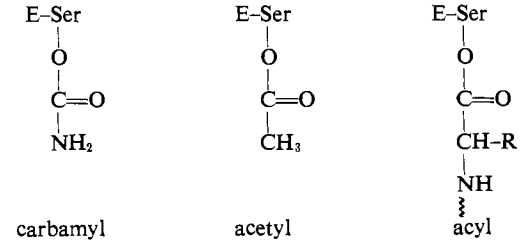
A Chemical and Crystallographic Study of Carbamyl-Chymotrypsin A†

George T. Robillard,* James C. Powers,‡ and Philip E. Wilcox§

ABSTRACT: The reaction of *p*-nitrophenyl cyanate with chymotrypsinogen A and chymotrypsin A has been studied to determine the potential of this reagent in the field of enzyme modifications. These experiments have shown that *p*-nitrophenyl [^{14}C]cyanate can react at specific loci on the enzyme under mild conditions. These conditions can be controlled in such a manner as to virtually eliminate nonspecific side reactions. *p*-Nitrophenyl [^{14}C]cyanate reacts differently with the various species of chymotrypsin A. The reagent reacts simultaneously at two sites in chymotrypsin A_π and A_δ, the active-site Ser-195 and the amino terminal of Cys-1, to give a dicarbamylated product. However, in chymotrypsin A_α, the reagent reacts principally at Ser-195 giving at first a monocarbamylated product. This difference in behavior has been shown to be due to a difference in the reactivity of the amino terminal of Cys-1 in the various enzyme species. Crystals of monocarbamyl-chymotrypsin A_α were readily obtained in a form isomorphous with native and tosyl-chymotrypsin A_α. The structure of the carbamyl derivative has been determined from crystallographic studies at 2.5-Å resolution. The carbamyl group occupies the same region as the carbonyl group of indoleacryloyl-chymotrypsin A_α and the sulfonyl group of the tosyl derivative. It is stabilized in a single conformation by a hydrogen-bonding network involving a water molecule, the carbonyl oxygen of the carbamyl group, the peptide carbonyl of Phe-41 and the amide nitrogen of Gly-193. Based on information taken from the carbamyl derivative, the structure of acetyl-chymotrypsin A_α has been proposed. A detailed knowledge of the orientation of the carbonyl groups of the acyl moieties in the acetyl-enzyme and the indoleacryloyl-enzyme has led to a clearer understanding of the stereochemistry of the deacylation mechanism.

Two other laboratories have tried to obtain specifically carbamylated derivatives of serine proteases using potassium cyanate (Shaw *et al.*, 1964; Svendsen, 1967). However, considerable amounts of nonspecific side reaction occurred during the inhibition of the enzymes, reflecting an inability to successfully control the reactivity of the reagent. Recently a new class of cyanates, aryl cyanates, have been developed (Grigat and Pütter, 1967) whose reactivity can be controlled by the nature of the substituents on the aromatic ring. Their usefulness has already been recognized in synthetic organic chemistry, but they have not yet been introduced into the field of enzyme modification. The initial purpose of the present investigation was to study the usefulness of these compounds as reagents for protein chemistry. In the studies described here, *p*-nitrophenyl cyanate was used to prepare a stable, inhibited derivative of bovine chymotrypsin A with no nonspecific side reaction. Identification of the sites of reaction was accomplished, in part, by isolation of soluble peptides after chemical degradation of the derivative. The labeled active-site residue responsible for loss of enzymatic activity was not stable to chemical degradation and, therefore, this site was identified by X-ray crystallographic analysis of monocarbamyl-chymotrypsin A_α. We are deeply indebted to Dr. David Blow who provided us with the structure factor amplitudes and phase information for the parent structure, tosyl-chymotrypsin A_α.[1] Without his contribution this crystallographic study could not have been accomplished.

By means of fingerprints of peptide digests Shaw *et al.* (1964) had presented evidence that the inhibition of chymotryptic activity by potassium cyanate could be linked to a carbamylation of the active-site Ser-195. Our crystallographic study proved this to be the case. Further investigation indicated that the detailed structure of the derivative would have an important bearing on the mechanism of enzyme action. Since the carbamyl derivative is structurally similar to the acetyl-enzyme and the acyl-enzyme itself, it could provide information



concerning the relationship between the orientation of the carbonyl group in the acyl-enzyme intermediate and the rate of deacylation.

The structure of indoleacryloyl-chymotrypsin A_α (Henderson, 1970) in combination with crystallographic investigations by Steitz *et al.* (1969) has led to a very attractive mech-

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received November 24, 1971.* This research was supported in part by a grant from the National Institutes of Health (GM 15163); G. T. R. was a National Institutes of Health predoctoral trainee (GM 5201) and J. C. P. held a Special Fellowship (GM 12588). This work has been presented in the dissertation of G. T. R. in partial fulfillment of the requirements for the Ph.D. degree.

* Present address: Bell Laboratories, Murray Hill, N. J. 07974. To whom correspondence should be addressed.

‡ Present address: Department of Chemistry, Georgia Institute of Technology, Atlanta, Ga. 30332.

§ Dr. Philip E. Wilcox died on November 2, 1971. This is a great loss to those who have known him personally, or followed his work in protein chemistry.

[1] Abbreviations used are: AcTyrOEt, acetyl-L-tyrosine ethyl ester; Dip-chymotrypsin, diisopropylphosphoryl-chymotrypsin; tosyl-chymotrypsin, toluenesulfonyl-chymotrypsin.

anism invoking a specific stereochemistry for the deacylation process.

The present investigation is, in part, an extension of this approach.

Materials

Chymotrypsinogen A. Five-times-recrystallized bovine pancreatic chymotrypsinogen A was obtained from Princeton Laboratories, Inc., Princeton, N. J. In order to reduce the chymotryptic activity to less than 0.2%, the protein was recrystallized from ammonium sulfate containing DFP.

Chymotrypsin A_α. Three-times-recrystallized chymotrypsin A_α (code CDI) was purchased from Worthington Biochemical Co. and used without further purification.

Acetyl-trypsin. Acetyl-trypsin was obtained from Mann Research Laboratories.

Collodion membrane filters used in enzyme concentration had a porosity of less than 5 mμ and were obtained from Carl Schielcher and Schuell, Keene, N. H.

Diaflo ultrafilters, also used in enzyme concentration, were purchased from the Amincon Corp., Lexington, Mass.

Carboxymethylcellulose, microgranular, CM-52, preswollen, was obtained from Reeve Angel, Clifton, N. J.

Sephadex was purchased from Pharmacia Fine Chemicals Inc., Piscataway, N. J.

Diisopropyl phosphofluoridate was a gift of Dr. Ross Tye.

[^{14}C]*Potassium cyanide* (45.2 mCi/mmole) was obtained from Amersham–Searle Corp., Arlington Heights, Ill.

p-Nitrophenol (Matheson Coleman & Bell), *succinic anhydride* (Mann Chemical Co.), and all other reagents were used without further purification.

Methods

Synthesis of p-Nitrophenyl [^{14}C]Cyanate. The method of synthesis was based on the work of Grigat and Pütter (1967). A solution containing 6 mmoles of [^{14}C]potassium cyanide (specific activity 0.05 mCi/mmole) in 5 ml of water was added dropwise to a solution of 6 mmoles of bromine in 0.31 ml of water. The reaction mixture was stirred at 25° until the bromine color was completely discharged. After stirring for an additional 5 min [^{14}C]cyanogen bromide was extracted from the mixture with four volumes (5 ml) of anhydrous ether. The combined ether extracts were dried over magnesium sulfate and filtered into a three-necked flask containing 6 mmoles of *p*-nitrophenol. The flask was sealed with a drying tube and cooled to 5° in an ice bath. A solution of 6 mmoles of distilled triethylamine in 5 ml of anhydrous ether was added dropwise to the reaction mixture over a 0.5-hr period at 5°. After addition of the triethylamine, the reaction mixture was diluted with 50 ml of anhydrous ether and stirred vigorously for 5 min. The mixture was filtered and the precipitate of amine hydrobromide was washed with anhydrous ether. The combined ether extracts were evaporated at room temperature under vacuum. The residue was taken up in hot carbon tetrachloride, filtered, and crystallized. Upon cooling, 410 mg (40–50% yield) of white plates crystallized with a melting point of 75.5–77° [Grigat and Pütter (1967) report mp 75–76°]. The specific activity was 0.0498 mCi/mmole.

Synthesis of p-Nitrophenyl Carbamate. A solution containing 500 mg of *p*-nitrophenyl cyanate in 20 ml of acetate was added to 20 ml of 2.0 N HCl. It was warmed to 50° and then allowed to cool. The product crystallized as white plates in 82% yield (413 mg) with a melting point of 164–165° (Dittert and Higuchi (1963) report mp 161°).

Carboxymethylcellulose Chromatography. A 60 × 2 cm column of CM-cellulose-52 packed at 5 psi of N_2 was used. The column was reequilibrated before each run with 3 l. of 0.075 M potassium phosphate (pH 6.2). The sample was pumped on the equilibrated column at 5 psi of N_2 and eluted with a linear potassium phosphate gradient of ascending ionic strength. The gradient was made from 2 l. each of 0.075 and 0.225 M potassium phosphate, both at pH 6.2. The chromatography was carried out at 5°.

