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MODIFICATION OF CHYMOTRYPSIN WITH THE SPECIFIC CYANATE REAGENT *N*-ACETYL-*p*-CYANATO-L-PHENYLALANINE ETHYL ESTER

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Summary

The specific cyanate reagent *N*-acetyl-*p*-cyanato-L-phenylalanine ethyl ester (compound 1) was synthesized in an attempt to selectively modify the binding pocket of chymotrypsin (EC 3.4.21.1) while leaving the catalytic residues untouched. The reagent reacts with chymotrypsin to yield chiefly an inactive derivative 3a, with the active site Ser-195 carbamylated and the α-amino group of Cys-1 linked to the inhibitor via an isoureido (—NHC (=NH)O—) linkage. Reaction of chymotrypsin, blocked by a carbamyl group on the α-amino group, with the reagent 1 yields a modified enzyme (compound 4) with an additional carbamyl group on Ser-195. Neither derivative 3a nor 4 reacts with diisopropylfluorophosphate under conditions where chymotrypsinogen is modified, indicating that Ser-195 is altered. Both derivatives 3a and 4 are retained on a 4-phenylbutylamine affinity column demonstrating that the substrate binding pocket is intact in both derivatives. The results indicate the potential value of aryl cyanates as protein reagents for the selective modification of nucleophilic sites. However, it is apparent that reaction at unreactive residues in the binding pocket of chymotrypsin with cyanates or similar reagents will require blockage of the more nucleophilic catalytic residues.

Introduction

The serine proteases are probably the most well-characterized of all enzyme families studied. For chymotrypsin (EC 3.4.21.1) in particular, the X-ray structure has been determined [1], aspects of the catalytic mechanism are becoming established [2,3], and the extended binding site has been investigated using

Abbreviations: *i*Pr*P*-F, diisopropylfluorophosphate; DIP-chymotrypsinogen, complex formed between *i*Pr*P*-F and chymotrypsinogen; Ac-Tyr-OEt, ethyl ester of *N*-acetyltyrosine; Tos-PheCH$_2$Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Ac-Phe(OCN)-OEt, *N*-acetyl-*p*-cyanato-L-phenylalanine ethyl ester.

substrates and inhibitors [4,5]. While the serine proteases possess a remarkable similarity among their active site geometries and catalytic mechanisms, there are structural differences in the binding region to accommodate their distinct substrate specificities. The binding pocket at the S_1 subsite determines the enzyme's primary substrate specificity. In chymotrypsin this so-called "tosyl hole" [4] accounts for its specificity towards residues with aromatic side chains. The phenyl group of a substrate P_1 residue fits snugly into this hydrophobic pocket which is surrounded by Ser-189, Ser-190, and parts of the peptide backbone.

The purpose of this investigation was to create an enzyme derivative with altered substrate specificity by selective chemical modification of residues in the S_1 subsite of chymotrypsin. We choose to investigate the reaction of the substrate analog *N*-acetyl-*p*-cyanato-L-phenylalanine ethyl ester (Ac-Phe-(OCN)-OEt)(1). Aryl cyanates (Ar-OCN) are gaining utility as reagents in synthetic organic chemistry [6,7]. One in particular, *p*-nitrophenyl cyanate, has also been used as an effective enzyme modification reagent in specifically attacking two sites on chymotrypsin including Ser-195, the catalytic residue [8]. The aryl cyanate reagent (compound 1) was designed with a reasonably reactive electrophilic center and a substrate-like structure, which upon bonding should position the cyanate group deep within the binding pocket of the enzyme. In this paper, we report the characterization of the derivatives formed by reaction of Ac-Phe(OCN)-OEt with chymotrypsin.

Materials and Methods

Reagents and Materials. Chymotrypsin A_α (lot CDI1BK) was obtained from Worthington Biochemical Co. and used without further purification; its substrate, Ac-Tyr-OEt, was synthesized by standard methods and had m.p. 80—81°C. Chymotrypsinogen A (lot 111C-8170) and acetyl-trypsin (lot 90C-2200) were obtained from Sigma Chemical Co. and used without further purification. Diaflo ultrafilters used in enzyme concentration were purchased from Amicon Corp. Carboxymethyl cellulose, CM-52, microgranular, was purchased from Whatman Biochemicals Ltd. Sephadex G-25 and Sepharose 4B were obtained from Pharmacia Fine Chemicals. $K^{14}CN$ (11.5 Ci/mol, lot 553-199) and diiso-[^{14}C]propylfluorophosphate (110 Ci/mol, lot 669-016) were obtained from New England Nuclear. CNBr, *p*-nitrophenol, 4-phenylbutylamine, and all other reagents and solvents used were analytical grade. Mass spectra were taken on a Perkin-Elmer Hitachi RMU-7L instrument and nuclear magnetic resonance (NMR) spectra were taken on a Varian T-60 instrument. Enzyme inhibition kinetics were performed on a Radiometer automated pH-stat (model (TTT11). Chymotrypsin activity was measured by the potentiometric assay method using Ac-Tyr-OEt as the substrate [9]. *p*-Nitrophenyl cyanate was synthesized from *p*-nitrophenol and CNBr according to the method of Robillard et al. [8].

