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## Substrate Binding Site in Bovine Chymotrypsin A<sub>γ</sub>. A Crystallographic Study Using Peptide Chloromethyl Ketones as Site-Specific Inhibitors\*

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**ABSTRACT:** The crystallographic determination of the structure of bovine chymotrypsin A<sub>γ</sub> at high resolution has opened the way to a study of the extended binding site of the enzyme using the difference Fourier method. Peptide chloromethyl ketones have been chosen as specific, irreversible inhibitors of the enzyme. A number of these compounds were synthesized and tested for inhibition of tetragonal crystals of A<sub>γ</sub> at pH 5.6 in high concentrations of salt. Three compounds were selected for the preparation of inhibited crystals that were used in the collection of X-ray data at a resolution of 2.7 Å: acetyl-L-phenylalanine chloromethyl ketone (APCK), acetyl-L-alanyl-L-phenylalanine chloromethyl ketone (AAPCK), and acetyl-L-alanylglycyl-L-phenylalanine chloromethyl ketone (AAGPCK). Although inhibition caused no changes in unit cell dimensions greater than 0.5 Å or 0.6%, the inhibited crystals were not isomorphous with crystals of the native enzyme. However, pairs of crystals inhibited with two different chloromethyl ketones were found to be isomorphous, and gave interpretable electron density difference maps, when these were computed with phases for tosyl-chymotrypsin A<sub>γ</sub>. For each of the three pairs, the highest peak in the map represented the difference in structure of the two inhibitors. Kendrew skeletal models of the inhibitor-enzyme complexes were constructed with an optical comparator, placing the inhibitor moieties in the highest electron density of the difference maps, thereby

locating the ends of the chains. The C-terminal ends were fixed by a covalent bond to His-57, since it was known from amino acid analysis that the chloromethyl ketones reacted with a histidine residue. The phenyl ring was located in the hydrophobic pocket of the enzyme. The models show the peptide chain of the inhibitor bound to an extended segment of the main chain of the enzyme, consisting of Ser-214—Trp-215—Gly-216, in an anti-parallel β structure. One hydrogen bond links the C=O of Ser-214 (subsite S<sub>1</sub>) with the NH of residue P<sub>1</sub> (Phe) of the inhibitor, and two hydrogen bonds link the NH and C=O of Gly-216 (subsite S<sub>3</sub>) to the C=O and NH of residue P<sub>3</sub> (Ala in AAPCK) of the inhibitor. An examination of the model indicates that the enzyme should show stereospecificity for an L residue in position P<sub>2</sub>, and that a bulky, nonpolar residue at P<sub>2</sub> (e.g., Leu or Val) should interact with Ile-99 in subsite S<sub>2</sub>, in accord with previous reports in the literature. It is unlikely that the enzyme interacts with the main chain of a substrate beyond three residues on the N-terminal side of the cleavage point. Without changing the position of the chain of the tripeptide inhibitor, it is possible to break the bond to His-57 and to form an ester bond between the hydroxyl group of Ser-195 and a carboxyl group placed on the phenylalanine residue of the inhibitor. This structure would correspond to the postulated acyl intermediate of enzyme catalysis.

The crystallographic determinations of the structure of chymotrypsin A<sub>α</sub><sup>1</sup> (Matthews *et al.*, 1967; Sigler *et al.*, 1968; Birktoft *et al.*, 1970) and the structure of chymotrypsin A<sub>γ</sub>

(Davies *et al.*, 1969) at high resolution have opened the way to investigations of the structural basis for specificity of this serine protease. Studies carried out with A<sub>α</sub> (Steitz *et al.*, 1969; Birktoft *et al.*, 1970; Henderson, 1970) have defined the

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<sup>1</sup> The forms of chymotrypsin produced by activation of bovine chymotrypsinogen A are designated by the symbols A<sub>γ</sub>, A<sub>α</sub>, etc. (Wilcox, 1970). A<sub>γ</sub> and A<sub>α</sub> correspond to the alternative designations, γ- and α-chymotrypsin, respectively. Other abbreviations used in this paper are: Z, benzyloxycarbonyl; Boc, *tert*-butoxycarbonyl; Ac, acetyl; tosyl, *p*-tolylsulfonyl; APCK, acetyl-L-phenylalanine chloromethyl ketone; AAPCK, acetyl-L-alanyl-L-phenylalanine chloromethyl ketone; AAGPCK, acetyl-L-alanylglycyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride.

specific, aromatic binding site and have shown how binding at this site is involved in the interaction of substrate with the catalytic residues of the enzyme. In this paper we report on an extension of these studies to an investigation of the peptide binding site of the enzyme using chymotrypsin A $\gamma$  crystals. As will be shown in a later communication, the relative positions of residues in the catalytic region of chymotrypsin A $\gamma$  are essentially the same as those reported for A $\alpha$ .

The binding site of a proteolytic enzyme for a polypeptide substrate may be conveniently defined in terms of a sequence of subsites, as shown in Figure 1. It has long been known that the specificity of subsite S<sub>1</sub> in chymotrypsin is directed toward aromatic side chains and also requires an unsubstituted amido nitrogen. A crystallographic study of the binding of formyl-L-tryptophan to the enzyme in monoclinic crystals of A $\alpha$  showed that the aromatic ring is inserted into a hydrophobic pocket with the carboxyl group held near the catalytic residues, Ser-195 and His-57 (Steitz *et al.*, 1969). The N-H bond of the formamido group is pointed toward the carbonyl oxygen of Ser-214 but is too far removed for H-bond formation. This observation led these authors to speculate that when a true peptide substrate is bound, a hydrogen bond to Ser-214 would contribute to the specific interaction at subsite S<sub>1</sub> and would explain the requirement for an unsubstituted nitrogen atom.

When the packing of molecules in the crystals of A $\alpha$  was examined, it became apparent that crystallographic studies of binding to other subsites would be impossible in this crystal form (Birktoft *et al.*, 1970). The two molecules in the asymmetric unit are related by a noncrystallographic twofold axis which passes within a few angstroms of the S<sub>1</sub> subsite. Much of the binding region in each molecule is occluded by the proximity of amino acid residues around the axis. On the other hand, the molecular packing in tetragonal crystals of A $\gamma$  is such that the region around subsite S<sub>1</sub> is somewhat more open and it appeared that binding of a short peptide sequence in adjacent subsites might be feasible.

For this study of the binding site in A $\gamma$ , we have used irreversible inhibitors which are close analogs of peptide substrates. In each peptide, the carboxyl group of the terminal L-phenylalanine residue is replaced by a chloromethyl ketone group. Thus they are related to the site-specific reagent, L-(1-tosyl-amido-2-phenyl)ethyl chloromethyl ketone, which was designed by Schoellmann and Shaw (1963) and which was shown to react with His-57 in chymotrypsin A $\alpha$ . The advantages of using an irreversible inhibitor are twofold: it makes possible the achievement of high occupancy at the binding site in the crystal and it also permits straightforward chemical and enzymatic characterization of the inhibited enzyme. The use of inhibitors that are close analogs of good substrates increases the prospect that the observed three-dimensional structures will resemble the productive mode of enzyme-substrate interaction. Therefore, in the design of the inhibitors we have been guided by specificity studies of peptide substrates (Neil *et al.*, 1966; Yamamoto and Izumiya, 1966; Yoshida *et al.*, 1968).<sup>2</sup> A preliminary report on the inhibition of chymotrypsin crystals by these peptide chloromethyl ketones has recently been published (Powers and Wilcox, 1970).

This paper describes the synthesis of the inhibitors, their

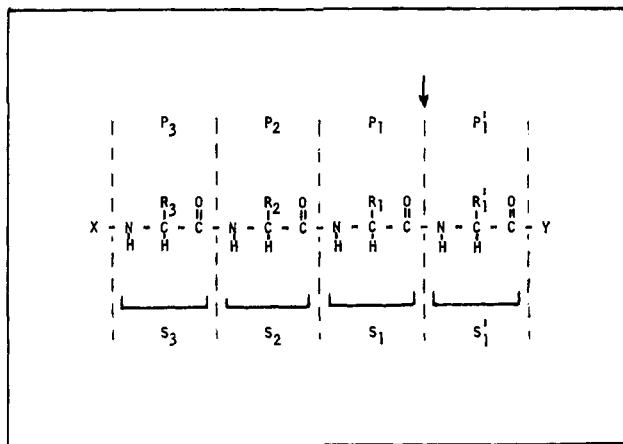


FIGURE 1: Scheme for depicting the positioning of a polypeptide chain in the binding site of a protease. Amino acid residues, P<sub>3</sub>, P<sub>2</sub>, P<sub>1</sub>, and P<sub>1</sub>', are located at subsites S<sub>3</sub>, S<sub>2</sub>, S<sub>1</sub>, and S<sub>1</sub>', respectively. Side chains are designated by R. Cleavage occurs between residues P<sub>1</sub> and P<sub>1</sub>'. No assumptions about interactions between substrate and particular subsites are implied. Additional subsites may exist for residues at either end, X or Y. This scheme was originally proposed by Schechter and Berger (1967).

reactions with crystals of chymotrypsin, and crystallographic studies of the derivatives by means of difference Fourier syntheses. An interpretation of the data, together with the building of the inhibitors into an atomic model of the enzyme, lead to the identification of subsites S<sub>2</sub> and S<sub>3</sub>, and confirms the nature of subsite S<sub>1</sub> as proposed by Steitz *et al.* (1969).