Determination of ^{14}C Specific Activity. The specific activity of the *p*-nitrophenyl [^{14}C]cyanate was determined by measuring the ^{14}C content of a sample using a Packard Tri-Carb liquid scintillation spectrometer (Model 3003) and relating the ^{14}C content to the *p*-nitrophenolate ion absorption (ϵ at 400 nm = 1.8×10^4 l./mole) in a duplicate sample. The scintillant used in these determinations and all enzyme measurements contained 7.5 g of 2,5-diphenyloxazole, 375 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene, and 125 g of naphthalene in 1.0 l. of 1,4-dioxane. A counting efficiency of 75 to 85% was always obtained as determined by using a [^{14}C]toluene internal standard.

Assay for Enzymatic Activity. Chymotryptic activity was determined by the potentiometric assay (Wilcox, 1970) using *N*-acetyl-L-tyrosine ethyl ester as substrate.

Preparation of Monocarbamyl-chymotrypsinogen A. Lyophilized chymotrypsinogen A was dissolved in cold water to a concentration of 20 mg/ml. It was brought to 0.1 M in sodium acetate with a small amount of 4 M sodium acetate and the pH was adjusted to 4.5 with 1 N acetic acid. A total of 5 equiv of *p*-nitrophenyl [^{14}C]cyanate over zymogen was dissolved in dry dioxane, added directly to the protein solution and stirred for 2 hr at 5°. In all preparations the final dioxane concentration was kept below 3%. The pH was then lowered to 3.0 and the product was exchanged into 0.001 N HCl by dialysis at 5°. The sample was subjected to a high pH treatment in 0.1 M carbonate (pH 10.0) to remove any *p*-nitrophenol which remained bound to the zymogen amino groups as *o*-(*p*-nitrophenyl)isoureido derivatives. The pH was lowered and the solution was dialyzed against 0.001 N HCl for 24 hr at 5° and lyophilized. The reaction product was chromatographed on CM-cellulose and eluted as one major peak containing 70% of the total applied protein (Figure 1B). In separate experiments, the ^{14}C:zymogen molar ratio ranged from 0.9 to 1.0.

Preparation of Dicarbamyl-chymotrypsin A_π. To a solution of 1.1 g of chymotrypsinogen A in 40 ml of cold distilled water was added 10 ml of 0.5 M sodium β-phenylpropionate and the standard rapid activation was carried out (Wilcox, 1970). After 90 min, the pH was lowered to 3.0 and the sodium β-phenylpropionate was removed by centrifugation at 5° followed by gel filtration in 0.001 N HCl. The enzymatic activity of the preparation ranged from 100 to 120% in separate runs. After bringing the solution to 0.1 M in sodium acetate (pH 4.5) 10 equiv of *p*-nitrophenyl [^{14}C]cyanate was added in dioxane and allowed to react at 5° for 2 hr, giving a final activity of 2–7%. Excess reagent was removed by dialysis at 5° against 0.001 N HCl and the residual enzymatic activity was destroyed by treating the preparation to an excess of DFP for 2 hr at 5° and pH 7.8. This resulted in a derivative which was 0.1–0.5% active. The *p*-nitrophenol bound to the enzyme amino groups was released by high pH treatment as described above. After dialysis at pH 3 the product was chromatographed on CM-cellulose (Figure 1C). The major peak, con-

taining 70–80% of the applied protein had a ^{14}C:enzyme ratio of 1.8 to 2.0 in separate runs.

Preparation of Dicarbamyl-chymotrypsin A_δ. This derivative is prepared in essentially the same manner as the dicarbamylchymotrypsin A_π derivative with the exception that sodium β-phenylpropionate was not added to the activation mixture. The major peak from the CM-cellulose chromatography of the reaction product (Figure 1D) had a ^{14}C:enzyme ratio of 1.8 to 2.0 in separate runs. As can be seen by comparing Figure 1C,D, dicarbamyl-chymotrypsin, A_π and A_δ can be separated from each other by CM-cellulose chromatography.

Preparation of Monocarbamyl-chymotrypsin A_α. Lyophilized chymotrypsin A_α with an enzymatic activity of 80–90%, based on AcTyrOEt assays, was dissolved in cold distilled water to a concentration of 20 mg/ml and brought to 0.1 M sodium acetate by adding a small amount of 4 M sodium acetate. The pH was adjusted to 4.5 and 1.2 equiv of *p*-nitrophenyl [^{14}C]cyanate was added in dioxane. The inhibition proceeded for 2 hr, at which time the enzymatic activity had decreased to between 4 and 7%. Excess reagent was removed by gel filtration and the derivative was isolated by CM-cellulose chromatography (Figure 1E). The major peak, containing 70–80% of the applied protein, had a ^{14}C:enzyme ratio of 0.75 to 0.90 in separate runs.

Cyanate Amino-Terminal Analysis. Amino-terminal analyses were performed using the standard cyanate method (Stark, 1967).

Amino Acid Analyses. Amino acid analyses were performed on a Beckman automatic amino acid analyzer, Model 120C. The samples were prepared for analysis by hydrolyzing 1 mg of sample in 2 ml of 6 N HCl in an evacuated ignition tube for 24 hr at 100°. The samples were then evaporated, taken up in a citrate buffer, and analyzed.

X-Ray Data Collection. A Picker Nuclear/FACS-1 automatic four circle diffractometer with an on-line Digital PDP-8/I computer and teletype print out were used to collect intensity data. The program to direct data collection was the Picker/FACS-1 system utilizing an $\omega - 2\theta$ scan. Copper radiation with a wavelength of 1.5418 Å was generated by a Dunlee fine-focus copper tube.

A 2.5-Å resolution set of data composed of 15,500 reflections has been collected from one quadrant of the sphere of reflection for both native and monocarbamyl-chymotrypsin A_α. Of these reflections, 14,800 were unique. The crystals were mounted with the *b* axis parallel to the φ axis.

During data collection, 3 reflections of relatively high intensity were measured after every 200 reflections and 8 were measured after every 1200 reflections to determine the deterioration of the crystals from exposure to X-rays. Crystals of the native and the derivative enzyme deteriorated only 12–13% during the course of collecting a full set of data (approximately 240-hr exposure to X-ray for each crystal). This unusually low level of deterioration in protein crystals is due, in part, to filtering out the white radiation before it reaches the crystal by placing the nickel filter between the X-ray source and the diffracting crystal.

Absorption curves were determined for both crystals after collecting a full set of data. The scaling factors for deterioration of the crystal, the absorption correction (North *et al.*, 1968) and the lorentzian and polarization factors were applied to the raw intensity data during the reduction to structure factor amplitudes. After sorting the data, all duplicate measurements and equivalent reflections were averaged. The agreement in duplicate measurements has been calculated

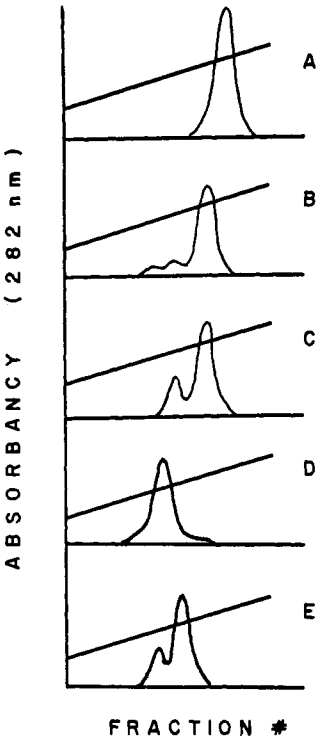


FIGURE 1: CM-cellulose chromatograms of chymotrypsinogen and carbamyl-chymotrypsin A derivatives. The chromatographies were carried out as stated in the section on Methods. (A) Chymotrypsinogen A eluting at 0.1175 M K^+, (B) monocarbamyl-chymotrypsinogen A eluting at 0.1125 M K^+, ^{14}C/enzyme = 0.9–1.0, (C) dicarbamyl-chymotrypsin A_π eluting at 0.1125 M K^+, ^{14}C/enzyme = 1.8–2.0; (D) dicarbamyl-chymotrypsin A_δ eluting at 0.100 M K^+, ^{14}C/enzyme = 1.8–2.0, (E) monocarbamyl-chymotrypsin A_α eluting at 0.104 M K^+, ^{14}C/enzyme = 0.75–0.90. The stated elution points refer to the center tube of the major peak in each chromatogram. In each case 70–80% of the total protein applied to the column eluted in the major peak.

using the equation: $R = [\Sigma(|F_1| - |F_2|)]/\Sigma|F_1|$. The value of *R* for the duplicate measurements in the carbamyl structure was 0.024 and in the native was 0.008.

Preparation of Electron Density Maps. Fourier syntheses and generation of electron density maps were carried out on a CDC 6400 computer using the X-ray 67′ system provided by Stewart (1967). Values of electron density were printed out by the computer on a specified grid. Levels of electron density were then contoured by hand and the contours were transferred to sheets of cellulose acetate or Plexiglass in arranging the three-dimensional maps. The electron density maps were contoured at increments of 0.25 e/Å^3 with the lowest contour at 0.8 e/Å^3. The electron density difference maps were contoured at increments of 0.16 e/Å^3 with the lowest contour at 0.32 e/Å^3. Contours below these levels produced no increase in the integrated electron density.

Integration of Electron Density. Integrated electron density measurements were placed on an absolute scale by comparison to the integrated electron density of the imidazole ring (35 electrons) of His-57 in the electron density Fourier map of monocarbamyl-chymotrypsin A_α.

