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Isolation and Partial Characterization of a Covalent Intermediate between α-Chymotrypsin and o-Hydroxy-α-toluenesulfonic Acid Sultone

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Reaction of α-chymotrypsin with o-[^{35}S]hydroxy-α-toluenesulfonic acid sultone (I) at pH 7 produces an intermediate which substantially decomposes over a period of 24 hr at pH 3 as determined by gel chromatography. Isolation of the intermediate at pH 3 followed by denaturation in urea, reduction of disulfide bonds, and SDS–urea gel electrophoresis resulted in separation of the three peptides which compose the enzyme. Radioactivity (^{35}S) was associated with the peptide which contains serine 195 and not with the peptide containing histidine 57. These results indicate that a covalent linkage is established between sultone I and α-chymotrypsin, presumably at serine 195.

INTRODUCTION

In the preceding paper we presented evidence which demonstrates that on incubation of o-hydroxy-α-toluenesulfonic acid sultone (I) with α-chymotrypsin, an intermediate accumulates to the extent of 70 to 75% of the total enzyme under steady-state conditions (*1*). We believe this intermediate to be a covalent "sulfonyl–enzyme" compound which is a result of transesterification of the sultone with serine 195 of the enzyme active site. Such an interpretation is reasonable, given the proclivity of α-chymotrypsin to form covalent compounds with certain acyclic esters (*2*). But this particular cyclic ester (*1*) is unusual in that it is a highly strained five-membered ring system (*3–5*), and the covalent enzyme–cyclic ester intermediate which accumulates appears to revert readily to the cyclic ester and free enzyme (*1*). Consequently, if the intermediate is indeed o-hydroxy-α-toluenesulfonylchymotrypsin, it must reclose the five-membered sultone ring with the attending complement of strain energy.

Since the interpretation of ring-strain accommodation by the enzyme rests entirely on whether transesterification occurs between I and α-chymotrypsin, it is essential that the nature of the accumulating intermediate be defined. The extent to which the linkage between enzyme and sultone can chemically be defined is, of course, limited to the stability of the intermediate. Though its half-life at pH 7 is

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only about 3.5 min, the intermediate appears to be much more stable under slightly acidic conditions (*1*). The work presented here is the result of our attempts to isolate and characterize the intermediate which has been kinetically defined by proflavin displacement and inhibition of *N*-acetyl-L-tryptophan ethyl ester hydrolysis.

MATERIALS AND METHODS

Tritiated diisopropyl fluorophosphate (1 mCi/mmol) and [^{35}S]sodium sulfite (43 mCi/mmol) were purchased from New England Nuclear. Dithioerythritol (DTE), Tris:HCl, diisopropyl fluorophosphate (DFP), Triton X-114, and sodium dodecylsulfate (SDS) were obtained from Sigma Chemical Company and except for recrystallization of SDS with 80% ethanol, all compounds were used without further purification.[2] Scintoprep 3, toluene, and xylene all of scintiquality grade were purchased from Fisher Scientific, while Sephadex G-25 and G-75 were from Pharmacia. Urea (ultrapure) and Coomassie brilliant blue were obtained from Schwartz-Mann. Reagents for polyacrylamide gel electrophoresis (acylamide, bisacrylamide, ammonium persulfate, and *N,N,N'N'*-tetramethylethylenediamine (TEMED)), all from Bio-Rad Laboratories, were of electropurity grade. *o*-Hydroxybenzyl alcohol was purchased from Aldrich Chemical Company, α-toluenesulfonic acid sultone was from Eastman Kodak, and all other reagents were from MCB.

Standards for molecular weight determination (cytochrome *c* and myoglobin, untreated and cyanogen bromide fragments) and all reagents for gel electrophoresis were kindly donated by Dr. P. A. Hargrave.

α-Chymotrypsin (Sigma Chemical Co.) was further purified on a Sephadex G-75 column by elution with 1 m*M* HCl at 4°C (modified procedure of Yapel *et al.* (*6*)). The peak fractions were lyophilized and stored in sealed vials at 4°C.

Dry acetonitrile (MCB spectroquality) was prepared by distillation over P_2O_5 in a nitrogen atmosphere.