### Materials

Optically active amino acids were obtained from commercial sources: L-phenylalanine from International Chemical and Nuclear Corp., L-alanine from Aldrich Chemical Co., and L-leucine from Mann Research Laboratories. Benzyloxycarbonyl chloride was a product of Peninsula Chemresearch, Inc., and isobutyl chloroformate was purchased from Aldrich Chemical Co. *N*-tert-Butyloxycarbonylglycine (mp 94–95°) and *N*-tert-butyloxycarbonyl-L-alanine (mp 82–85°) were prepared by the procedure of Schnabel (1967). Phenylmethanesulfonyl fluoride (mp 91–92°) was synthesized according to the procedure described by Fahrney and Gold (1963) and was purified by sublimation. Substrates for chymotrypsin, benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester and benzoyl-L-tyrosine ethyl ester, were commercial samples obtained from Mann Research Laboratories. All other chemicals and solvents were reagent grade.

Bovine chymotrypsinogen A was obtained as a gift from Princeton Laboratories, N. J. Acetyl-trypsin (lot U4046), and chymotrypsins A $\alpha$  (lot COI 8LK) and A $\gamma$  (lot CDG-6204-5) were purchased from Worthington Biochemical Corp., N. J.

*N*-Benzyloxycarbonyl-L-phenylalanine chloromethyl ketone (ZPCK) was prepared from L-phenylalanine by the method of Shaw (1967). The product (mp 106–108°, recrystallized from ethyl acetate-ether) was obtained in 44% yield from benzyloxycarbonyl-L-phenylalanine,  $[\alpha]_D^{25} -47.2^\circ$  (methanol).

Anal. Calcd for C<sub>18</sub>H<sub>18</sub>ClNO<sub>3</sub>: C, 65.16; H, 5.47; Cl, 10.68. Found: C, 65.15; H, 5.71; Cl, 10.55.

*L*-Phenylalanine Chloromethyl Ketone Hydrobromide. To a saturated solution of HBr in acetic acid was added 500 mg (1.51 mmoles) of ZPCK. After 20 min, ether was added and the resulting crystals filtered. Recrystallization from ethyl

<sup>2</sup> Independently from the present work, Morihara and Oka (1970) designed similar inhibitors for subtilisin BPN'. They found that their compounds also inhibited chymotrypsin A $\alpha$ , benzyloxycarbonyl-L-alanyl-glycyl-L-phenylalanine chloromethyl ketone being the most reactive.

acetate-methanol gave 290 mg (69% yield) of product with mp 180–181° dec.

**Acetyl-L-phenylalanine Chloromethyl Ketone (AAPCK).** A solution of 10 mmoles (1.02 g) of acetic anhydride in tetrahydrofuran was cooled to 5°. Deblocked ZPCK (from 10 mmoles of ZPCK) was added, followed by 10 mmoles (1.1 ml) of *N*-methylmorpholine. The reaction mixture was stirred 1 hr and several volumes of water added. The crude product thus obtained was recrystallized from ethanol-water to give 1.2 g (50% yield), mp 164.5–165.0°.

*Anal.* Calcd for  $C_{12}H_{14}ClNO_2$ : C, 60.12; H, 5.88; N, 5.83. Found: C, 59.93; H, 5.84; N, 5.72.

**Acetyl-L-alanyl-L-phenylalanine Chloromethyl Ketone (AAPCK).** Benzoyloxycarbonyl-L-phenylalanine chloromethyl ketone (0.32 g, 10 mmoles) was deblocked with HBr in acetic acid. The crude product, obtained by precipitation with a large volume of cold ether, was washed several times with ether to remove benzyl bromide and was then used directly in the coupling reaction.

Acetyl-L-alanine (prepared by acetylation of L-alanine with 1 equiv of acetic anhydride in hot glacial acetic acid) was activated using the mixed-anhydride procedure (Anderson *et al.*, 1967). Acetyl-L-alanine (10 mmoles) was dissolved in tetrahydrofuran and cooled to –10 to –20° in a three-necked flask equipped with a stirrer, drying tube, and addition funnel. *N*-Methylmorpholine (10 mmoles; 1.1 ml) was added, followed by isobutyl chloroformate (10 mmoles; 1.3 ml); mixed-anhydride formation was practically instantaneous. Crude deblocked ZPCK was added, followed by 10 mmoles of *N*-methylmorpholine. The reaction mixture was allowed to warm to room temperature and stirring was continued for 1 hr. Water was added, the organic solvent was evaporated, and the aqueous solution was extracted with ethyl acetate. Aqueous citric acid and bicarbonate washes and evaporation of the ethyl acetate gave a white solid. Recrystallization from ethyl acetate resulted in 1.5 g (48%) of product with mp 191–191.5°.

*Anal.* Calcd for  $C_{18}H_{22}ClN_2O_5$ : C, 57.97; H, 6.16; N, 9.01. Found: C, 58.01; H, 5.88; N, 8.94.

**Acetylglycyl-L-phenylalanine Chloromethyl Ketone (AGPCK).** The procedure was similar to that used to prepare AAPCK. Upon work-up, the crude ketone was obtained as an oil which solidified. Purification was effected by chromatography on 0.05–0.02-mm silica gel G. The product was eluted with 5% methanol in chloroform and was crystallized from ethyl acetate in chloroform, giving 17% yield, mp 147–148°.

*Anal.* Calcd for  $C_{14}H_{17}ClN_2O_3$ : C, 56.65; H, 5.77; N, 9.44. Found: C, 56.81; H, 5.73; N, 9.24.

***N*-tert-Butyloxycarbonyl-L-alanylglycyl-L-phenylalanine Chloromethyl Ketone.** *N*-tert-Butyloxycarbonyl-L-alanylglycine methyl ester was prepared from *N*-tert-butyloxycarbonyl-L-alanine and glycine methyl ester hydrochloride using the mixed-anhydride procedure of Anderson *et al.* (1967). Alkaline hydrolysis gave a 26% yield of *N*-tert-butyloxycarbonyl-L-alanylglycine.

*N*-tert-Butyloxy-L-alanylglycine was converted into *N*-tert-butyloxycarbonyl-L-alanylglycyl-L-phenylalanine chloromethyl ketone by a procedure similar to that used to prepare AAPCK. The product, obtained in 62% yield, was recrystallized from ethyl acetate-petroleum ether (bp 30–60°), mp 129–132°.

*Anal.* Calcd for  $C_{20}H_{26}ClN_3O_5$ : C, 56.40; H, 6.63; N, 9.87. Found: C, 56.30; H, 6.60; N, 9.29.

**Acetyl-L-alanylglycyl-L-phenylalanine Chloromethyl Ketone (AAGPCK).** Acetyl-L-alanylglycine was converted into AAGPCK by a procedure similar to that used to prepare

AAPCK except that mixed-anhydride formation was allowed to proceed for 30 min at –15° due to the insolubility of acetyl-L-alanylglycine in tetrahydrofuran. After the reaction mixture had been stirred for 3 hr at room temperature, the solvent was removed and a partially crystalline oil obtained. This was dissolved in chloroform and chromatographed on Merck silica gel 0.05–0.02 mm. The product eluted with 10% methanol in chloroform and was crystallized from methanol to give a 29% yield of AAGPCK. An analytical sample was obtained by recrystallization from methanol-water and had mp 174–174.5°. The product thus obtained was homogeneous by tlc on silica gel G plates eluted with 10% methanol in chloroform.

The mass spectrum of AAGPCK showed peaks at *m/e* 367 (molecular ion), 369 ( $M + 2$ ,  $^{37}Cl$ AAGPCK), 331 ( $M - HCl$ ), 290 ( $M - COCH_2Cl$ ), 245 ( $M - HCl - CH_3CONHCHCH_3$ ), 240 ( $M - HCl - C_7H_7$ ), 203 ( $CH_3CONHC(CH_3)C_6H_5HCOCH_2$ ), 171 ( $CH_3CONHC(CH_3)HCONHCH_2CO$ ), 161 ( $203 - CH_2CO$ ), 143 ( $CH_3CONHC(CH_3)HCONHCH_2$ ), 120 ( $NHCHCH_2C_6H_5$ ), 114 ( $CH_3CONHC(CH_3)HCO$ ), 91 ( $C_7H_7$ ), and 86 ( $CH_3CONHCHCH_3$ ). The mass of the *m/e* 290 peak ( $M - COCH_2Cl$ ) was exactly measured as 290.151 (290.150 calculated for  $C_{15}H_{20}N_3O_3$ ).