Results

Reaction of p-Nitrophenyl Cyanate with Chymotrypsin A. In aqueous solutions, *p*-nitrophenyl cyanate undergoes hydrolysis according to reaction 1 and reaction 2. Therefore it

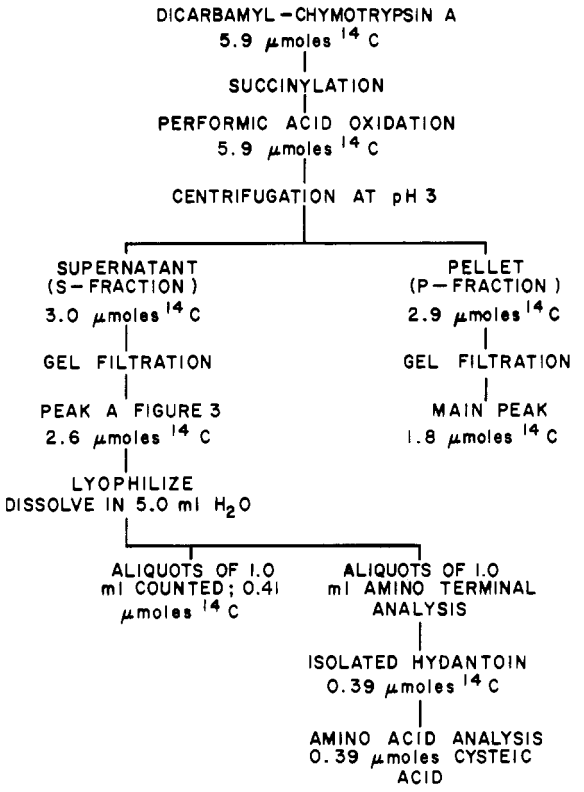
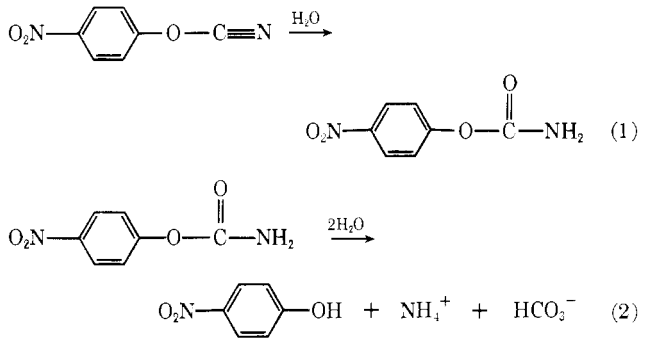


FIGURE 2: Flow diagram of the steps used in the isolation of the ^{14}C-labeled A chain and residue 1. The yield of the work-up of the S fraction from the centrifugation step to the amino acid analysis was 69% as determined by total ^{14}C content. The yield of the work-up of the P fraction from the centrifugation step to the gel filtration was 60% as determined by the total ^{14}C present.



was essential to determine the pH range within which the protein could successfully compete with water for the reagent. Approximate rate constants were determined from spectrophotometric data for the conversion of the cyanate to the carbamate. They showed that at pH 4 the cyanate possessed a half-life of approximately 5 min, whereas at higher pH's the conversion was too rapid to measure by these techniques. The inhibition of chymotrypsin A_δ with *p*-nitrophenyl cyanate was then studied in the pH range 3–7. We observed that the rate of inhibition increased with increasing pH, but that even at pH 4, 95% inhibition could be achieved in a short time. Similar studies with *p*-nitrophenyl carbamate showed no inhibition of enzymatic activity.

Cyanates can react with a wide range of nucleophiles including protein α- and ϵ-amino groups and the rate of reaction is strongly dependent on the state of ionization of the nucleophile. The criteria used in assessing the efficacy of *p*-nitrophenyl cyanate as a specific reagent for chymotrypsin were (1) reaction to yield a derivative which was 90–95% inactive, (2) using the lowest pH, and (3) with the smallest amount of reagent possible in order to minimize nonspecific side reactions.

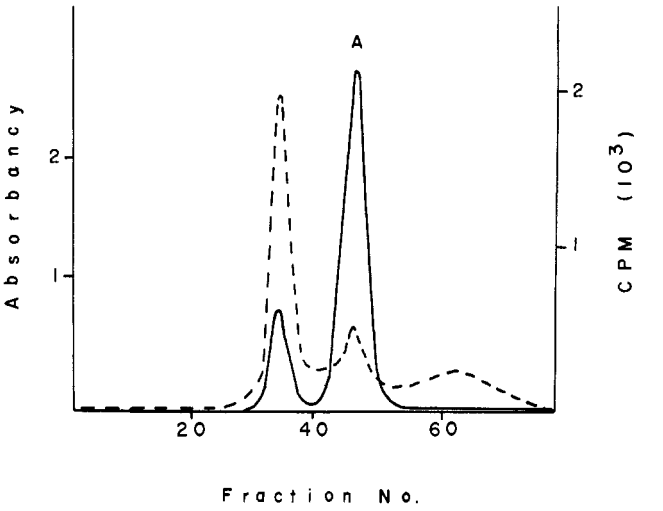


FIGURE 3: Gel filtration pattern from a 107 × 2 cm column of Sephadex G-25-fine in 0.1 N acetic acid; effluent fractions of 4–5 ml were collected. The sample was the supernatant from the acid precipitation of succinylated-oxidized dicarbamyl-chymotrypsin A_π. The solid line represents the ^{14}C counts per minute. The dashed line represents optical density at 230 nm.

Chemical Identification of Sites of Reaction on Dicarbamyl-chymotrypsin A_π. The intent of this portion of the investigation was to positively identify all the sites of reaction, by quantitatively recovering the ^{14}C label in various peptides. This could be achieved best by working with peptides which were soluble in aqueous systems. Therefore the experiments were patterned after those delineated by Hapner and Wilcox (1970).

One of the sites of reaction in dicarbamyl-chymotrypsin A_δ is the α-amino group of Cys-1. This has been demonstrated in the following experiments outlined by the flow diagram in Figure 2. They were designed to separate the A chain from the rest of the enzyme and to isolate the ^{14}C moiety which was thought to be attached to Cys-1.

Dicarbamyl-chymotrypsin A_π was first succinylated by the method of Hapner and Wilcox (1970). No loss of ^{14}C occurred during this step. The succinylated enzyme was then dissolved in 5.0 ml of 99% formic acid and oxidized for 4 hr at 5° in 10 ml of performic acid (Hirs, 1967). The product, lyophilized from 400 ml of water, was titrated into solution with 0.1 M ammonium acetate buffer. The pH of the final solution was 5.6. The total ^{14}C present at this time was 5.92 μmoles. After determining the ^{14}C content, the pH of the solution was lowered to 2.5 by adding 20 ml of glacial acetic acid. A precipitate formed immediately which was removed by centrifugation for 30 min at 8000 rpm. The A chain was soluble at this low pH and remained in the supernatant. Since ϵ-amino groups were masked due to succinylation, the rest of the protein was found in the pellet. The supernatant and the pellet were separated and the pellet was dissolved in water and adjusted to pH 6.0 with ammonium acetate buffer. Both fractions were counted in order to determine their ^{14}C content and then lyophilized.

The product obtained from the supernatant (S fragment) was chromatographed on Sephadex G-25 fine giving the elution pattern shown in Figure 3. The recovery in this step of the purification, as determined by ^{14}C content, was 85%. Peak A containing most of the ^{14}C was lyophilized and ana-

TABLE I: Amino Acid Analysis of the A Chain.

Residue	Predicted[a]	Found[b]
Lys	0.0	0.07
His	0.0	0.03
Arg	1.0	0.76
Cysteic acid	1.0	0.60
Asp	0.0	0.25
Thr	0.0	0.23
Ser	2.0	1.78
Glu	1.0	1.14
Pro	2.0	1.80
Gly	2.0	2.00
Ala	1.0	1.00
Val	2.0	2.05
Ile	1.0	0.95
Leu	2.0	1.90
Tyr	0.0	0.04
Phe	0.0	0.06

[a] Values predicted from the amino acid sequence of bovine chymotrypsinogen A (Hartley, 1964, and Hartley and Kauffman, 1966). [b] Values reported as μmoles of amino acid/μmole of peptide; calculations based on Ala = 1.0 μmole/μmole of peptide.

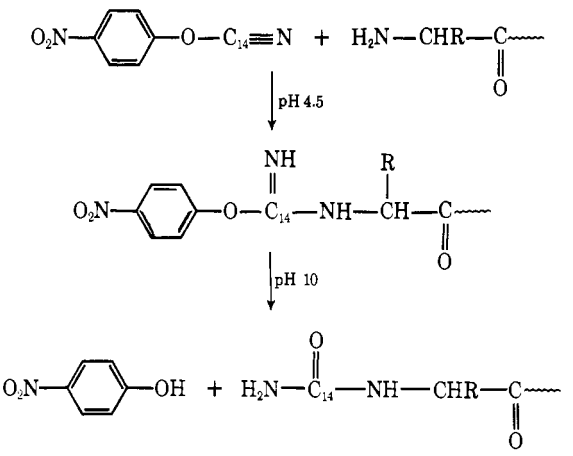
lyzed. Its amino acid composition is listed in Table I and compared to the theoretical amino acid composition of the A-chain (Hartley, 1964; Hartley and Kauffmann, 1966). The observed values agree closely with the values given by Hartley. The presence of significant amounts of threonine and aspartic acid indicates that some of the derivative had been converted to the A_γ form with the release of the threonyl-asparagine dipeptide, residues 147 and 148. This would result from the presence of a small amount of uninhibited enzyme. The dipeptide would be expected to co-chromatograph with the A chain during gel filtration.

