [Met(O)]chymotrypsin is approximately 10–15% larger than for α-chymotrypsin. This is consistent with Fersht's estimate of about 15% inactive α-chymotrypsin at neutral (7) pH.

[10] It has been suggested by Valenzuela and Bender (1971) that Ala-149 also plays a role in the high pH conformation of α-chymotrypsin. This point is under investigation.

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Recording Spectrophotometric Method for Determination of Dissociation and Phosphorylation Constants for the Inhibition of Acetylcholinesterase by Organophosphates in the Presence of Substrate†

Geoffrey J. Hart and Richard D. O'Brien*

ABSTRACT: An experimental procedure, based on the use of a recording spectrophotometer, has been developed for determination of affinity, phosphorylation, and bimolecular reaction constants for the inhibition of an esterase by organophosphates *in vitro*. The theoretical background and derivation of the relevant equations are discussed. The method has been applied to a study of the effect of temperature on the inhibition of acetylcholinesterase by paraoxon, and to an investigation of the influence of carbon chain length on the inhibitory potencies of five *O,O*-diethyl (*ω-tert*-butalkyl) phosphates. Results are discussed in terms of the relative contributions of affinity and phosphorylation enhancement to overall inhibitory power.

Studies on the inhibition of cholinesterase by organophosphates have led to the recognition of the formation of a reversible enzyme–inhibitor complex (Aldridge, 1950) that can be related to the overall inhibition process as follows

$$E + PX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EPX \xrightarrow{k_2} EP + X \qquad (1)$$

where E represents the enzyme, PX the organophosphate with its leaving group X, EPX the reversible complex, and EP the covalently phosphorylated enzyme. Despite the implications of reaction 1, in much of the subsequent work kinetic treatments were used which ignored the formation of EPX and yielded a simple bimolecular rate constant (k_i'), suggesting that the reaction is a one-step process of the type

$$E + PX \xrightarrow{k_i'} EP + X \qquad (2)$$

Main and Dauterman (1963) have described a procedure, using a pH-Stat, for the determination of k_i' in the presence of substrate.

The development of more definitive kinetic treatments (Wilson, 1960; Main, 1964) allowed the determination of the dissociation constant, K_d (*i.e.* k_{-1}/k_1), and the phosphorylation constant, k_2, for reaction 1. These two parameters are related to overall inhibitory potency by the expression $k_i = k_2/K_d$, where k_i may conveniently be described as a "bimolecular reaction constant" (Main, 1964) since it is a combination of an equilibrium and a rate parameter and does not have the same meaning as the bimolecular rate constant, k_i'. Only a limited number of organophosphates have been subjected to this affinity *vs.* phosphorylation analysis because of the difficulties inherent in the present experimental procedures and the consequent need to use special reaction vessels (Main and Iverson, 1966; Chiu and Dauterman, 1969a,b; Chiu *et al.*, 1969; Lee and Metcalf, 1972). The difficulties arise due to the desirability of using inhibitor concentrations approaching K_d (Main, 1969a). Consequently, readings have to be taken over

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