Synthesis of [^{35}S]*o-Hydroxy-*α*-Toluenesulfonic Acid Sultone*

In the synthesis of [^{35}S]sultone I the method of Zaborsky and Kaiser (*7*) was followed with minor modification. A sample of $Na^{35}{}_2SO_3$ was dissolved in 0.05 ml of water–isopropanol mixture (20:1). The solution was combined with 128 mg of nonradioactive Na_2SO_3, 250 mg *o*-hydroxybenzyl alcohol, and 18 ml of water, and titrated to pH 4.0 with 6% sulfurous acid. After refluxing for 8 hr the solvent was removed by rotary evaporation under vacuum. The solid residue was extracted with 95% ethanol in a soxhlet apparatus. Evaporation of ethanol gave white crystals of the sodium salt of *o*-[^{35}S]hydroxy-α-toluenesulfonic acid. This compound was then refluxed in 10 ml of $POCl_3$ for 4 hr. Most of the $POCl_3$ was

[2] Abbreviations used: diisopropylphosphorylchymotrypsin (DIP-Ct), diisopropyl fluorophosphate (DFP), sodium dodecylsulfate (SDS), dithioerythritol (DTE), *N,N,N',N'*-tetramethylethylenediamine (TEMED), cyanogen bromide (CNBr)

removed by distillation; the remaining solution was cooled in an ice bath and combined slowly with crushed ice. After 5 hr in the ice bath, a light yellow precipitate was separated from the reaction mixture by filtration. Attempts to dry the residue by evaporation resulted in formation of an oil. The preparation was further purified on silica gel tlc plates with fluorescent background by elution with chloroform–benzene (1:1). The bands with the same mobility as the standard (nonradioactive) sultone were removed from the plates and extracted with acetonitrile. Evaporation of the acetonitrile resulted in a white powder of [^{35}S]sultone which was stored in sealed vials at −20°C.

Formation of Covalent Enzyme–Substrate Compounds

The basic incubation mixture for formation of sulfonyl–enzyme which was used for gel-filtration chromatography and gel electrophoresis experiments was prepared in the following manner. Acetonitrile (0.05 ml) containing [^{35}S]sultone (total activity 1–2 × 10^6 counts/min) in the presence of 0.5–0.75 mg of nonradioactive sultone was added with stirring to 0.6 ml of α-chymotrypsin solution (13–17 mg/ml in 0.03 *M* citrate buffer, pH 7.0). This mixture was incubated for 25 to 30 min prior to use in either gel-filtration chromatography or SDS–urea gel electrophoresis.

The incubation mixture to form diisopropylphosphorylchymotrypsin (DIP-Ct) was prepared by a 2-hr incubation of 0.5 ml solution of α-chymotrypsin (20 mg/ml) in 0.5 *M* phosphate buffer, pH 7.7, with 0.02 ml of [^{3}H]diisopropyl fluorophosphate ([^{3}H]DFP) (2 × 10^{-5} *M*) in isopropanol (*8*). A control incubation mixture using nonradioactive DFP was also used in the SDS–gel electrophoresis experiments.

Gel-Filtration Chromatography

Except as indicated, all chromatographic experiments involving sulfonyl–enzyme were performed by diluting the incubation mixture (30 min incubation time, see above) with 0.6 ml of 1 m*M* HCl to stabilize the compound. The entire solution was applied to the Sephadex G-25 column which had been equilibrated with HCl pH 3.

All columns were eluted at the rate of 50 ml/hr with 1 m*M* HCl and 1.5-ml fractions were collected. Since column size varied as indicated in the figure legends, direct comparison of elution volumes for each of the figures is not possible. In comparing the elution of a particular compound on columns of different length it is necessary to use the parameter V_e/V_t, where V_e is the elution volume of the compound and V_t is the total column-bed volume. The parameter is a constant, independent of column dimensions but characteristic of the compound being eluted (*9*).

SDS–Gel Electrophoresis

There are three types of preparations which were used in the SDS–gel electrophoresis experiments, marker peptides for molecular weight calibrations,

denatured and reduced tritiated DIP-Ct, and ^{35}S-denatured and reduced sulfonyl-chymotrypsin.