The ^{14}C:A chain ratio was determined by correlating the ^{14}C specific activity of an aliquot of the material from peak A with the alanine content of this peptide determined by the amino acid analysis of an identical aliquot. The ^{14}C:A chain ratio was 0.80.

Having suspected that the [^{14}C]carbamyl group on the A chain was bound to the amino terminal of residue Cys-1, the isolated A chain was carried through a standard cyanate aminoterminal analysis (Stark, 1967). However, the initial treatment of the peptide with potassium cyanate was omitted. The [^{14}C]hydantoin was quantitatively isolated from a column of Dowex 1-×8. All of the ^{14}C in the applied sample eluted as a symmetrical peak. An aliquot of this peak was hydrolyzed in base for 24 hr and analyzed by amino acid analysis. A second aliquot was counted for its ^{14}C content. The only amino acid present in the analysis was cysteic acid, and it was present in a 1:1 ratio with the ^{14}C in the hydantoin peak.

Similar fragmentation experiments were performed to identify the labeled active-site residue which was responsible for the loss of enzymatic activity. Dicarbamyl-chymotrypsin A_π was first succinylated; the disulfide bonds were broken by reduction and carboxyamidomethylation (Hapner and Wilcox, 1970) and peptide bond cleavage was obtained at Met-180 and -192 with cyanogen bromide in 70% formic

SCHEME I



acid. During the course of this fragmentation procedure, however, the ^{14}C label was found to be unstable. Therefore the identification of this site was accomplished through X-ray crystallographic investigation.

Difference in the Reactivity of Chymotrypsin A_δ and A_α with p-Nitrophenyl Cyanate. The methods for preparing the various carbamyl-chymotrypsin derivatives clearly indicate that chymotrypsin A_α does not react with *p*-nitrophenyl cyanate in exactly the same manner as chymotrypsin A_π and A_δ or chymotrypsinogen A. Inhibitor (10 equiv) is necessary to obtain over 90% inhibition of chymotrypsin A_π or A_δ and the resulting product is carbamylated at both the active site and the α-amino group of Cys-1. On the other hand, the same degree of inhibition of chymotrypsin A_α can be achieved with 1.2 equiv of inhibitor, resulting in a high yield of product carbamylated only at the active site. Since there are two sites at which *p*-nitrophenyl cyanate reacts with chymotrypsin A, it would be useful to establish parameters which would indicate immediately whether site 1 or site 2 or both had reacted with the reagent. Such parameters were established by the following experiments.

Chymotrypsinogen A (20 mg/ml) was reacted with 5 equiv of ^{14}C-labeled reagent at pH 4.5 and 5° for 2 hr and subsequently dialyzed at pH 3 for 24 hr to remove excess reagent. An aliquot was counted and a value of 0.95 was calculated for the ^{14}C:protein ratio.

The pH of another aliquot was raised to 10, and after 1 hr the amount of released *p*-nitrophenolate ion was measured spectrophotometrically. The *p*-nitrophenolate ion:protein ratio was 0.95, the same as the ^{14}C:protein ratio. A reasonable explanation for the observed sequence of reactions is shown in Scheme I. *p*-Nitrophenyl cyanate reacting with the α-amino group of Cys-1 at pH 4.5, forms an *o*-(*p*-nitrophenyl)isoureido intermediate which is stable at low pH. When the pH is raised, the *p*-nitrophenolate ion is released and a ^{14}C-labeled carbamyl group is left attached to the α-amino group.

After converting monocarbamyl-chymotrypsinogen A to active chymotrypsin A_δ with acetyl-trypsin, it could be inhibited by 1.2 equiv of *p*-nitrophenyl [^{14}C]cyanate, resulting in the incorporation of a second equivalent of ^{14}C. However, no further release of *p*-nitrophenol could be detected by a second high pH treatment. From these observations it is clear that the reaction at the active site must occur with the rapid formation of the carbamyl derivative directly. If any *p*-nitrophenyl intermediate is formed it must be very unstable.

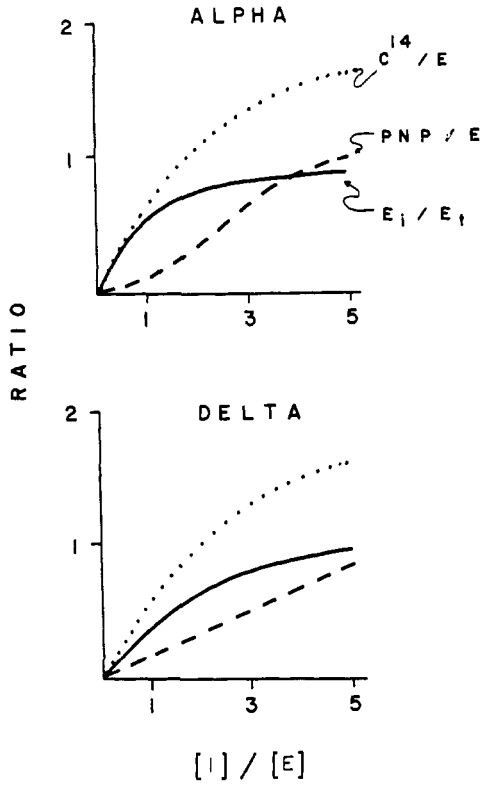


FIGURE 4: Reaction of *p*-nitrophenyl [^{14}C]cyanate with chymotrypsin A_α and A_δ. Enzyme concentration was 20 mg/ml. The reaction was carried out at 5° at pH 4.5 for 2 hr. The inhibitor was introduced into solution in dioxane so that the final dioxane concentration in the reaction mixture was never more than 2%. At the end of the reaction the solutions were dialyzed *vs.* 0.1 M formate (pH 3.0) and then *vs.* 0.001 N HCl. Methods for measuring various parameters have been described in the section on Methods. The dotted line represents the molar ratio of ^{14}C to enzyme. The dashed line represents the molar ratio of *p*-nitrophenolate ion to enzyme released after high pH treatment. The solid line represents the ratio of inhibited enzyme to total enzyme measured by the standard AcTyr-OEt assay.

Therefore, the release of *p*-nitrophenol from the purified derivative at pH 10 can be used to monitor reaction solely at the amino-terminal cysteine.

The experiment outlined above shows that once the amino terminal is blocked in chymotrypsin A_δ, there is no difference between chymotrypsin A_δ and A_α with respect to the rate of inhibition at the active site since both enzymes can now be inhibited with 1.2 equiv of reagent. These results suggest that the difference in the behavior of chymotrypsin A_δ *vs.* A_α toward *p*-nitrophenyl cyanate must lie in the reactivity of the α-amino group of Cys-1 in the respective enzymes.

Since we have separate parameters to monitor the reactions of *p*-nitrophenyl [^{14}C]cyanate at each site, these two reactions were studied concurrently in chymotrypsin A_δ and A_α. The reaction at the active site is measured as the ratio of inhibited enzyme to total enzyme and the reaction at the amino terminal is measured as the ratio of *p*-nitrophenolate ion released to total enzyme. The results are plotted in Figure 4. It is clear that there is a definite lag in the reaction of the α-amino group of Cys-1 in chymotrypsin A_α at low concentrations of reagent. At these levels of inhibitor essentially all reagent is reacting at the active site. It is only at higher inhibitor concentrations that the α-amino group begins to react. However, in chymotrypsin A_δ both the α-amino group of Cys-1 and the active-site residue react at very low inhibitor concentrations and these two sites continue to react at about the same relative rates as the inhibitor concentration is raised. This experiment confirms the conclusion that the difference in the reaction of chymotrypsin A_δ and A_α with *p*-nitrophenyl cyanate lies in the lower reactivity of the α-amino group of Cys-1 in the A_α enzyme.

TABLE II: Unit Cell Dimensions of Native and Inhibited Crystals of Chymotrypsin A_α.

	Native	Monocarbamyl	Tosyl[a]
a (Å)	49.1	49.1	49.3
b (Å)	67.4	67.3	67.3
c (Å)	65.9	65.9	65.9
β (°C)	101.5	101.5	101.8

[a] Sigler *et al.* (1966).

Crystallization of Carbamyl-chymotrypsin A_α and Native Enzyme. Lyophilized monocarbamyl-chymotrypsin A_α was dissolved in water to a concentration of approximately 30 mg/ml. It was concentrated on a collodion membrane concentrator to 80 mg/ml. Volumes (1 ml) were desalted by gel filtration on a 0.9 × 20 cm column of Sephadex G-25. The sample was eluted at pH 4.2 with a citrate buffer consisting of 0.053 M trisodium citrate–0.051 M citric acid, 3% in dioxane. The enzyme was crystallized from this buffer after bringing it to 1.65 M in ammonium sulfate. After reaching a length of 0.7–0.8 mm and a thickness of 0.4–0.5 mm, the crystals were transferred to a solution of the same sodium citrate–citric acid concentration, but 2.6 M in ammonium sulfate containing no dioxane. The crystals were standard monoclinic rhombs characteristic of native chymotrypsin A_α (Massey and Hartley, 1956). The dioxane was essential in obtaining crystals which had no visible twinning, similar to the situation for native chymotrypsin A_α.