N-Acetyl-p-cyanato-L-phenylalanine ethyl ester. KCN (0.32 g, 5 mmol) was dissolved in 5 ml of water and added dropwise to bromine (0.26 ml, 5 mmol) under 0.5 ml water at 10°C, insuring that the bromine color was completely discharged. The CNBr was extracted into 4 times 5 ml of ether and dried over $MgSO_4$. A solution of Ac-Tyr-OEt (1.35 g, 5 mmol) in 20 ml of acetone was

stirred at 0°C, while the solution of CNBr (approx. 5 mmol) in 20 ml of ether was added. Triethylamine (0.70 ml, 5 mmol, in 5 ml acetone) was added dropwise over a 0.8-h period, 5 ml of ether was then added, and the mixture was stirred at 0°C for 1 h. After filtration of the reaction mixture, the filtrate was evaporated in vacuo at 25°C. Treatment of the yellow oil twice by adding anhydrous ether, stirring, filtering, and evaporating the ether yielded a crude white solid. The product was crystallized with difficulty from ethyl acetate/cyclohexane (3 : 5—3 : 8, v/v) to give 0.42 g (30%) of a white solid, m.p. 86—87°C, $[\alpha]_D^{25}$ = + 16° (c 1, ethanol). The mass spectrum showed a major peak at *m/e* 276 (M^+), and the IR spectrum showed a cyanide band at 2240 and 2280 cm^{-1}. The NMR spectrum ([^{2}H]chloroform) had peaks at 7.3 δ (4 H, s, C_6H_4), 6.3 (1 H, d, NH), 4.7 (1 H, m, CH), 4.2 (2 H, q, CH_2CH_3), 3.2 (2 H, d, CH_2Ph), 2.0 (3 H, s, CH_3CO), and 1.3 (3 H, t, CH_3CH_2). Anal. Calculated for $C_{14}H_{16}N_2O_4$: C, 60.86; H, 5.84; N, 10.14. Found: C, 61.16; H, 5.92; N, 10.22. Ac-Phe(O^{14}CN)-OEt (compound) 1 was prepared by an identical procedure, beginning with K^{14}CN (specific activity approx. 0.1 Ci/mol). The product yield was 0.46 g (33%) of a white solid, with a specific activity of 0.088 Ci/mol.

CM-cellulose chromatography. A 70 × 1.2 cm column of carboxymethyl cellulose CM-52 was re-equilibrated before each run with 1 l of 0.05 M potassium phosphate (pH 6.2). All operations were performed at 4°C. The protein sample was pumped onto the column and eluted at 0.5 ml/min with a linear salt gradient of increasing ionic strength. This gradient consisted of 0.6 l each of 0.05 and 0.12 M potassium phosphate (pH 6.2).

Affinity chromatography. An affinity column of 4-phenylbutylamino Sepharose was prepared according to the method of Stevenson and Landman [10] with slight modification. The 50 × 1.2 cm column of 4-phenylbutylamino Sepharose was re-equilibrated before each run with 1 l of 0.1 M Tris (pH 8.0). All operations were performed at 4°C. The protein sample was pumped onto the column and eluted at 0.6 ml/min with 0.1 M Tris (pH 8.0). Protein bound to the column was removed by eluting with 0.1 M Tris (pH 8.0) containing 1 M tetraethylammonium bromide.

Reaction of chymotrypsin A_α with p-nitrophenyl cyanate. *O*-Carbamyl-Ser-195 chymotrypsin A_α was prepared using the general method of Robillard et al. [8]. Lyophilized chymotrypsin A_α (100 mg, 4 μmol) was dissolved in 10 ml of 0.05 M acetate buffer (pH 4.5) at 25°C. A solution of *p*-nitrophenyl cyanate (0.95 mg, 6 μmol, 1.5 equiv.) in 0.30 ml of dioxane was added in increments to the enzyme solution. After the inhibition reaction proceeded for 1 h at 25°C and pH 4.5, the enzymatic activity decreased to 6.5%. The pH was raised to 7.6 and phenylmethanesulfonyl fluoride (0.70 mg, 4 μmol, 1 equiv.) in 0.25 ml of dioxane was added; after 1 h at 25°C and pH 7.6 chymotrypsin was 100% inactivated. Excess reagent was removed by dialysis at pH 3. The protein mixture was subjected to CM-cellulose chromatography and two major derivatives, *O*-carbamyl-Ser-195 chymotrypsin A_α (compound 2a) and a disubstituted chymotrypsin A_α (compound 2b), which eluted at 0.105 and 0.095 M K^+ were obtained in a 50 : 50 ratio. After concentration the proteins were dialyzed at pH 3. *O*-carbamyl-Ser-195 chymotrypsin A_α (compound 2a) was chromatographed on the affinity column in a second purification step and was isolated as 9.0 mg of a lyophilized solid.