[^{35}S]Sulfonylchymotrypsin was prepared by dilution of 0.65 ml of an incubation mixture pH 7 (30 min incubation time) with 0.6 ml of acidified 10 *M* urea solution to give a final pH of 3. This solution was incubated at 40°C for 0.5 hr. An aliquot (0.1 ml) of this solution was mixed with 0.1 ml of dissociation medium containing 5% DTE, and incubated at 37°C for half an hour. The solution was then applied (0.025- to 0.1-ml volumes) to the SDS–gel. (The dissociation mixture contained 1% SDS, 5.5 m*M* H_3PO_4 and sufficient Tris-HCl to adjust the pH to 6.8). Controls of nonradioactive sulfonylchymotrypsin and chymotrypsin alone were prepared for electrophoresis in the same manner.

Nonradioactive and tritiated DIP-Ct were prepared for electrophoresis by mixing 0.5 ml of the incubation mixture (described previously, 2 hr incubation time) with 1 ml of 10 *M* urea (pH 3) and the pH was then readjusted to 3. The mixture was heated in a water bath at 60°C for 1 hr, then 0.1 ml of the solution was mixed with an equal volume of dissociation medium (described above) containing 5% DTE. This resulting solution was further incubated for 0.5 hr at 50°C followed by application of 0.02- to 0.1-ml aliquots to the SDS–gels.

Myoglobin (5 mg/ml) and cytochrome *c* solutions (5 mg/ml) both of which had been partially cleaved with CNBr were prepared for electrophoresis by mixing 0.05 ml of each solution with 0.025 ml of SDS dissociation medium (as described above) followed by 0.025 ml of 10 *M* urea. These solutions were incubated for 30 min at 50°C and applied (0.02- to 0.1-ml volumes) to SDS gels.

All gel electrophoresis experiments were run for 12 hr on 10-cm gels (12% polyacrylamide SDS–urea gels prepared according to the method of Swank and Munkres (*10*)) at 4 V/cm of gel. Bromophenol blue was used as the tracking dye. After electrophoresis, the gels were removed from the tubes by breaking the glass and then stained with Coomassie brilliant blue.

Some gels containing radioactive material were stained for proteins to locate the enzyme peptides. The remaining radioactive gels were frozen with powdered dry ice for 15 min and cut into 1-mm slices with a Mickle gel slicer. The slices were placed in scintillation vials with 0.2 ml of 70% perchloric acid and 0.4 ml of 30% hydrogen peroxide. The caps were closed tightly and the vials were incubated in a water bath at 60°C for 3–6 hr until the slices were completely dissolved (method of Mahin and Lofberg (*11*)). Counting was done in 10-ml portions of liquid scintillation cocktail (Scintiprep 3, Triton X-114, and toluene or xylene) for 2 or 10 min. The counting was performed in a Mark III Searle scintillation counter.

RESULTS

Recently we presented kinetic evidence which suggests that a covalent compound is formed between α-Ct and sultone I at pH 7.0 (*1*). It was further shown that the decomposition of the putative sulfonyl enzyme is strongly pH dependent, resisting hydrolysis at low pH. This discovery indicated the possibility of