The nmr spectrum of AAGPCK was difficult to obtain due to low solubility in common solvents. A spectrum in  $Me_2SO-d_6$  showed peaks at 7.3 (aromatic protons), 1.9 (s,  $CH_3CO$ ), and 1.3 ppm (d, alanyl  $CH_3$ ).

*Anal.* Calcd for  $C_{17}H_{22}N_3O_4Cl$ : C, 55.51; H, 6.03; Cl, 9.64; N, 11.42. Found: C, 56.20; H, 5.51; Cl, 9.10; N, 11.49.

The initial sample of AAGPCK which was used for the crystallographic work was prepared from Boc-Ala-Gly-Phe $CH_2Cl$ .

***N*-tert-Butyloxycarbonyl-L-alanylglycyl-L-phenylalanine chloromethyl ketone** (426 mg, 1 mmole) was dissolved in 1 ml of trifluoroacetic acid and allowed to stand for 1 hr at room temperature. Excess trifluoroacetic acid was removed by evaporation under reduced pressure and 1 ml of acetic anhydride and 0.11 ml (1 mmole) of *N*-methylmorpholine were added to the residue. Crystals separated from the solution after 1.5 days at room temperature. The product was recrystallized from methanol-water, chromatographed on silica gel G (elution with methanol), and again recrystallized from methanol-water to yield 55 mg of material with mp 173.5–178.5°.

Subsequent examination of the mass spectrum of this sample showed that it was contaminated with *N*-trifluoroacetyl-L-alanylglycyl-L-phenylalanine chloromethyl ketone. Two spots, one corresponding to AAGPCK, were observed in a thin-layer chromatogram of the above material on silica gel plates (10% methanol in chloroform). Examination of the Fourier difference maps in the regions corresponding to the acetyl group of AAGPCK did not reveal the high electron density expected for three fluorine atoms, but since a water molecule is displaced by the inhibitor in this region, the observed density is not a true representation of the numbers of electrons present in the constituent atoms of inhibitor.

**Acetyl-L-leucyl-L-phenylalanine Chloromethyl Ketone.** The procedure was similar to that used to prepare AAPCK. The crude product was recrystallized from ethanol-water and washed with 25% ethanol-water to yield 1.5 g (43% yield) of needles with mp 164.5–165.5° (sealed tube).

*Anal.* Calcd for  $C_{18}H_{25}ClN_2O_3$ : C, 61.26; H, 7.14; N, 7.84. Found: C, 61.06; H, 7.32; N, 7.80.

***N*-tert-Butyloxycarbonylglycyl-L-phenylalanine Chloromethyl Ketone.** This compound was prepared by a mixed-

anhydride procedure from *N*-*tert*-butyloxycarbonylglycine (2 g, 11.4 mmoles). The crude product was chromatographed on 0.50–0.2-mm silica gel G. Elution with ethyl acetate–petroleum ether (1:1, v/v) gave several crystalline fractions. These were crystallized from ethyl acetate–cyclohexane to give 0.99 g (24% yield) of product with mp 101–103°.

*Anal.* Calcd for C<sub>17</sub>H<sub>23</sub>ClN<sub>3</sub>O<sub>4</sub>: C, 57.54; H, 6.53; N, 7.89. Found: C, 57.54; H, 6.60; N, 7.89.

*Benzoyloxycarbonylglycylglycyl-L-phenylalanine Chloromethyl Ketone.* Benzoyloxycarbonylglycylglycine was prepared from glycylglycine and benzoyloxycarbonyl chloride. Recrystallization from ethyl acetate–methanol gave material with mp 182–183.5° (Bergmann and Zervas, 1932, report mp 178–179°).

Benzoyloxycarbonylglycylglycine was converted into benzoyloxycarbonylglycylglycyl-L-phenylalanine chloromethyl ketone by a procedure similar to that used for the synthesis of AAPCK. The product, obtained in 20% yield, was recrystallized from methanol, mp 98–115°.

*Anal.* Calcd for C<sub>22</sub>H<sub>24</sub>ClN<sub>3</sub>O<sub>5</sub>: C, 59.31; H, 5.42; N, 9.42. Found: C, 59.30; H, 5.40; N, 9.40.

## Methods

*Preparation of Crystals of Chymotrypsin for Inhibition Experiments.* The crystals of A $\gamma$  used in a survey of the various inhibitors were grown from enzyme freshly derived from zymogen by rapid activation. Chromatographically pure chymotrypsinogen (1.0 g) was treated with acetyl-trypsin (40 mg) at 5° and pH 7.5 in the presence of 0.005 M CaCl<sub>2</sub>. After 90 min, the mixture of enzyme species was separated by chromatography at pH 6.2 on CM-cellulose. Details of the preparation have been published elsewhere, along with a description of the conditions which lead to the isolation of the various forms of chymotrypsin (Wilcox, 1970). The major fraction consisting of chymotrypsin A $\delta$  was collected, the pH was adjusted to 4.0, and the protein was concentrated to 60–90 mg/ml in the Amincon Diaflo apparatus, using UM 20 membranes. During this stage of the preparation, it has been shown that the A $\delta$  species is largely converted into A $\gamma$ .

Crystallization leading to a limited number of single crystals of uniform size was carried out as follows. The concentrated enzyme solution (2 ml) was filtered through a 0.9 × 18 cm column of G-25 fine Sephadex which had been thoroughly prewashed with distilled water. An effluent fraction of 4.0 ml containing most of the protein was collected in a dust-free beaker, the pH was adjusted to 5.6–5.9, and the concentration brought to 30 mg/ml. Aliquots of this solution were mixed in small crystal dishes with equal volumes of salt solution, 4.0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>–0.02 M cacodylate buffer (pH 5.6). Care was taken not to nucleate the solution during mixing. The dishes were seeded by touching the liquid surface with a glass fiber that had been dipped into the mother liquor of a stock preparation of tetragonal A $\gamma$  crystals. Each dish was then sealed and stored at 20°. Well-formed tetragonal bipyramids appeared within 2–4 days. When these had reached the size of 0.3 or 0.5 mm in the longest dimension, they were used in the inhibition experiments.

Monoclinic crystals of chymotrypsin A $\alpha$  were prepared by the method of Sigler *et al.* (1966), using final concentrations of 2.0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>–0.10 M citrate buffer (pH 4.2) and 4% dioxane. The block-shaped crystals were used in the inhibition experiments when they had reached 0.5 mm in the longest dimension.

*Survey of Inhibitors.* Crystal inhibition experiments were carried out in small filter tubes prepared by bonding a small

disk of nylon screen (Pharmacia column screening material) to the lower ends of fire-polished glass tubes (5 × 50 mm), using Dow Corning silicon rubber sealer. Chymotrypsin A $\gamma$  crystals in mother liquor were transferred to a filter tube by means of a pipet. The mother liquor was allowed to drain and was replaced with 2.4 M sodium phosphate buffer (pH 5.6). Ammonium sulfate was replaced by sodium phosphate in order to preclude a possible competing reaction of ammonia with the chloro ketones. After 1 day in the phosphate solution, the crystals were immersed in an inhibitor solution by dipping the end of the tube into a saturated solution of the inhibitor. This was prepared by suspending finely divided solid inhibitor in a few milliliters of the 2.4 M phosphate buffer. For most experiments a small percentage of acetonitrile or dioxane was added in order to increase the solubility of the inhibitor. The nylon screen allowed inhibitor solution, but not solid inhibitor, to come in contact with the crystals. Periodically, the filter tubes were removed, allowed to drain, and then replaced in the heterogeneous mixture in order to allow fresh inhibitor solution to bathe the crystals. Crystals of chymotrypsin A $\alpha$  were treated with inhibitors by the same procedure, but for these experiments the pH of the salt solution was adjusted to 4.5–5.0.

After inhibition, the crystals were washed thoroughly by passing fresh buffer solution through the filter tubes. The crystals were then dissolved in enough 0.001 M HCl (2–4 ml) to give a protein concentration of 0.1–0.7 mg/ml (determined spectrophotometrically, using a molar extinction coefficient of 5.00 × 10<sup>4</sup>). Enzymatic activity was then measured with benzoyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester as substrate in the spectrophotometric assay (Wilcox, 1970). The activities of inhibited crystals in each set of experiments were related to the activity of chymotrypsin crystals which had been treated in an identical fashion but without the addition of inhibitor.