Crystals of native chymotrypsin A_α were obtained by the method outlined by Sigler *et al.* (1966). Solutions of enzyme (10 mg/ml), buffered with 0.053 M trisodium citrate and 0.051 M citric acid (pH 4.2), were grown from 1.95 M ammonium sulfate–4% dioxane at 20°. The only difference in crystallizing native *vs.* monocarbamyl-chymotrypsin A_α is the concentration of ammonium sulfate in the mother liquor. Native chymotrypsin A_α crystallized optimally at 1.95 M ammonium sulfate whereas monocarbamyl-chymotrypsin A_α crystallized optimally at 1.65 M ammonium sulfate.

Unit Cell Dimensions. The unit cell dimensions for monocarbamyl-chymotrypsin A_α and native chymotrypsin A_α were determined by least-squares calculations on the PDP-8 digital computer using strong reflections in the crystals. They are listed in Table II and compared to the published unit cell dimensions for tosyl-chymotrypsin A_α, the parent structure (Sigler *et al.*, 1966). The unit cell dimensions for native and monocarbamyl-chymotrypsin A_α are almost identical with those for tosyl-chymotryspin A_α.

Preparation of Electron Density Maps. Electron density F_2 maps (Luzzati, 1953) of native and monocarbamylchymotrypsin A_α were generated using coefficients

$$M(2|F_{nat}|_{hkl} - |F_{tos}|_{hkl})\exp i\alpha_{tos}(hkl) \quad (3)$$

$$M(2|F_{carb}|_{hkl} - |F_{tos}|_{hkl})\exp i\alpha_{tos}(hkl) \quad (4)$$

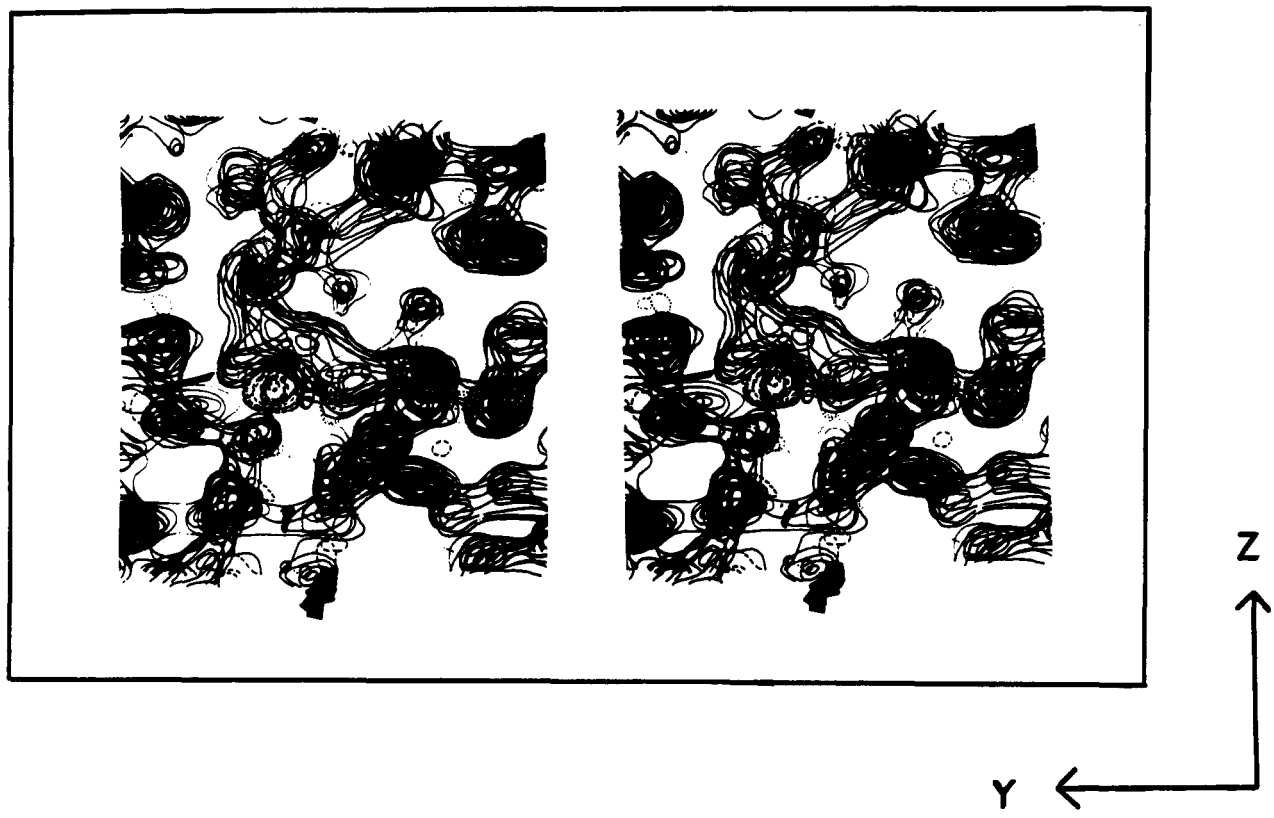


FIGURE 5: Stereocomposite photograph of the electron density map of the active site region of native chymotrypsin A_α (molecule 2) with a difference map of monocarbamyl minus native chymotrypsin A_α superimposed. The resolution is 2.5 Å. The solid lines are the electron density of the native molecule. The dashed lines are peaks of positive electron density in the difference map, and the dotted lines are peaks of negative electron density in the difference map. The sections shown in Figures 5 and 6 correspond to $x = 17/64$ to $24/64$, $y = -18/87$ to $7/87$, and $z = 38/86$ to $64/86$ unit cell fractions. The projections are parallel to the x axis with the positive x direction going into the plane of the paper. The y and z axes are as indicated in the figures. The arrow indicates the region of negative electron density in the difference map resulting from displacing the serine hydroxyl group. The contours directly above the arrow represent the electron density of the imidazole of His-57.

where $|F_{nat}|$, $|F_{carb}|$, and $|F_{tos}|$ are the structure factor amplitudes for the native, monocarbamyl- and tosyl-chymotrypsins A_α, respectively. M is the figure of merit assigned by the Cambridge group to the phase angle for a particular reflection and α_{tos} is the phase angle for that reflection determined for tosyl-chymotrypsin A_α. The structure factor amplitudes, phase angles, and figures of merit for tosyl-chymotrypsin A_α were supplied by D. M. Blow (personal communication). A difference map of the carbamyl minus native chymotrypsin A_α structure was also prepared. The coefficients used in the Fourier syntheses were obtained by a direct subtraction of expressions 3 and 4

$$2M(|F_{carb}|_{hkl} - |F_{nat}|_{hkl})\exp i\alpha_{tos}(hkl) \qquad (5)$$

Figure 5 shows a stereocomposite photograph of the F_2 electron density map of native chymotrypsin A_α (molecule 2)[2] with the difference map of monocarbamyl minus native chymotrypsin A_α superimposed. The positive peaks in the difference map are represented by dashed lines and the negative peaks by dotted lines. From these contours it is apparent that, during the conversion from native to monocarbamyl-chymotrypsin A_α, new electron density has been added which appears to be bonded to Ser-195 through the oxygen atom. These observations are confirmed in the electron density F_2 map of monocarbamyl-chymotrypsin A_α in Figure 6. Here the electron density associated with the side chain of Ser-195 in the native enzyme has diminished and a new region of electron density appears which has the same coordinates as the large peak of positive electron density in the difference map (arrow in Figure 6). The black dots represent approximate positions of the atoms for the imidazole ring of His-57 and the Ser-195–carbamyl group complex. When the peak height of this new region of electron density assigned to the carbamyl group was compared with peak heights of other peptide bonds in the same region, it was calculated that the carbamyl group was present in 70–80% occupancy. This correlated well with the ^{14}C content of dissolved crystals which had a ^{14}C:enzyme ratio of 0.80.

Information Obtained from the Electron Density Maps. A Kendrew skeletal model of chymotrypsin A_α had been constructed previously from published coordinates of the averaged electron density for molecules 1 and 2 (Birktoft *et al.*, 1969). The F_2 map of monocarbamyl-chymotrypsin (molecule 2) was prepared in our laboratory on a scale of 2 cm/Å and the region of the active site and the substrate binding site (Segal *et al.*, 1971) was fitted to this model of chymotrypsin A_α using an optical comparator (Richards, 1968). Eventhough the model, as it was built, was derived from the average electron density of molecule 1 and 2, whereas our map represented only molecule 2, very little adjustment

[2] The distinction between molecules 1 and 2 has been presented by Blow *et al.* (1964). The two molecules form the asymmetric unit and are related by two noncrystallographic axes, A and B.