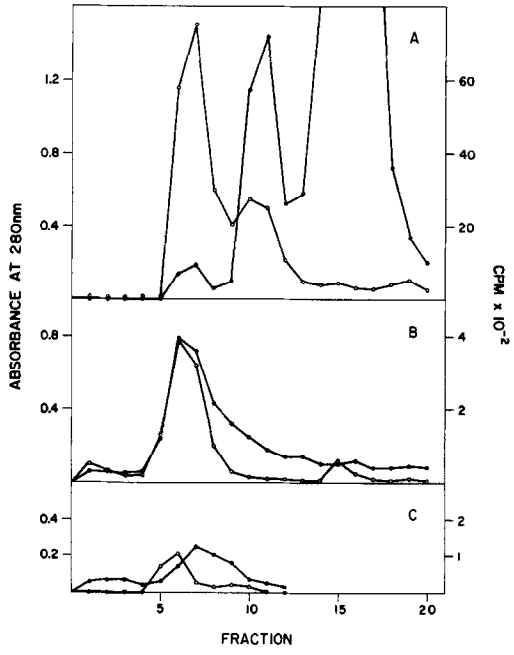

FIG. 1. Gel filtration of sultone I-α-chymotrypsin intermediate. [^{35}S]Sultone I (final concentration, 4.5 mM) incubated at 25°C with 0.5 mM α-chymotrypsin in 8% (v/v) CH_3CN, 30 mM citrate buffer, pH 7.0. After 30 min incubation the pH was adjusted to 3.0 and the mixture was eluted at room temperature on a Sephadex G-25 column (1 × 20 cm) equilibrated with HCl, pH 3.0. (○) 280-nm Absorbance, (●) radioactivity in counts/min. (A) Chromatography 40 min after initiating the incubation mixture. (B) Rechromatography of pooled fractions 6 and 7 from column A 3.5 hr after initiating the incubation mixture. (C) Rechromatography of fraction 6 from column B 24 hr after initiating the reaction mixture.

obtaining evidence for such a complex by physical isolation and characterization at low pH.

Basically two lines of investigation will be presented. One deals with the isolation and characteristics of the enzyme–sultone intermediate using gel-filtration chromatography, and the second involves degradation of the intermediate in an attempt to establish whether covalent attachment occurs and if so, the possible site of linkage.

The gel-filtration chromatography experiments were performed by incubating α-chymotrypsin with [^{35}S]sultone for 30 min at pH 7.0 (see experimental section) and then titrating the incubation mixture, with rapid stirring, to pH 3 with 1 N HCl. The resulting solution was then chromatographed (using pH 3 HCl) on Sephadex G-25 preequilibrated with 1 mM HCl. The results of a typical column profile are given in Fig. 1A.[3] Figure 1B was obtained by mixing fractions 6 and 7 described in Fig. 1A and rechromatographing them on the same column. Elution of this second column took place 3.5 hr after the original incubation mixture had

[3] The small 280-nm absorbance peak observed at fraction 10 of Fig. 1A is in large part due to absorbance by o-hydroxy-α-toluenesulfonic acid.

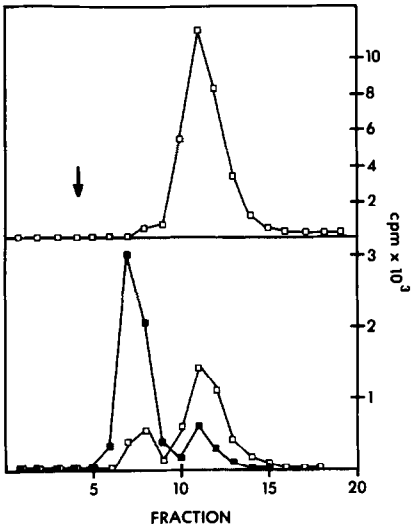

FIG. 2. Gel filtration of [^{35}S]sultone I. [^{35}S]Sultone was incubated under the same conditions as in Fig. 1, but without enzyme. Upper panel, the mixture was chromatographed on Sephadex G-25 (1 × 14 cm) at room temperature and eluted with HCl, pH 3.0. Arrow indicates elution position of α-chymotrypsin. Lower panel, the peak fraction from the above column was divided and one-half was rechromatographed immediately (□). The other half was treated with NaOH for 20 min before rechromatography (■).

been prepared. Figure 1C was likewise obtained on the same column by rechromatographing fraction 6 of the second chromatographic run. This final chromatography was initiated 24 hr after forming the incubation mixture.

Though part of the ^{35}S cochromatographed with the primary protein peak ($V_e/V_t = 0.45$) in Fig. 1A, two other ^{35}S species with V_e/V_t values of 0.80 and 1.18 were also eluted. The occurrence of the additional radioactive species required controls to determine the nature of these compounds. Figure 2 (upper panel) gives the elution profile of [^{35}S]sultone ($V_e/V_t = 1.18$) alone on Sephadex G-25, and the arrow indicates the elution volume expected for protein ($V_e/V_t = 0.45$). Figure 2 (lower panel) was obtained by dividing the peak sultone fraction of Fig. 2 (upper panel) into equal parts, one of which was rechromatographed immediately (2 hr had lapsed from beginning the elution of column 2 (upper panel)) and the other was set aside for subsequent use. This second half of the sample was treated with a small amount of concentrated NaOH for 20 min to induce partial sultone hydrolysis before G-25 chromatography. Both rechromatographic profiles (Fig. 2, upper and lower panels) exhibited two species with V_e/V_t values of 0.82 and 1.18.