*Amino Acid Analysis of Inhibited Enzyme.* A sample of chymotrypsin A $\gamma$  was treated with a 10 molar excess of acetyl-L-alanylphenylalanine chloromethyl ketone (AAPCK) at pH 7.0. After 2 hr, inhibition was complete and the protein was dialyzed thoroughly against 10<sup>-3</sup> M HCl. About 10 mg was hydrolyzed with 6 N HCl at 110° for 24 hr. The hydrolysate was analyzed in a Beckman Model 120C amino acid analyzer coupled to an Infotronics integrator, using the 3.5-hr acidic and neutral column system and the 1.3-hr basic column system.

*Preparation of Inhibited Crystals for X-Ray Analysis.* Chymotrypsin A $\gamma$  crystals, grown from 50% saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (pH 5.6), were slowly transferred to a portion of stock salt solution which was 65% saturated in (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and contained 5% dioxane and 0.01 M sodium cacodylate buffer (pH 5.6). Reaction of the crystals with each of the three inhibitors, APCK, AAPCK, and AAGPCK, was carried out by the following procedure. Some of the chloro ketone was dissolved in dioxane to give a concentration of about 0.05 M. One part of this solution was mixed with 100 parts of the stock salt solution which was used to bathe the crystals. Some of the inhibitor precipitated from the mixture, indicating saturation. A slurry was maintained by frequent stirring and was exchanged slowly with the solution which bathed the enzyme crystals, leaving always about 2 ml of solution over 10–20 mg of crystals. The reaction was allowed to proceed at room temperature with occasional mixing and additions of fresh inhibitor solution. After approximately 3-weeks soaking, the crystals were transferred to a solution which was 65% saturated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and contained 0.01 M sodium cacodylate buffer (pH 5.6), and were washed several times with this solution to

TABLE I: Unit Cell Dimensions of Native and Inhibited Crystals of Chymotrypsin A<sub>γ</sub> (Space Group, P4<sub>2</sub>2<sub>1</sub>2).

Inhibitor <sup>a</sup>	<i>a</i> = <i>b</i> (Å)	<i>c</i> (Å)
None <sup>b</sup>	69.6	97.4
APCK	69.9	96.9
AAPCK	70.0	97.0
AAGPCK	69.8	97.2

<sup>a</sup> See first footnote 1 for abbreviations. <sup>b</sup> Native crystals in 65% saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (pH 5.6).

remove excess inhibitor and dioxane. The crystals were then used directly for X-ray analysis, after a few had been removed for measurement of activity.

For the activity assays, several crystals were centrifuged and washed two more times. The ammonium sulfate solution was replaced by a small amount of 10<sup>-3</sup> N HCl in which the crystals dissolved rapidly. The resultant solution was then assayed for esterolytic activity using benzoyl-L-tyrosine ethyl ester by the spectrophotometric method (Wilcox, 1970). The activity was compared with that of a stock solution of chymotrypsin A<sub>γ</sub> prepared by dissolving commercial lyophilized powder in 10<sup>-3</sup> N HCl. Enzymes concentrations were determined spectrophotometrically.

**X-Ray Analysis.** For each of the three inhibited derivatives, a single set of unique data was collected in overlapping shells to a resolution of 2.7 Å with Cu Kα radiation, using a Picker FACS1 automatic diffractometer. Four, nearly isometric crystals, typically 0.3–0.4 mm on an edge, were employed for each set of data, and the data for each crystal were brought to the same scale through a comparison of the overlapping reflections together with a 2.7-Å set of *h*0*l* data.

Cell dimensions were computed using a least-squares routine and the angular coordinates of ten reflections. The cell dimensions of native and inhibited crystals differed by only a few tenths of an Ångström (see Table I).

Reflections were measured by scanning 0.8° through ω, and by measuring background at the beginning and the end of each scan. Absorption corrections and preliminary data processing were carried out essentially as described by Cohen *et al.* (1969).

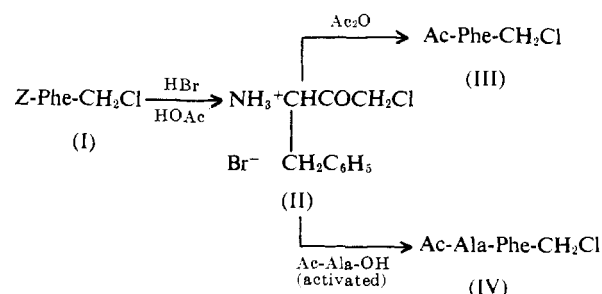
**Fourier Calculations.** Difference Fourier summations were carried out as described by Cohen *et al.* (1969). Each set of data from a given derivative was scaled to a single set of native chymotrypsin A<sub>γ</sub> data. *R* factors of substitution were computed between the three derivatives and the native enzyme, as well as between the derivatives themselves. The results are shown in Table II, where it can be observed that the average

TABLE II: "R" Factors of Substitution<sup>a</sup> for Chloromethyl Ketone Derivatives of Chymotrypsin A<sub>γ</sub>.

	Native	APCK	AAPCK
APCK	0.247		
AAPCK	0.276	0.109	
AAGPCK	0.286	0.140	0.131

$$^a R = \sum_{h,k,l} (|F_1| - |F_2|) / \frac{1}{2} \sum_{h,k,l} (|F_1| + |F_2|).$$

SCHEME I



amplitude differences between inhibited and native crystals are about twice as large as those between pairs of inhibited crystals.

Amplitude differences were then used to calculate difference Fourier summations. The phases used in the calculations were those found by D. R. Davies *et al.* (unpublished data) in the determination of the structure of tosyl-chymotrypsin A<sub>γ</sub> (Davies *et al.*, 1969).

Electron densities were initially calculated over one whole asymmetric unit at 1.4-Å intervals. Densities over individual regions of the asymmetric unit which showed interesting features were then calculated at 0.7-Å intervals. Where desired, electron densities were plotted with the aid of a Cal Comp plotter, and the results were compared to the 2.7-Å electron density map of the native protein.

**Model Building.** A complete atomic model of chymotrypsin A<sub>γ</sub> had been constructed in the laboratory using Kendrew skeletal models, 2 cm to the Å (D. R. Davies *et al.*, 1971, unpublished data). For each of the chloromethyl ketone derivatives, the model was modified by inserting the atoms of the inhibitor moiety according to the electron density found on the difference maps. An optical comparator of the kind described by Richards (1968) was used to position the atoms in the model.

## Results

**Synthesis of Chloromethyl Ketones.** The key intermediate for the synthesis of all of the peptide chloromethyl ketones was benzyloxycarbonyl-L-phenylalanine chloromethyl ketone (I). Deblocking of this compound with HBr in anhydrous HOAc yielded the crystalline hydrobromide (II) which could be used without further purification in the preparation of III and IV. The synthetic procedure following Scheme I has been found to be quite facile and has resulted in the preparation of nearly a dozen peptide chloromethyl ketones in 17–62% yield from I.

**Inhibition of Crystals of Chymotrypsin with Peptide Chloromethyl Ketones.** Preliminary trials showed that several peptide chloromethyl ketones were effective inhibitors of chymotrypsin in solution. Experiments were then designed to test the effectiveness of the various compounds as inhibitors of enzyme in the crystalline state. The results of a series of tests on chymotrypsin A<sub>γ</sub> are summarized in Table III. Since the spectrophotometric assay using the *p*-nitrophenyl ester gives optimal results with as little as 1 μg of fully active enzyme in the cuvet, it was possible to make accurate determinations of activities down to 1% with 0.1 mg of inhibited enzyme.

In the first series of experiments, the salt solution bathing the crystals contained 2% acetonitrile in order to increase the solubility of the inhibitors. We found that the crystals bathed

TABLE III: Per Cent Inhibition of Chymotrypsin A<sub>γ</sub> Crystals.

Inhibitor <sup>a</sup>	Series I, <sup>b</sup>		Series II, <sup>b</sup>		Series III, <sup>c</sup>		Series IV, <sup>d</sup>	
	14 Days	7 Days	7 Days	7 Days	7 Days	7 Days	9 Days	9 Days
PMSF	100							
Z-PheCH <sub>2</sub> Cl	30							
Boc-Gly-PheCH <sub>2</sub> Cl	100	99		51			45	
Ac-PheCH <sub>2</sub> Cl		65						
Ac-Gly-PheCH <sub>2</sub> Cl		75						
Ac-Ala-PheCH <sub>2</sub> Cl		71		49				
Ac-Leu-PheCH <sub>2</sub> Cl		17						
Boc-Ala-Gly-PheCH <sub>2</sub> Cl							13	
Z-Gly-Gly-PheCH <sub>2</sub> Cl							27	

<sup>a</sup> The amino acid residues Phe, Ala, and Leu have the L configuration. <sup>b</sup> Inhibitions were carried out in a saturated solution of inhibitor in 2.4 M phosphate containing 2% CH<sub>3</sub>CN (pH 5.6). <sup>c</sup> Inhibitions were carried out in a saturated solution of inhibitor in 2.4 M phosphate (pH 5.6). <sup>d</sup> Inhibitions were carried out on crystals larger than usual, 0.5 mm instead of 0.3 mm long, in 2.4 M phosphate (pH 5.6).

in a saturated solution of Boc-Gly-Phe-CH<sub>2</sub>Cl were completely inhibited after 2 weeks. A control experiment was run with phenylmethanesulfonyl chloride, since it was known that this compound inhibits crystals of both A<sub>α</sub> and A<sub>γ</sub> (Sigler *et al.*, 1966; Kraut *et al.*, 1967). The compound, benzyloxycarbonyl-L-phenylalanine chloromethyl ketone (ZPCK), reduced the activity only to 70%, and did not appear to be useful for X-ray studies.