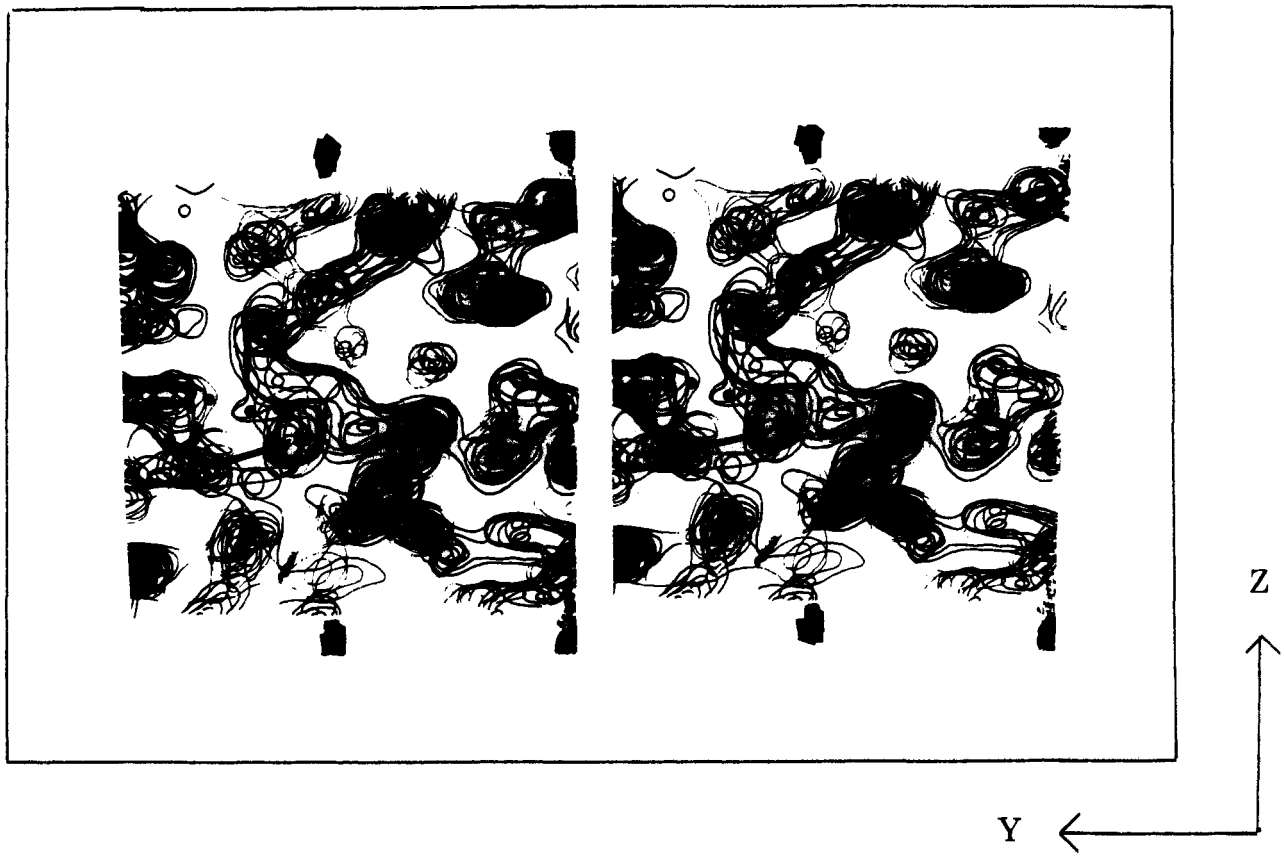


FIGURE 6: Stereocomposite photograph of the electron density F_2 map of molecule 2 of monocarbamyl-chymotrypsin A_α. The coefficients for the Fourier syntheses were prepared using expression 4. The arrow points to the peak of carbamyl electron density.

was necessary to make this portion of the map and the model coincide. Atomic coordinates for the active site and the substrate binding site components of monocarbamyl-chymotrypsin A_α (molecule 2) were then measured from this Kendrew skeletal model. Table III contains the coordinates for the carbamyl serine and the oxygen of the water molecule hydrogen bonded to it.

Our interpretation of the electron density map F_2 of monocarbamyl-chymotrypsin A_α in the region of the active site can be seen in Figure 7. Here we have rotated 90° around the y axis from the viewpoint in Figures 5 and 6 and are looking down the z axis. In this figure, a stereocomposite photograph of the electron density maps of the active-site region of monocarbamyl-chymotrypsin A_α molecule (2) is superimposed on a stereoscopic drawing of the residues at the active site of this derivative. The coordinates for the ORTEP drawing are those taken directly from the model. The photograph of the map and the orientation of the drawing is made from the exact same viewpoint. The perspective of what is seen in the stereocomposite is exactly equivalent to what is seen when the map and the model are superimposed with the optical comparator.

A stereocomposite drawing (Figure 8) shows the location of the carbamyl group and its orientation near the active site. The carbamyl group is covalently bonded through its carbonyl carbon to the oxygen of Ser-195. The orientation of the carbonyl group is stabilized by a hydrogen-bond network involving a water molecule, the carbonyl oxygen of the carbamyl group, the peptide carbonyl oxygen of Phe-41 and the amide nitrogen of Gly-193.

Besides these features which are very clear in the electron density maps, there is a diffuse region of electron density on the solvent side of His-57. It could represent two or three partially ordered water molecules, but it is impossible to be certain.

Information Obtained from the Electron Density Difference Maps. The large positive peak in the difference map associated with the carbamyl group has a center of gravity, x = 15.6 Å, y = 0.5 Å, and z = 6.1 Å, which corresponds almost exactly with the center of gravity of the carbamyl peak in the electron density F_2 map, x = 15.2 Å, y = 0.5 Å, and z = 6.1 Å. The coordinates are measured according to the cartesian coordinate system defined by Birktoft *et al.* (1969). When the electron density in this region is integrated, it gives a value of 24 electrons, exactly the value obtained when the carbamyl group in the F_2 map is integrated.

There is a significant negative peak very close to the positive carbamyl group peak. When integrated, this region has an electron content of 8 and represents the displacement of a water molecule by the carbamyl group.

There is only a small positive peak in the difference map for the water molecule which participates in the hydrogen-bond network stabilizing the carbamyl group. However, in the F_2 map, the electron density in this region is quite prominent. Since this region is exposed to solvent in the native molecule, it is reasonable to assume that there is loosely ordered water present. Therefore, when the native structure is subtracted from the carbamyl derivative, this loosely ordered water in the native will cause the positive peak for the water in the difference maps to be lower than the value for one molecule.

Only a small negative peak can be found in the difference

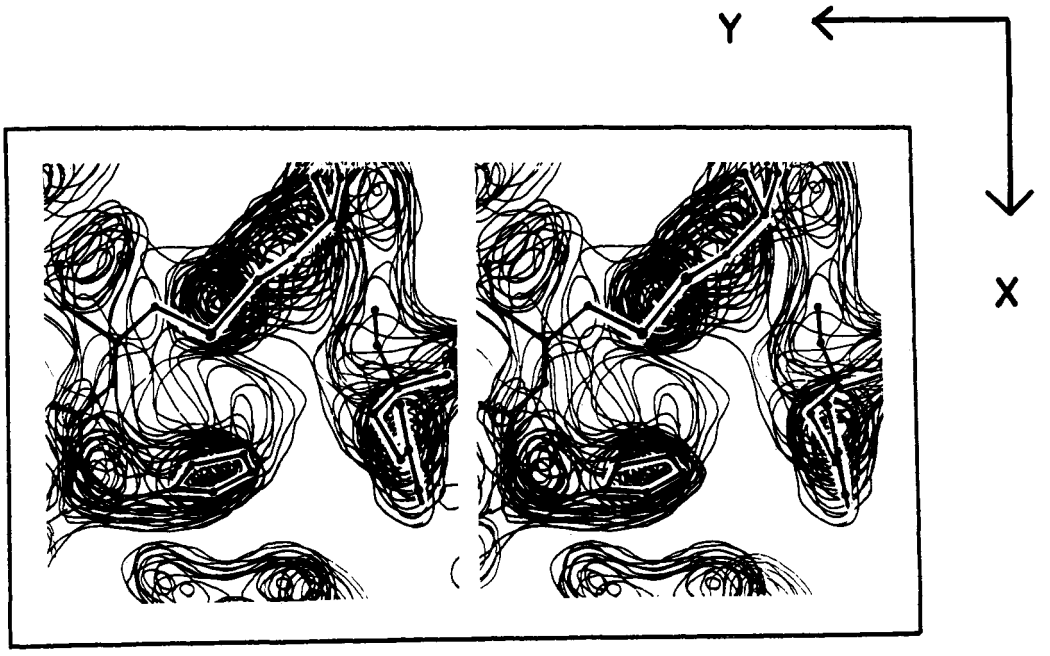


FIGURE 7: Stereocomposite photograph of the electron density maps of molecule 2 of monocarbamyl-chymotrypsin A_α superimposed on stereo ORTEP drawings of the active site residues of this derivative. The coordinates for the ORTEP drawing are those taken from the Kendrew skeletal model after it was fit to the electron density maps of this derivative. The orientation for photographing the maps and that used in making the drawings is the same.

map representing the position of the hydroxyl group of Ser-195 in the native enzyme. Figure 5 shows the electron density map of native-chymotrypsin A_α (molecule 2) with the electron density difference map of carbamyl minus native chymotrypsin A_α superimposed. The carbamyl group stands out prominently as a positive peak of electron density. However, there is barely any negative electron density representing the hole left by the serine hydroxyl when it changes positions in going from the native enzyme to the monocarbamyl derivative. A single contour on two levels is all that is seen. In contrast to our observations, Sigler *et al.* (1968), Steitz *et al.* (1969), and Henderson (1970) observe a large negative peak representing movement of the serine hydroxyl group in the difference map of tosyl minus native chymotrypsin A_α and indoleacryloyl minus native chymotrypsin A_α, respectively. The discrepancy between these data resides in the method of preparing the electron density maps. The difference map of monocarbamyl minus native chymotrypsin A_α represents only molecule 2, whereas those just mentioned are averaged maps in which the electron density of molecule 1 is rotated 180° around the noncrystallographic twofold axis (Dyad A) and averaged with the corresponding regions of electron density in molecule 2.