To further investigate the nature of the complex formed between α-Ct and [^{35}S]sultone, two incubation mixtures were prepared which were identical in α-chymotrypsin and total sultone concentrations but different in that one was incubated at pH 3, while the other was incubated at pH 7.[4] After one-half hour of

[4] The sultone solutions in the incubation mixtures were prepared by adding microliter amounts of [^{35}S]sultone I stock solution to 3 m*M* nonradioactive sultone I. The total number of counts in the two incubation mixtures differed.

incubation at 25°C the incubation mixtures were adjusted to pH 3 (if necessary) and applied to Sephadex G-25 columns. The results of these two elution profiles are given in Fig. 3.

It becomes clear from the gel-filtration experiments (Fig. 1) that the complex formed between α-Ct and sultone at pH 7 was somewhat stable at pH 3 but not sufficiently to permit attempting total degradation of the protein to identify the specific site of attachment. However, attempts were made to identify at least the protein fragment to which the sultone was attached. These experiments made use of the fact that α-Ct is composed of three peptide chains (A, B, C), which are held together by disulfide linkages. By denaturation of the ^{35}S intermediate which accumulates, and reduction of the disulfide bonds, it should be possible to separate the three peptides and determine if the sulfonyl moiety is attached.

Separation of the three peptides by molecular weight was achieved by SDS–urea gel electrophoresis, and marker peptides of known molecular weight were used to calibrate a standard molecular-weight plot (*10*). Further identification of the peptide containing serine 195 of α-Ct was accomplished by denaturation, reduction, and SDS–urea gel electrophoresis of [^{3}H]diisopropylphosphoryl α-Ct (DIP-Ct). SDS–urea gel electrophoretic migration of the three α-Ct peptides and [^{3}H]DIP peptide appear on the standardized molecular-weight plot in Fig. 4.

Identification of the peptide containing the sulfonyl ^{35}S label was accomplished by first incubating enzyme with [^{35}S]sultone solution as described in Fig. 1A for 30 min, adjustment of the pH to 3, denaturation of the sulfonyl–enzyme with urea for 30 min, dilution of the mixture by SDS–urea–dithioerythritol solution, and gel

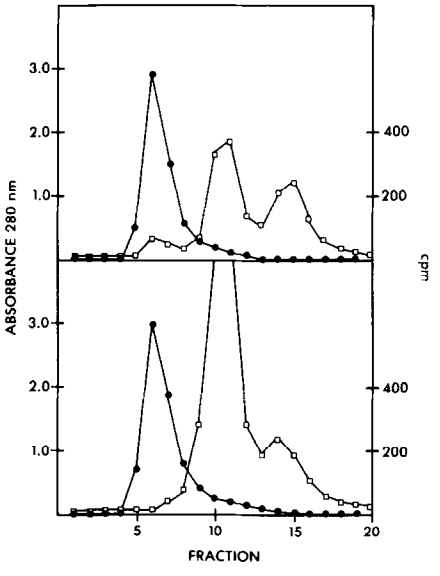

FIG. 3. Formation of α-chymotrypsin–sultone I intermediate at pH 7.0 and pH 3.0 (see footnote 4). Upper panel, α-chymotrypsin–sultone I incubated at pH 7.0 under conditions as in Fig. 1, titrated to pH 3.0 and chromatographed at room temperature on Sephadex G-25 (1 × 20 cm), using HCl, pH 3.0. Lower panel, α-chymotrypsin–sultone I incubated under the same conditions as above but at pH 3.0 instead of 7.0. Chromatography proceeded as above; (●) 280-nm absorbance, (□) ^{35}S counts/min.