In the second series of experiments, the Boc compound was used as a reference, and the effectiveness of acetyl-L-phenylalanine chloromethyl ketone (APCK) and acetyl dipeptide inhibitors with side chains varied in the P<sub>2</sub> position were compared. Although each reacted less rapidly than the Boc compound, the extent of reaction was satisfactory if the group attached to the phenylalanine residue was small in bulk. The larger side chain of leucine, however, clearly interfered with reaction in the crystal. In the third series, the effect of leaving out organic solvent during the reaction with inhibitor was tested. It was apparent that a small amount of organic solvent was indeed beneficial in increasing the rate of reaction. Finally, the inhibition of crystals with two blocked tripeptides was tested. The longer chains attached to the phenylalanine residue further reduced the rate of reaction, but the fact that Z-Gly-Gly-Phe-CH<sub>2</sub>Cl reacted to the extent of 27% in 9 days without organic solvent present appeared to indicate that blocked tripeptides with residues of small bulk could be accommodated in the crystal lattice. This observation led us to synthesize AAGPCK.

The crystals of inhibited chymotrypsin A<sub>γ</sub> which were prepared for X-ray diffraction analysis were almost completely substituted at the active site. No activity was found in the assay of the AAPCK and AAGPCK crystals, and only 8% activity was found in the APCK crystals. We believe that the relatively high extent of inhibition of these crystals was due to increased solubility of inhibitor in the 65% saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-5% dioxane solvent used in these experiments. These results also demonstrated that ammonium ion has very little effect on reactivity of chloromethyl ketones at pH 5.6.

TABLE IV: Amino Acid Analysis of Chymotrypsin A<sub>γ</sub> Inhibited with Ac-Ala-Phe-CH<sub>2</sub>Cl (AAPCK).

Residue	Theory <sup>a</sup>	Found
Lys	14	13.7
His	1	1.0
Arg	3	2.7
Asp	22	23.0
Thr	22	22.8 <sup>b</sup>
Ser	27	25.2 <sup>b</sup>
Glu	15	15.0
Pro	9	9.3
Gly	23	22.1
Ala	23	22.0
Val	23	19.6 <sup>b</sup>
Met	2	1.9
Ile	10	8.8 <sup>b</sup>
Leu	19	19.0
Tyr	4	3.6 <sup>b</sup>
Phe	6	6.0

<sup>a</sup> Values predicted from amino acid sequence of chymotrypsinogen minus the two dipeptides removed during activation and the one histidine residue which reacted with the inhibitor, and plus one alanine residue introduced by inhibitor. <sup>b</sup> Uncorrected for destruction or incomplete yield in acid hydrolysis.

In the experiments with chymotrypsin A<sub>α</sub> crystals, several different solvent compositions were used: 3.4 M sodium phosphate (pH 4.5)-3.9% dioxane, 2.0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-0.1 M acetate buffer (pH 4.5)-3% dioxane, and 60% saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-0.1 M citrate buffer (pH 5.6). No substantial amount of inhibition was found in any experiment, some of which extended to 19 days.

It was no surprise that the peptide chloromethyl ketones did not react with the monoclinic form of A<sub>α</sub> since steric hindrance in the active-site region was clearly evident in the crystal structure (Birktoft *et al.*, 1970). However, it was known that steric hindrance did not prevent the binding of formyl-L-tryptophan in subsite S<sub>1</sub>. We therefore decided to test A<sub>α</sub> crystals for inhibition by the analogous halo ketones, formyl-L-phenylalanine chloromethyl ketone and formyl-L-phenylalanine iodomethyl ketone. No inhibition by the chloro ketone was observed up to 19 days at pH 4.5 in a variety of solvents, nor was there any inhibition in 0.1 M citrate buffer (pH 5.6) and 60% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> during 19 hr. However, the much more reactive iodo ketone reduced the enzyme activity to 61% after 19 days in 3.6 M phosphate (pH 4.5). This latter observation may correspond in some degree to the report that formyl-L-phenylalanine bromomethyl ketone reacts readily with A<sub>α</sub> crystals (Birktoft *et al.*, 1970). Nevertheless, the lack of reaction with chloro ketone suggests that in A<sub>α</sub> crystals the reactivity of His-57 toward these reagents is much less than the reactivity that is manifest in solution, for reasons that are not clear at this time.

*Amino Acid Analysis of Inhibited Chymotrypsin A<sub>γ</sub>.* The amino acid analysis of enzyme inhibited with AAPCK is presented in Table IV. The results are quite similar to those reported by Schoellmann and Shaw (1963) for reaction with A<sub>α</sub> with TPCK, and likewise indicate that one histidine residue has been alkylated by the inhibitor, AAPCK.

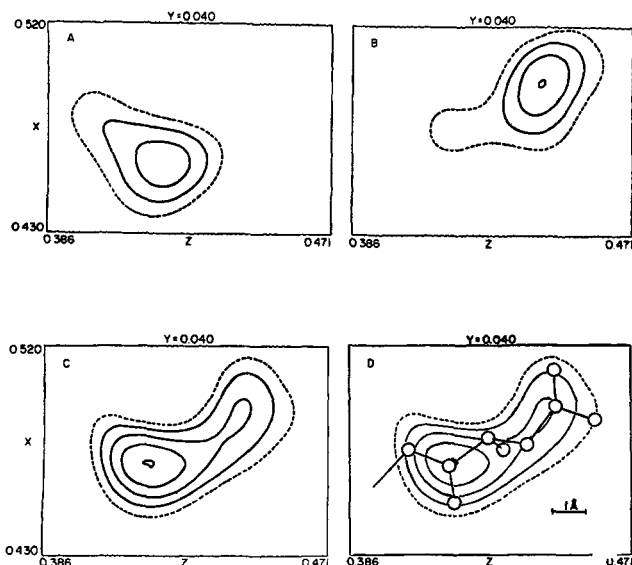


FIGURE 2: Electron density contours of difference maps between chloromethyl ketone derivative pairs. Lowest (dashed) contour is at  $0.110 \text{ e}/\text{\AA}^3$  and contours are drawn at  $0.055\text{-e}/\text{\AA}^3$  intervals. (A) AAPCK minus APCK difference map. (B) AAGPCK minus AAPCK difference map. (C) AAGPCK minus APCK difference map. (D) AAGPCK minus APCK difference map with corresponding portion of model projected upon it.

**Difference Fourier Synthesis between Inhibited and Native Chymotrypsin  $A_\gamma$ .** Electron density difference maps between native enzyme and enzyme inhibited with APCK, AAPCK, and AAGPCK were calculated using data collected to  $2.7\text{-\AA}$  resolution. All three difference maps were very similar. Numerous peaks and holes, all well above background were distributed for the most part over the entire molecule. There were, in fact, too many features on the difference maps to have arisen solely from the electron density of the inhibitors themselves, indicating substantial deviations from isomorphism between native and inhibited crystals. The source of these deviations cannot be readily defined; clearly the observed changes are more widespread than could be accounted for in terms of a single adjustment of the enzyme to the inhibitor in the vicinity of the active site. In view of the ubiquitous nature of electron density shifts over the whole asymmetric unit, and since the inhibitors are located very close to the twofold axis relating two molecules within the unit cell, it seems more likely that these shifts have arisen as a result of a slight rotation of each enzyme molecule within the crystal lattice when the enzyme reacts with inhibitor. In any case these deviations from iso-

TABLE V: The Three Highest Electron Density Peaks in the AAGPCK Minus APCK, AAGPCK Minus AAPCK, and AAPCK Minus APCK Different Maps of Inhibited Chymotrypsin  $A_\gamma$ .<sup>a</sup>

Peak	AAGPCK - APCK	AAGPCK - AAPCK	AAPCK - APCK
1	0.330	0.274	0.258
2	0.161	0.159	0.157
3	0.153	0.152	0.152

<sup>a</sup> Electron density expressed as electrons per  $\text{\AA}^3$ .