Early in our investigations, the choice was made not to average the electron densities of the two molecules because, in the region of primary interest, the active site, the electron density of molecule 2 seemed to be interpretable by itself. But this was not entirely correct. When the difference maps of molecules 1 and 2 for tosyl minus native chymotrypsin A_α were examined separately, the size of the negative peak for the serine hydroxyl group was comparable to that seen in the carbamyl difference maps. However, the size of the negative peak in molecule 1 is greater than that of molecule 2 in the difference maps of both the tosyl and the carbamyl derivatives. Therefore, the large negative electron density in the averaged difference map has its origin mostly in molecule 1. Since we were dealing primarily with molecule 2, a large negative peak for the shift of the serine hydroxyl was not observed.

Small positive and negative regions of electron density indicate some movement of the disulfide 191–220, the carbonyl oxygen of Ser-190, the hydroxyl group of Ser-189, and the peptide carbonyl of Val-17. There is also some movement of the C_α of Asp-194, the peptide bond of residues 141–142, and possibly of His-57. No movement was observed for Met-192.

TABLE III: Atomic Coordinates[a] for Carbamylated Ser-195 in Monocarbamyl-chymotrypsin A_α.

Atom	*X*	*Y*	*Z*
Ser-195			
CA	13.5	−0.3	9.2
CB	14.0	0.7	8.2
OG	15.2	0.3	7.4
C	13.5	0.1	10.2
N	13.2	1.5	8.4
O	11.3	0.1	10.0
Carbamyl group			
C	14.8	0.4	6.1
O	13.6	0.6	5.7
N	15.7	0.5	5.2
H1	15.7	0.5	4.7
H2	16.4	0.3	5.5
Water[b]			
O	11.9	0.6	4.3

[a] The coordinates, in Å, are reported in the cartesian coordinate system defined by Birktoft *et al.* (1969). [b] Water molecule involved in the hydrogen-bonding network with the carbamyl group (see text).

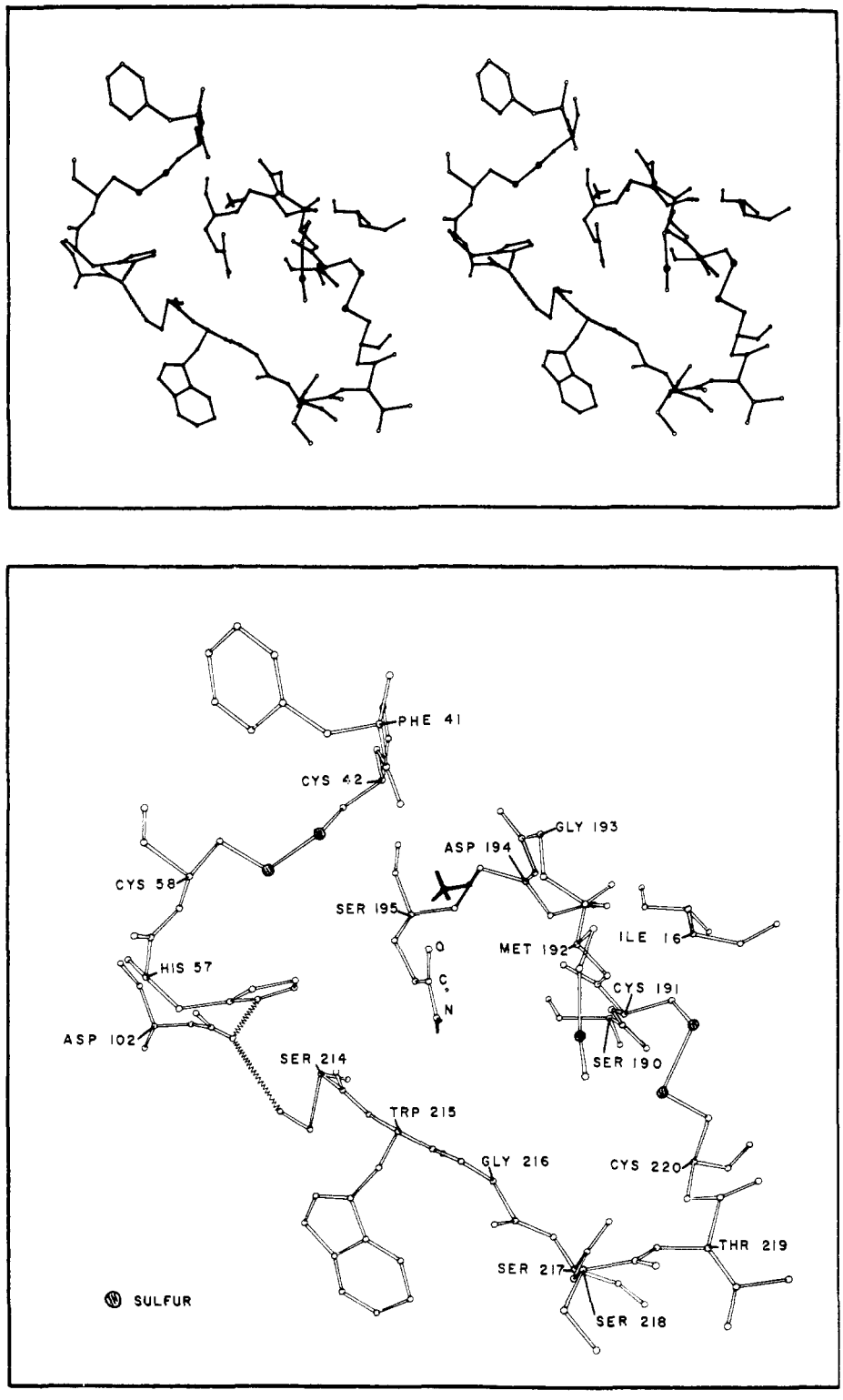


FIGURE 8: Stereo ORTEP drawing of the active-site residues of monocarbamyl-chymotrypsin A_α. The coordinates used as input in the ORTEP program were measured directly from the Kendrew skeletal model of monocarbamyl-chymotrypsin A_α.

Discussion

Chemical Modification with p-Nitrophenyl Cyanate. These studies of the carbamylation of chymotrypsin A by reaction with *p*-nitrophenyl cyanate have indicated that *p*-nitrophenyl cyanate and possibly other aryl cyanates have the reactivity, stability and versatility necessary to rate them as important reagents in protein chemistry.

Aryl cyanates are electrophilic compounds which readily add to nucleophilic groups in proteins. Their reactivity can be controlled in two ways; internally, by the nature of the substituents on the aromatic ring which can activate or deactivate the cyanate grouping, and externally, by the pH of the reaction medium. At neutral or high pH they can react indiscriminately with all unprotonated nucleophiles including hydroxide ion. At lower pH, where most nucleophiles

exist in the protonated state they can react with specific, activated residues.

Reactivity of Chymotrypsin A_δ and A_α toward p-Nitrophenyl Cyanate. The large excess (10 equiv) of reagent needed to inhibit the A_δ enzyme probably results from the circumstance that *p*-nitrophenyl cyanate is involved in two competing reactions. The first is reaction with the enzyme to form a covalent derivative. The second process is hydrolytic destruction of the reagent possibly catalyzed by the enzyme. If the hydrolysis reaction were more rapid than reaction of the amino group, it is clear that a large amount of reagent would be necessary to obtain complete carbamylation of the α-amino group of Cys-1.

The differences in the reactivities of the A_α and A_δ enzyme can be explained by assuming that the α-amino group of Cys-1 in the A_α enzyme is less available than in the A_δ enzyme, possibly due to dimer formation in solution. Another explanation could be a difference in the specific binding of *p*-nitrophenyl cyanate to the Cys-1 site in the A_δ *vs.* the A_α enzyme. It is this difference in behavior of the α-amino group of the two enzymes which has allowed us to prepare a monocarbamylated chymotrypsin A_α, but not the corresponding A_δ derivative. There is no other chemical or crystallographic evidence yet available to suggest that the α-amino group of Cys-1 or the surrounding environment is different in the two species. However, small differences in the orientation of neighboring residues or the orientation of the α-amino group itself could alter the reactivity of this group.

Interpretation of the Electron Density Maps. The major change in the structure of the active site upon carbamylation with *p*-nitrophenyl cyanate is a 120° rotation of the hydroxyl group of Ser-195 about the C_α–C_β bond. This movement is characteristic of other covalently linked acyl-enzyme analogs of chymotrypsin A_α which have been studied crystallographically (Sigler *et al.*, 1968) and (Henderson, 1970).

Comparison of Carbamyl-chymotrypsin A_α and Acetyl-chymotrypsin A_α. As was indicated in the introduction, the carbamyl derivative bears close resemblance to the acetyl-enzyme, differing only in the replacement of a methyl group with an amino group. From the structure of the carbamyl derivative as shown in Figure 8 it is clear that there are no hydrogen bonding interactions between the carbamyl group and neighboring amino acid side chains of the enzyme. It is difficult to propose any reasons why the acetyl group of acetyl-chymotrypsin A_α should not occupy the same space and have the same orientation as the carbamyl group. Therefore we feel that the structure of carbamyl-chymotrypsin A_α provides us with an accurate picture of the structure of the more labile acetyl-enzyme.