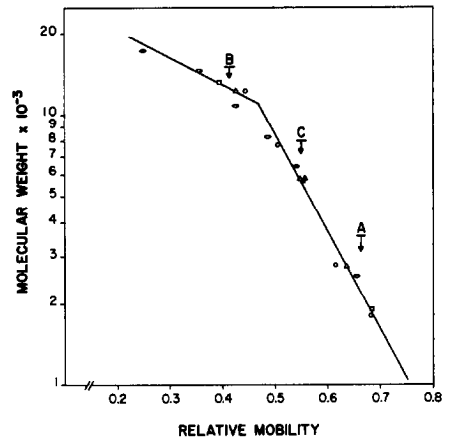

FIG. 4. Electrophoretic mobility of oligopeptides on SDS-urea gels. (●) Peptides from myoglobin partially cleaved with cyanogen bromide; (○) peptides from cyanogen bromide-treated cytochrome *c*(*10*). Other symbols signify peptides of reduced α-chymotrypsin previously treated with substrates: DFP (△), ^{3}H-DFP (▲), sultone I (□), and [^{35}S]sultone I (■). All open symbols indicate protein bands stained with Coomassie brilliant blue, and filled symbols show the positions of radioactive bands.

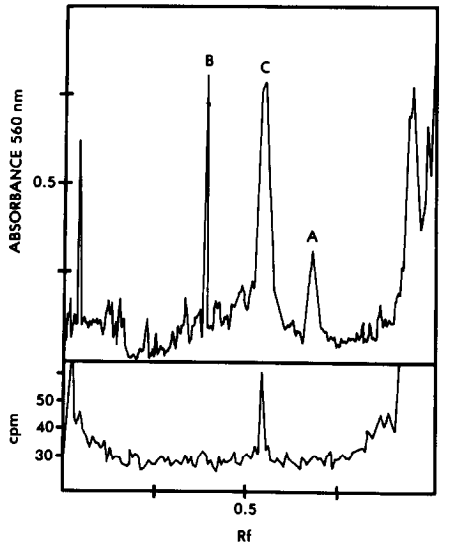

FIG. 5. Absorbance and radioactivity scans of peptides produced from α-chymotrypsin (0.40 μmol) which was previously incubated with (4.58 μmol) [^{35}S]sultone I at pH 7.0, denatured, reduced, and electrophoresed (100-μl aliquot) as described. Upper panel, spectrophotometric scan of SDS–urea gel stained with Coomassie brilliant blue for peptides. Lower panel, radioactivity profile of the same gel sliced into 1-mm sections.

electrophoresis as described in Fig. 4. The result of one such experiment is given in Fig. 5.

DISCUSSION

Upon mixing α-chymotrypsin with excess sultone I at pH 7.0 an intermediate accumulates to an extent representing about 70% of the total enzyme as detected by kinetic analysis (*1*). In the absence of excess sultone the intermediate decomposes within minutes at pH 7 but much more slowly at lower pH. By use of [^{35}S]sultone we have been able to form the intermediate at pH 7 and then remove excess sultone at pH 3 by gel chromatography to observe decomposition of the intermediate at low pH. Consecutive molecular sieve chromatography of the peak protein fractions demonstrates that it takes 24 hr to substantially remove the ^{35}S label from the protein peak (Fig. 1). This slow rate of decomposition is indicative of a covalent α-Ct–sultone I compound which spontaneously degrades at pH 3. The additional ^{35}S species indicated in Fig. 1A at V_e/V_t values of 0.80 and 1.18 represent *o*-hydroxy-α-toluenesulfonic acid and sultone I, respectively. Identification of these compounds is demonstrated in Fig. 2, which shows that sultone I alone chromatographs with V_e/V_t of 1.18. With time, or on base hydrolysis, the sultone is converted to the corresponding sulfonic acid with V_e/V_t equal to 0.82. The number of ionized carboxylate groups on the Sephadex gel matrix (*12*) is apparently sufficient to exclude the sulfonate anions more efficiently than the uncharged sultone. It is also possible that sultone I for some reason interacts more strongly with the gel matrix than the hydrolyzed product. Regardless of the reason, an unanticipated separation of the two small-molecular-weight species on Sephadex G-25 is observed in this system.

Incubations of α-chymotrypsin with sultone I at pH 3 and 7 followed by gel chromatography at pH 3 give results which differ in that the intermediate that is formed at pH 7 is not formed in detectable amounts at pH 3 (see Fig. 3). These results also preclude the possible interpretation of Fig. 1 that sultone I coelutes with α-chymotrypsin due to strong noncovalent interaction of enzyme and I at pH 3.