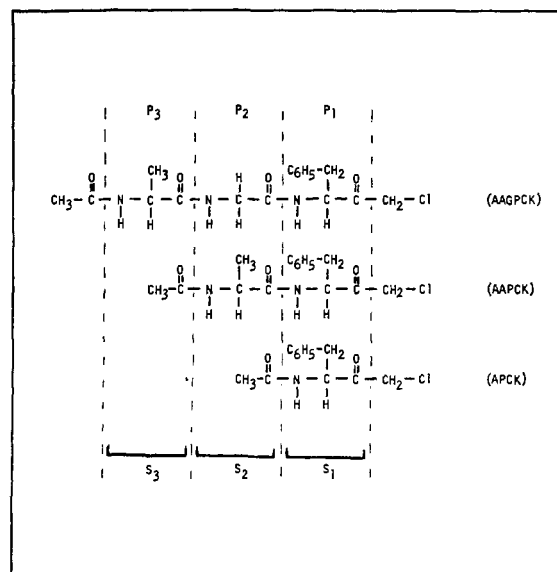


FIGURE 3: Alignment of inhibitors in the binding site of chymotrypsin. See Figure 1 for definition of subsites.

morphism within protein molecules had the consequence of making each of the three maps of inhibited enzyme minus native enzyme virtually uninterpretable.

**Difference Electron Density Maps between Pairs of Inhibited Crystals.** The APCK, AAPCK, and AAGPCK derivatives all seemed to show similar shifts in electron density when compared with uninhibited crystals and it therefore seemed possible that the effects of gross deviations from isomorphism might be avoided by computing difference Fourier maps using pairs of data sets from inhibited crystals. Such maps would be expected to show electron density only in positions occupied by residues present in one inhibitor and absent in the other.

Difference maps were therefore computed between the derivative pairs, AAGPCK minus APCK, AAPCK minus APCK, and AAGPCK minus AAPCK. It was immediately apparent that each map had only one major site of electron density, and that the maxima at these sites were approximately twice the electron density found in any other part of the molecule. This is illustrated in Table V, where the magnitudes of the maxima of the three largest peaks in the asymmetric unit have been listed in decreasing order, for each of the three difference maps. We believe this to be strong evidence supporting the contention that the crystals of all three derivatives are mutually isomorphous.

In Figure 2, electron density contours in  $xz$  sections at  $y = 0.04$  for all three difference maps are shown. Note that the electron density seen on the AAGPCK minus AAPCK map does not overlap that of the AAPCK minus APCK map but is displaced in the  $z$  direction. The electron density of the AAGPCK minus APCK map is essentially the sum of electron densities of the other two.

Figure 3 depicts the chemical structures of the three inhibitors aligned in register with respect to the subsites in the enzyme. In the derivatives the chlorine atom would be replaced by  $N^\epsilon$  of His-57. AAGPCK differs from AAPCK by a  $\text{CH}_3\text{C}(=\text{O})\text{NH}$  group at the N-terminal end of the chain and a methyl side chain at subsite  $S_3$ . The electron density on the difference map of the derivative pair, AAGPCK minus AAPCK, can therefore be equated with these additional parts



of the AAGPCK molecule. The small hole which might be expected from the methyl group present in AAPCK at subsite S<sub>2</sub> but missing from AAGPCK evidently does not appear on the difference map. Similarly the electron density on the map of AAPCK minus APCK represents the CH<sub>3</sub>C(=O)NH group and methyl side chain present at the N terminus of AAPCK, but not in APCK. Finally, the density in the third difference map, AAGPCK minus APCK, represents CH<sub>3</sub>C(=O)N(H)C(CH<sub>3</sub>)(H)C(=O)N(H) which is just the sum of the other two differences. (See Figure 2D.)

When the electron density is examined on the optical comparator, the planes of the two peptide units between P<sub>2</sub> and P<sub>3</sub> and between P<sub>3</sub> and P<sub>4</sub> are immediately apparent and can be fitted with an atomic model having an extended  $\beta$  configuration. Low electron density appears in the region of the terminal methyl group, which is near a peak that we believe corresponds to a water molecule in A $\gamma$  crystals. This water molecule would be displaced in the AAGPCK derivative.

## Discussion

The formulation of a mechanism for proteolysis based upon a solid understanding of steric interactions between enzyme and substrate has long been a primary objective of crystallographic investigations on chymotrypsin. In both  $\alpha$ - and  $\gamma$ -chymotrypsins, considerable effort has been expended in the search for crystals of enzyme-substrate or enzyme-inhibitor complexes which would be amenable to difference Fourier analyses. The ultimate purpose of such analyses would be to show the mode of binding of substrate or inhibitor molecules to the active site, and to indicate their steric relationships to catalytically important functional groups on the enzyme.

In order for the direct difference Fourier technique to be effective, the inhibitor must bind without altering the protein molecule, both with respect to its position within the crystal lattice, and with respect to its general internal structure, except for changes that are localized in a few specific regions. Substantial deviations in either respect would result in an uninterpretable electron density difference map. The survey of the inhibition of chymotrypsin crystals by peptide chloromethyl ketones showed that the tetragonal form of A $\gamma$  accommodated tripeptides with small side chains in positions P<sub>2</sub> and P<sub>3</sub>. In spite of this result and the fact that inhibition caused no change in the unit cell dimensions greater than 0.6%, the inhibited crystals were not closely isomorphous with native crystals.

We have been able partially to circumvent this difficulty through the use of three mutually isomorphous enzyme-inhibitor complexes. These were prepared by reacting chymotrypsin A $\gamma$  crystals with three structurally similar inhibitors, and the resulting difference maps allowed us to locate specific groups of the inhibitor moieties with a fair degree of precision. On the other hand, as a result of the distortion of the crystal lattice by the inhibitors, the position of the protein molecule itself is known with less certainty. Although it seems likely that inhibition has caused some displacement of the molecules in the crystal, probably by a small rotation, it is noteworthy that useful difference maps between inhibited enzyme crystals could be computed using the phases which had been obtained for tosyl-chymotrypsin A $\gamma$ . It follows that a vast majority of the groups in the protein as a whole have probably not been significantly displaced when viewed at a resolution of 2.7 Å. We have therefore felt justified in ignoring any small departures from the tosyl-chymotrypsin atomic

positions in our analysis of the three-dimensional relationship between protein and inhibitor.

In constructing a model of the enzyme-inhibitor complex, the stereochemistry of both the inhibitor and the protein molecule greatly limited the number of ways in which the inhibitor could interact with the protein. Moreover the difference maps located the N-terminal positions of the di- and tripeptide inhibitors, while we knew from chemical analysis that the methylene group of the chloromethyl ketone was attached to a nitrogen in the imidazole ring of His-57. These constraints allowed us to construct a model which we feel must be very close to reality in spite of our not being able to see electron density corresponding to the whole inhibitor.

In the completed model the inhibitor moiety was comprised of the chain *N*-acetyl-L-Ala-L-Ala-L-Phe-methylene attached to the ring of His-57. This chain contains all of the groups to be found in the peptide chains of APCK, AAPCK, and AAGPCK. The model accounts very well for the electron densities found in the difference maps, and a computer drawing of the active-site region of the enzyme containing the inhibitor is shown in Figure 4. Coordinates for A $\gamma$  are at present being refined, and the inhibitor coordinates will be published later with the refined A $\gamma$  coordinates.

The model shows the peptide chain of the inhibitor bound to an extended segment of the main chain in the enzyme, consisting of Ser-214-Trp-215-Gly-216. The two chains are anti-parallel and form essentially a  $\beta$  structure. Three hydrogen bonds are present in this structure: (1) a bond between the N—H of residue P<sub>3</sub> of the inhibitor and the C=O of Gly-216; (2) a bond between the C=O of P<sub>3</sub> and the N—H of Gly-216; and (3) a bond between the N—H of residue P<sub>1</sub> and the C=O of Ser 214. The N—H and C=O of Trp-215 point toward the interior of the enzyme molecule; at the same time N—H and C=O of P<sub>2</sub> point outward toward the solvent, as would be expected for an anti-parallel pleated-sheet conformation.

Steitz *et al.* (1969) have already called attention to the possible importance of the hydrogen bond in subsite S<sub>1</sub>. These investigators give a convincing argument for the view that the mode of binding of *N*-formyl-L-tryptophan, which they observed in crystals of A $\alpha$ , was not the productive mode of binding for the enzymatic hydrolysis of a substrate. They summarize a variety of chemical investigations that support this view. However, they suggest that a productive mode of binding would be obtained by a displacement of this "virtual substrate" so as to bring the N—H group into bonding distance of the C=O of Ser-214. This is indeed the binding interaction at subsite S<sub>1</sub> that best fulfills the stereochemical requirements of the present study with chymotrypsin A $\gamma$ .