The acetyl derivative deacetylates fairly rapidly whereas the carbamyl derivative is quite stable and, since we believe that their structures are the same, we must consider the differing chemical nature of the two groups in order to explain this variation in stability. Breakdown of an acyl-enzyme is initiated by general base catalysis (Bender, 1962) and (Bender and Kezdy, 1964), in which an activated water molecule attacks the electrophilic carbonyl carbon of the acyl moiety. It might be expected that the degree of electrophilicity of this carbon atom will govern, to some extent, the rate of deacylation. In the carbamyl derivative the pair of electrons from the amino moiety are involved in a resonance system with the π electrons of the carbonyl group, thus lowering the electrophilic nature of the carbonyl carbon atom and making it less susceptible to attack by the activated water molecule. The acetyl group does not possess this same resonance system and consequently its carbonyl carbon is more electropositive in nature. Therefore we would expect the acetyl-enzyme to be less stable than the carbamyl derivative. This interpretation is supported by studies on the base-catalyzed hydrolysis of model compounds, which show that the rate of hydrolysis of ethyl carbamate is two orders of magnitude lower than the rate for ethyl acetate (Dittert and Higuchi, 1963; Potts and Amis, 1949). The ability to explain the differences in the stability of the two derivatives on the basis of their chemical nature offers further support to our proposal that the structures of the two derivatives are the same.

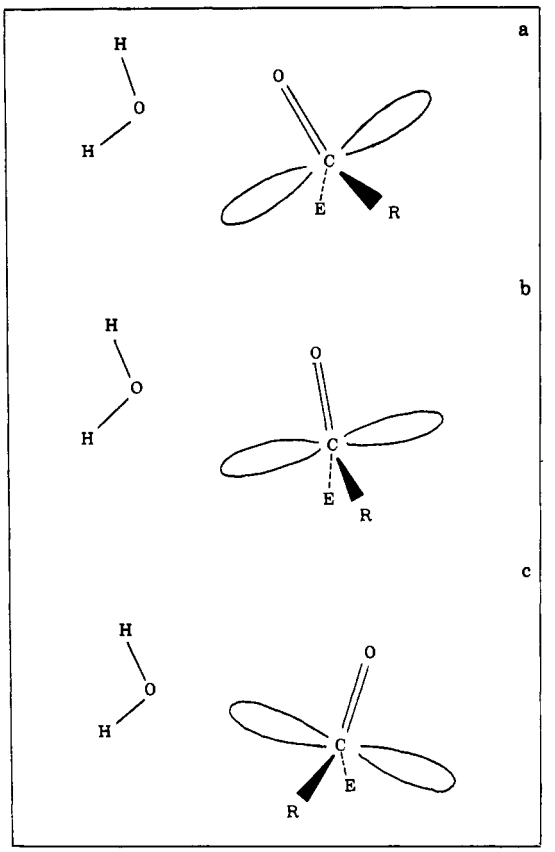


FIGURE 9: Plots of the orientations of the carbonyl groups and the p orbitals of the carbonyl carbon atoms for (a) indoleacryloyl-chymotrypsin A_α, (b) the monocarbamyl-chymotrypsin A_α derivative, and (c) the proposed true acyl-enzyme intermediate. The plane of the σ bond skeleton is perpendicular to the plane of the paper with the carbonyl carbon in the plane of the paper. In this orientation, the p orbitals also lie in the plane of the paper. The coordinates were taken from the literature (Henderson, 1970) or from the Kendrew skeletal model of monocarbamyl-chymotrypsin A_α and a proposed acyl-enzyme intermediate. The water molecule is situated so that it may be in a hydrogen-bonding position with the imidazole nitrogen of His-57.

Stereochemistry of Deacylation. Since attack occurs at the carbonyl carbon of the acyl moiety, the highest rate of deacylation should occur when the p orbital of the carbonyl carbon are in a position for maximum overlap with the orbitals of the attacking water molecule. Figure 9 is a plot of the orientation of the carbonyl moieties in the indoleacryloyl derivative (Henderson, 1970), the carbamyl or acetyl derivative, and a proposed true acyl-enzyme intermediate (Henderson, 1970). As the orientation of the p orbital is rotated into better position for overlap with the orbitals of the attacking water molecule, the rate of decomposition is increased. The rate of decomposition of the indoleacryloyl derivative (Fig-

ure 9a) is at least ten times slower than that of the acetyl derivative (Figure 9b), whereas the rate of decomposition of the acetyl derivative is 500–1000 times slower than that of a true acyl-enzyme intermediate (Figure 9c). The larger difference in the rates of deacylation between the acyl-enzyme intermediate and the acetyl derivative than between the acetyl and indoleacryloyl derivative supports the suggestion already made by Henderson (1970) that the carbonyl oxygen of the true acyl-enzyme intermediate may be hydrogen bonded to the amide nitrogen of Ser-195 and Gly-193. These hydrogen bonds would have the effect of increasing the electrophilicity of the carbonyl carbon, making it even more susceptible to attack by an incoming nucleophile.

Ingles *et al.* (1966) and Ingles and Knowles (1968) have clearly demonstrated that the presence of an aromatic side chain and an acyl amino group on the α-carbon of L-acyl-enzymes are essential for rapid deacylation. More recent chemical and crystallographic evidence on the hydrolysis of oligopeptide substrates and inhibition with peptide chloromethyl ketones has delineated an extended peptide binding site for the substrate in chymotrypsin (D. Segal, personal communication) and (Segal *et al.*, 1971). On the basis of the information provided by these investigators as well as our own findings we propose that one of the primary functions of these binding interactions, during the deacylation step, is to bring the p orbitals of the acyl carbon into proper orientation for maximum orbital overlap with the attacking water molecule.

Acknowledgments

We thank Dr. Lyle Jensen for his generosity in the use of his X-ray crystallographic facilities as well as his invaluable enthusiasm and insights during this study. We also express our thanks to Dr. Jon Herriott and Mr. Larry Sieker for their insights and assistance with the X-ray crystallographic portion of this investigation.

References

Bender, M. L. (1962), *J. Amer. Chem. Soc. 84*, 2580.

Bender, M. L., and Kezdy, F. (1964), *J. Amer. Chem. Soc. 86*, 3704.

Birktoft, J. J., Matthews, B. W., and Blow, D. M. (1969), *Biochem. Biophys. Res. Commun. 36*, 131.

Blow, D. M., Rossmann, M. G., and Jeffrey, B. A. (1964), *J. Mol. Biol. 8*, 65.

Dittert, L. W., and Higuchi, T. (1963), *J. Pharm. Sci. 52*, 852.

Grigat, E., and Pütter, R. (1967), *Angew. Chem. Int. Ed. Engl. 6*, 206.

Hapner, K. D., and Wilcox, P. E. (1970), *Biochemistry 9*, 4470.

Hartley, B. S. (1964), *Nature (London) 201*, 1284.

Hartley, B. S., and Kauffman, D. (1966), *Biochem. J. 101*, 229.

Henderson, R. (1970), *J. Mol. Biol. 54*, 341.

Hirs, C. H. W. (1967), *Methods Enzymol. 11*, 197.

Ingles, D. W., and Knowles, J. R. (1968), *Biochem. J. 108*, 561.

Ingles, D. W., Knowles, J. R., and Tomlinson, J. A. (1966), *Biochem. Biophys. Res. Commun. 23*, 619.

Luzzati, V. (1953), *Acta Crystallogr. 6*, 142.

Massey, V., and Hartley, B. S. (1956), *Biochim. Biophys. Acta 21*, 361.

Matthews, B. W., Sigler, P. B., Henderson, R., and Blow, D. M. (1967), *Nature (London) 214*, 652.

North, A. C. T., Phillips, D. C., and Matthews, F. S. (1968), *Acta Crystallogr. Sect. A 24*, 351.

Potts, Jr., J. E., and Amis, E. S. (1949), *J. Amer. Chem. Soc. 71*, 2112.

Reisfield, R. A., Lewis, U. T., and Williams, D. E. (1962), *Nature (London) 195*, 218.

Richards, F. M. (1968), *J. Mol. Biol. 37*, 225.

Segal, D. M., Powers, J. C., Cohen, G. H., Davies, D. R., and Wilcox, P. E. (1971), *Biochemistry 10*, 3728.

Shaw, D. C., Stein, W. H., and Moore, S. (1964), *J. Biol. Chem. 239*, PC 671.

Sigler, P. B., Blow, D. M., Matthews, B. W., and Henderson, R. (1968), *J. Mol. Biol. 35*, 143.

Sigler, P. B., Jeffery, B. A., Matthews, B. W., and Blow, D. M. (1966), *J. Mol. Biol. 15*, 175.

Stark, G. S. (1967), *Methods Enzymol. 11*, 125.

Steitz, T. A., Henderson, R., and Blow, D. M. (1969), *J. Mol. Biol. 46*, 337.

Stewart, J. M. (1967), Programs for X-ray Crystallography, Technical Report 67-58, Baltimore, Md., University of Maryland.

Svendsen, I. (1967), *C. R. Trav. Lab. Carlsberg 36*, 235.

Wilcox, P. E. (1970), *Methods Enzymol. 19*, 64.