While gel chromatography experiments at pH 3 do not unequivocally demonstrate that the enzyme–sultone I intermediate is covalent, it is certainly consistent with that conclusion. Furthermore, the experiments provide physical evidence for the existence of the intermediate detected by proflavin displacement and inhibition of *N*-acetyl-L-tryptophan methyl ester hydrolysis and places interpretation of the kinetic results on a firm foundation.

Direct evidence for the existence of a covalent linkage is provided by fragmentation of the sulfonylchymotrypsin into component peptides. Since α-chymotrypsin is composed of peptides A (residues 1–13), B (residues 16–146), and C (residues 149–245) linked by disulfide bonds (*13*), it is possible to reduce the disulfide linkages and separate the resulting peptides according to molecular weight using SDS–urea gel electrophoresis.

In terms of molecular weights the order of electrophoretic migration of the three

peptides on SDS–urea gels should be A, C, B, with the smallest peptide (A) migrating most rapidly. Gross assignments were made on this basis; but on checking expected molecular weights of the three bands with the apparent molecular weights from the gels, close agreement was not found for all peptides. Though peptide B gave reasonable agreement between its predicted and apparent molecular weights, peptides A and C were not as well behaved. Peptides with molecular weights as low as peptide A frequently do not migrate as expected, and this results in apparent molecular weights grossly different from their actual values (*10*). The band appearing on gels which we assigned as peptide C gave an apparent molecular weight some 40% lower than expected, outside the ±18% error in molecular weight reported for well-behaved proteins in similar SDS–urea gel systems (*10*). Again, there are known examples of peptides which fall well outside this error range (*10*); and since ionic change is a prime determinant of electrophoretic behavior in this molecular-weight range, the net (+5) charge on peptide C could be responsible for some of the aberrant behavior. Furthermore, in the interest of detecting any covalent linkage between I and α-chymotrypsin peptide before it was completely hydrolyzed, we reduced the electrophoresis time from the 15–20 hr used by Swank and Munkres to 12 hr in our experiments (*10*). This shorter time increases the error in our plots compared with the previous workers and undoubtedly contributes to the ambiguity in apparent molecular weight of peptide C.

Conclusive identification of peptide C on SDS–urea gels was provided by radioactively labeling the serine 195–containing peptide. Fully reduced [^{3}H] diisopropylphosphorylchymotrypsin was used in the SDS–urea gel experiments, and the label was found to comigrate with the band we had tentatively identified as peptide C. The rank order of electrophoretic migration of the peptides on SDS–urea gels along with positive identification of peptide C by use of the [^{3}H] diisopropylphosphoryl affinity label defines the peptides from reduced α-chymotrypsin.

The two primary candidates for attachment of affinity labels to α-Ct are the histidine 57 imidazole side chain located on peptide B and the serine 195 hydroxyl of peptide C (*2, 14*). Figure 4 illustrates that if [^{35}S]sultone I is used as substrate, peptide C becomes labeled. This is further illustrated in Fig. 5 where a specific activity of 20.2 counts/min/nmol for sultone I and 30 counts/min above background for peptide C gives 0.099 mol of ^{35}S label per mole of α-Ct. The 10% yield of sulfonylated peptide C is quite respectable, given the rate of decomposition of sulfonyl–enzyme at pH 3 and the 13.5 hr it takes to perform the denaturation, reduction, and electrophoretic separation of peptides. The results of these studies clearly show that the sulfonyl moiety is attached to the serine 195-containing peptide and not the peptide containing histidine 57.

As previously mentioned (*1*), a plausible explanation of the reversibility of the sulfonyl–enzyme at pH 7 would be formation of a sulfonyl (His 57) α-Ct. Since sulfonyl–imidazole compounds are known to be of relatively high free energy (*15*) such linkages could more readily revert to the highly strained sultone I substrate then sulfonate esters. Even though we isolated sulfonyl–enzyme at pH 3 which is clearly not attached to His 57, we cannot totally exclude a sulfonyl–imidazole

linkage to the enzyme at pH 7. This point is crucial to understanding the energetics of this system, and we are further investigating the nature of the linkage at neutral pH. However, at this point the weight of the evidence appears to be in favor of a sulfonyl–Ser 195 linkage rather than sulfonyl–His 57.

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