On the C-terminal side of Gly-216, the main chain of the enzyme makes a sharp bend away from the position of the inhibitor. It thus appears unlikely that chymotrypsin would recognize the main chain of a peptide substrate beyond three residues on the N-terminal side of the cleavage point.

We have noted from an inspection of our model that a prolyl residue would be accommodated in position P<sub>2</sub> in the enzyme-substrate complex without breaking hydrogen bonds, whereas if a prolyl residue were to be substituted into position P<sub>3</sub>, steric interference with the pyrrolidine ring would occur and at least one hydrogen bond would be ruptured. Thus one would expect, for example, that *K<sub>m</sub>* for the hydrolysis of Ac-Gly-Pro-Phe-OMe would be lower than *K<sub>m</sub>* for hydrolysis of Ac-Pro-Gly-Phe-OMe, and in fact kinetic studies which will be reported later show this to be the case.

It was found that Ac-Leu-Phe-CH<sub>2</sub>Cl reacted more rapidly



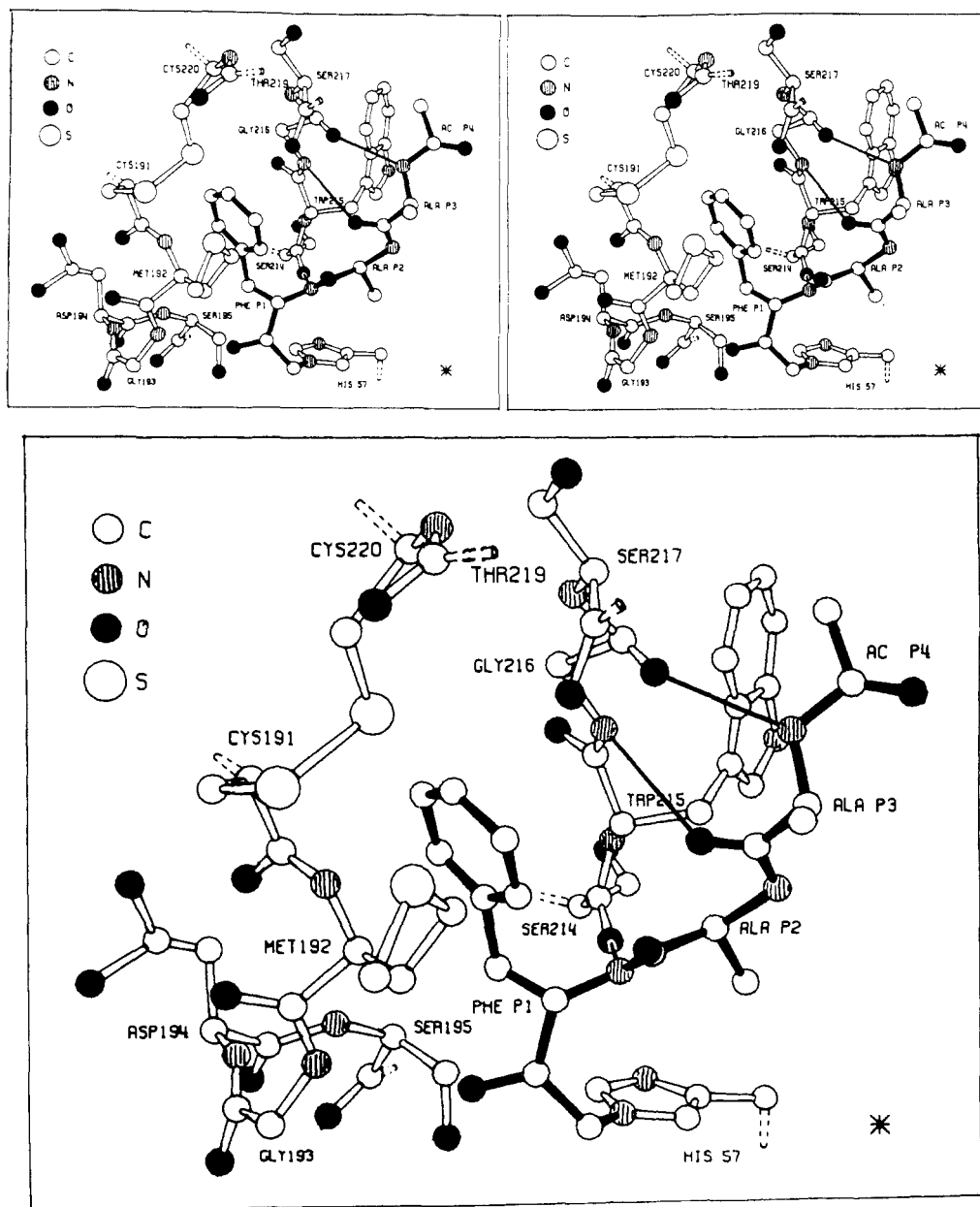


FIGURE 4: Illustration showing the proposed binding of the inhibitor moiety, *N*-acetyl-L-Ala-L-Ala-L-Phe-methylene to chymotrypsin  $A_7$ . Only pertinent portions of the protease molecule are shown. Upper two drawings are a stereo pair.

with the enzyme in solution than did Ac-Ala-Phe-CH<sub>2</sub>Cl, whereas the converse was true in the crystals of  $A_7$  (Table III). The relative rates in solution are in accord with the results of substrate studies by Yamashita (1960). From the kinetic data, the following values of  $k_{cat}/K_m$  (sec<sup>-1</sup> M<sup>-1</sup>) may be calculated for a series of glycylaminoacyltyrosinamides: Gly-Gly-Tyr-NH<sub>2</sub>, 26 ( $K_m$  = 0.041 M); Gly-Ala-Tyr-NH<sub>2</sub>, 110 ( $K_m$  = 0.045 M); Gly-Val-Tyr-NH<sub>2</sub>, 570 ( $K_m$  = 0.021 M); and Gly-Leu-Tyr-NH<sub>2</sub>, 400 ( $K_m$  = 0.021 M). Furthermore, our results may be related to an extensive survey of the specificity of chymotrypsin toward the polypeptide chains of nine denatured proteins, all of known sequence (Neil *et al.*, 1966). This study revealed, in addition to the "primary specificity" of subsite  $S_1$ , a pattern of "secondary specificity" for subsites  $S_2$ ,  $S_1'$ , and  $S_2'$ . Of particular interest here was the conclusion that Val, Ile, Lys, and Pro in positions  $P_2$  favor hydrolysis. This is especially evident when the residue in  $P_1$  is not favored

by the primary specificity, *i.e.*, when it is not aromatic or leucine. It was on the basis of such observations as those noted above that we designed the chloromethyl ketone containing leucine in position  $P_2$ . An examination of the model showed that a leucine residue in  $P_2$  comes into van der Waals contact with the side chain of Ile-99 in the enzyme. This may very well be a binding interaction in the  $S_2$  subsite. However, in the crystal it is clear that the leucine side chain of the inhibitor in one enzyme molecule would sterically overlap the corresponding side chain in the other inhibited molecule that is related to the first by the  $4_2$  axis. Thus the slow reaction of the crystal with an inhibitor containing a large side chain at  $P_2$  can be explained, although it is possible that the lower solubility of the leucine inhibitor may also affect the rate.

Inspection of the model also indicates that chymotrypsin should show a high degree of stereospecificity for position  $P_2$  in substrates. Placement of a  $\beta$ -carbon atom on the  $P_2$  residue in

the D configuration, for example, by replacing glycine with D-alanine in the inhibitor AAGPCK, results in steric hindrance with the  $\beta$ -carbon atom of Trp-215. The chain is forced away from the enzyme and the hydrogen bond in subsite S<sub>1</sub> cannot be formed. Rotation about the C $\alpha$ -CO bond of residue P<sub>2</sub> can alleviate this steric hindrance, but at the expense of breaking the hydrogen bonds in subsite S<sub>3</sub>. Stereospecificity at P<sub>2</sub> has been observed by Morihara *et al.* (1969) in the hydrolysis of the substrates, Z-L-Ala-L-Tyr-NH<sub>2</sub> and Z-D-Ala-L-Tyr-NH<sub>2</sub>.

Given the mode of binding of the inhibitor backbone to Ser-214-Trp-215-Gly-216 in the anti-parallel  $\beta$  structure, a covalent bond between the methylene group on residue P<sub>1</sub> and N $\epsilon$  atom of His-57 can be easily formed in the model. We have no X-ray data pertaining to this region of the complex, but we have been able to construct the model such that the position of His-57 has not moved by more than 0.5 Å from the native position.

The position of the phenyl ring in the inhibitor is approximately fixed relative to the enzyme by the covalent bond with His-57 and the anti-parallel  $\beta$  structure. As expected, this side chain of the inhibitor lies in the same general area as the phenyl ring in tosyl-chymotrypsin (Birktoft *et al.*, 1970; D. R. Davies *et al.*, 1971, unpublished data), and as the indole ring of N-formyl-L-tryptophan (Steitz *et al.*, 1969) and indoleacryloyl-chymotrypsin A $\alpha$  (Henderson, 1970). A more precise positioning of the phenyl ring is not possible by model building because of rotational freedom about the C $\alpha$ -C $\beta$  and C $\beta$ -C $\gamma$  bonds of the phenylalanyl residue. It should be possible to locate the ring more precisely by using a chloromethyl ketone that contains a marker on the aromatic side chain.

In the model which we have constructed, only two contacts are significantly shorter than would be expected from van der Waals radii. These are between the C $\beta$  of Ser-195 and each of the atoms in the carbonyl group of phenylalanine at P<sub>1</sub>. Since this region of the inhibitor does not show up on the difference maps, we have not attempted to move residues of the protein molecule to accommodate the inhibitor, although we believe that some small displacements must have occurred.

One may ask what relevance the structure of derivatives prepared with chloromethyl ketones has to the mechanism of proteolysis. A wide variety of studies has suggested that in an intermediate step of the catalysis a labile ester linkage is made between the O $\gamma$  of Ser-195 and the carboxyl group of residue P<sub>1</sub> in the substrate. In fact, if the bond which a chloro ketone forms with His-57 is broken and a carboxyl group substituted for the methyl carbonyl moiety, then an ester linkage can easily be made with Ser-195 without appreciably altering the orientation of the remaining portion of the inhibitor. We therefore, feel that the model which we have proposed for the inhibitor-enzyme complex most likely represents the mode of binding of acyl intermediates as well (*cf.* Henderson, 1970).

On the basis of the one study alone, it would not be possible to answer the skeptic who may ask whether or not the binding observed in the crystal is influenced in a significant way by intermolecular lattice contacts, and also perhaps the solvent used for crystallization. Indeed, evidence is presented above that binding does affect lattice contacts and causes movement of the molecules, and a reciprocal effect must be considered. This question would be most surely answered by crystallographic studies of other serine proteases such as elastase, trypsin, and subtilisin, using specifically designed peptide chloromethyl ketones or other specific inhibitors. In fact, such a study on subtilisin BPN' has been recently carried out (personal communication from J. Kraut, 1971), and the result leaves no doubt that the binding observed in chymotrypsin A $\gamma$

is essentially independent of the intermolecular packing. In subtilisin BPN' it was known that the structure of the catalytic site, including the residues His-64, Ser-221, Asp-32, and Ser-33, was analogous in three dimensions to the catalytic site of chymotrypsin (Alden *et al.*, 1970). A crystallographic study of the binding of Z-Ala-Gly-Phe-CH<sub>2</sub>Cl has now demonstrated that, in addition, there also exists in subtilisin a set of subsites, S<sub>1</sub>, S<sub>2</sub>, and S<sub>3</sub>, which are precisely analogous to those in chymotrypsin, including an extended segment of chain that forms a  $\beta$  structure with the inhibitor. Therefore, the binding observed in chymotrypsin A $\gamma$  is not unique to a particular crystalline form and can be predicted to be typical for the whole class of serine proteases.

Finally, it should be pointed out that the extended chain in chymotrypsin (residues 214-217) which we have shown to be functional in binding is more folded in the zymogen (Freer *et al.*, 1970). Activation of chymotrypsinogen results in the creation of the specific binding site, and it is now clear that this includes not only the opening of the hydrophobic pocket but also the positioning of the chain segment 214-217 for its role in forming a specific  $\beta$  structure with a natural substrate.

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## Effects of Traces of *n*-Alcohols on the Acid Denaturation of Horse Ferrihemoglobin\*

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**ABSTRACT:** No binding of  $^{14}\text{C}$ -labeled decanol by horse ferrihemoglobin ( $\text{Hb}^+$ ) is detected in equilibrium dialysis experiments. Nevertheless, decanol and other such alcohols (6–12 carbons) at extreme dilution markedly accelerate the rate of acid denaturation without changing the rate–pH profile. Plotting the rate constants of denaturation,  $k_{\text{obsd}}$ , against the square of the alcohol concentration yields straight lines which show no sign of leveling off. Since  $\text{Hb}^+$  is an  $\alpha, \beta$  dimer in the pH region of interest, an average of 1 equiv of alcohol must be bound per subunit to give the observed catalytic effect. The rate constant of the denaturation of the  $\text{Hb}^+$  alcohol complex is at least 250 times that of native  $\text{Hb}^+$ . The catalytic effectiveness increases greatly as the chain length of the alcohol is increased. From this observation it is proposed that hydro-

phobic interactions play a large part in the determination of the stability of the complex. Plots of  $\log k_{\text{obsd}}$  as a function of  $1/T$  are parallel both in the presence and absence of octanol. Thus, the change in the rate constant must be due to a change in the entropy of activation, consistent with the postulated hydrophobic interaction. Preliminary results show that octanol has no catalytic effect on the denaturation of either carbonyl- or oxyhemoglobin. The equilibrium pH profile for the denaturation of the octanol complex is shifted toward greater instability relative to that of  $\text{Hb}^+$ , and the number of protons required to initiate the denaturation is changed from five in the uncomplexed form to three in the complexed form: *i.e.*,  $\text{Hb}^+$  in the presence of octanol shows less cooperativity with respect to hydrogen ion than does  $\text{Hb}^+$  in its absence.

The binding of hydrocarbons and hydrocarbon derivatives to proteins has long been of interest to physical biochemists (Kauzmann, 1959). Bovine serum albumin (BSA),<sup>1</sup> for example, has been shown to possess a number of binding sites for simple hydrocarbons (Wishnia, 1962); in addition, 4–5 equiv of the alcohols dodecanol, decanol, and octanol are found to bind to BSA with association constants of  $1.5 \times 10^5$ ,  $7 \times 10^4$ , and  $3 \times 10^3$ , respectively (Ray *et al.*, 1966). BSA has also been shown to bind fatty acid and detergent anions (Reynolds *et al.*, 1967; Goodman, 1958). The binding of such ions produces perturbations in the tryptophan, tyrosine, and phenylalanine uv absorption and fluorescence spectra (Bigelow and Sonnenberg, 1962; Polet and Steinhardt, 1968).

With hemoglobin, however, few data are available. Wishnia (1962) showed that butane and pentane were bound to horse oxyhemoglobin. It will be shown here that ferrihemoglobin does not bind detectable amounts of those alcohols which are bound to BSA. Other results, however, indicated that the presence of traces of octanol markedly increased the rate of acid denaturation (Steinhardt *et al.*, 1966). The purpose of this study was, therefore, to examine the seeming paradox that even though  $\text{Hb}^+$  did not appear to bind alcohols, the presence of trace amounts of the latter markedly affected the rate of acid denaturation.

### Experimental Section

#### Materials

Horse COHb was prepared from blood of a single animal as described previously (Steinhardt *et al.*, 1966) and stored frozen in 5% solutions.  $\text{Hb}^+$  was prepared by oxidizing these solutions with 2 equiv of  $\text{K}_3\text{Fe}(\text{CN})_6$  and then dialyzing against first 0.2 M phosphate buffer (pH 6.8) and then distilled water. The alcohols and diols were obtained from the following sources: 1-dodecanol (Puriss), Fluka; 1-decanol and 1-octanol (Puriss), International Chemical and Nuclear; 1-heptanol, Aldrich; 1-hexanol, Fluka; 1,12-dodecanediol and 1,10-dodecanediol, K & K; 1,8-octanediol, Aldrich. [ $^{14}\text{C}$ ]Decanol was purchased from International Chemical and Nuclear.

#### Methods

Kinetic runs were performed by following the changes in optical density at 405 nm as in previous work (Polet and Steinhardt, 1969). Runs were carried out either on a Cary 14 spectrophotometer equipped with thermostated cell holders or on a Gibson-Durham stopped-flow apparatus. The temperature was controlled to  $\pm 0.1^\circ$ . Denaturation was effected by mixing  $\text{Hb}^+$  with acetate buffer, pH > 3.3 or HCl, pH < 3.3. Ionic strength was maintained at 0.02 M with sodium acetate or potassium chloride, respectively.

Solutions of the highest alcohol concentrations were prepared by using lambda micropipets. These solutions were then diluted to give the desired concentrations. Thus, while absolute accuracy of the concentrations is limited by the accuracy of the lambda pipets, the accuracy of the concen-

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<sup>1</sup> Abbreviations used are: BSA, bovine serum albumin; COHb, carbonylhemoglobin;  $\text{Hb}^+$ , ferrihemoglobin;  $\text{O}_2\text{Hb}$ , oxyhemoglobin.