Reaction of chymotrypsin A_α with Ac-Phe(OCN)-OEt. Lyophilized chymotrypsin A_α (150 mg, 6 μmol) was dissolved in 10 ml of 0.05 M citrate buffer (pH 6.5) and cooled to 0°C. A solution of Ac-Phe(OCN)-OEt (9.9 mg, 36 μmol, 6 equiv.) in 0.30 ml of dioxane was added in increments to the enzyme solution. After the inhibition reaction proceeded for 2.5 h at 0°C and pH 6.5, the enzymatic activity decreased to 1.5%. Excess reagent was removed by dialysis at pH 3. The protein mixture was promptly subjected to CM-cellulose chromatography and two major derivatives, designated compounds 3a and 3b, which eluted at 0.085 and 0.074 M K^+ were obtained in a 60 : 40 ratio. After concentration the proteins were dialyzed at pH 3. The two derivatives were chromatographed separately on the affinity column in a second purification step and were finally isolated as lyophilized solids, 28 mg of derivative 3a and 16 mg of derivative 3b.

The reaction of chymotrypsin A_α with Ac-Phe($O^{14}CN$)-OEt was run in an almost identical manner. Here 7 equiv. of Ac-Phe($O^{14}CN$)-OEt was used and the inhibition reaction proceeded for 4 h to reach 1.5% enzymatic activity. The two derivatives were isolated as lyophilized solids, 36 mg of ^{14}C-labeled derivative 3a and 16 mg of ^{14}C-labeled derivative 3b.

Preparation of N-carbamyl-Cys-1 chymotrypsin A_∂. *N*-Carbamyl-Cys-1 chymotrypsinogen A was synthesized on a 24 μmol scale from chymotrypsinogen A and 5 equiv. of *p*-nitrophenyl cyanate according to the method of Robillard et al. [8]. This intermediate was isolated as a lyophilized solid. *N*-Carbamyl-Cys-1 chymotrypsinogen A was activated by treating the zymogen (300 mg, 12 μmol, in 14 ml) with acetyl-trypsin (12 mg in 1 ml) in 10 mM $CaCl_2$, for 1.5 h at 0°C and pH 7.6. The chymotryptic activity reached 110% by the standard assay. The protein mixture was immediately subjected to CM-cellulose chromatography and a single major product was obtained which contained 70% of the protein applied. After dialysis at pH 3 the enzyme solution was concentrated to 10 mg/ml and used in further inhibition reactions.

Reaction of N-carbamyl-Cys-1 chymotrypsin A_∂ with Ac-Phe(OCN)-OEt. Chromatographed *N*-carbamyl-Cys-1 chymotrypsin A_∂ (105 mg, 4 μmol) in 10 ml of solution was adjusted to pH 6.5 by adding 1 ml of 0.5 M citrate buffer (pH 6.5) and cooled to 0°C. A solution of Ac-Phe(OCN)-OEt (6.8 mg, 24 μmol, 6 equiv.) in 0.30 ml of dioxane was added in increments to the enzyme solution. After the inhibition reaction proceeded for 3 h at 0°C and pH 6.5, the enzymatic activity decreased to 1.6%. Excess reagent was removed by dialysis at pH 3. The protein mixture was promptly subjected to CM-cellulose chromatography, and a single major derivative, designated compound 4, was obtained in a peak (0.083 M K^+) with a slight left shoulder. After concentration the protein was dialyzed at pH 3. Derivative 4 was chromatographed on the affinity column to aid in characterization and was isolated as 30 mg of a lyophilized solid.

The reaction of *N*-carbamyl-Cys-1 chymotrypsin A_∂ with Ac-Phe($O^{14}CN$)-OEt was run in an almost identical manner. Here 6 equiv. of Ac-Phe($O^{14}CN$)-OEt was used and the inhibition reaction proceeded for 3 h to reach 1.6% enzymatic activity. The lone derivative was isolated as a lyophilized solid, 20 mg of ^{14}C-labeled compound 4.

^{14}C specific activity radioassay. The specific activity of ^{14}C-labeled reagents and proteins was determined by measuring the ^{14}C content of a sample using a

Packard Tri-Carb liquid scintillation spectrometer (model 3375), and relating the ^{14}C content to the sample concentration as measured by weight basis (reagents) or ultraviolet absorbance at 282 nm (proteins). An aqueous sample solution (1.0 ml) was added to 10 ml of scintillant and duplicate sample groups were counted at 4° C. The scintillant used for the radioassays contained 7.50 g of 2,5-diphenyloxazole (PPO), 375 mg of 1,4-bis(2-(5-phenyloxazolyl)) benzene (POPOP), and 125 g of naphthalene made up to 1.0 l in 1,4-dioxane. A counting efficiency of approx. 80% was always maintained as indicated by a [^{14}C]toluene internal standard.

Reaction of chymotrypsinogen A and enzyme derivatives with diiso[^{14}C]-propylfluorophosphate. DIP-Chymotrypsinogen A was prepared based on the general method of Morgan et al. [11]. Diiso[^{14}C]propylfluorophosphate (*i*Pr*P*-F) possessed a nominal specific activity of 0.188 Ci/mol. [^{14}C]*i*Pr*P*-F (7.3 mg, 40 μmol, 100 equiv.) in ethylene glycol was added to a solution of chymotrypsinogen A (10 mg, 0.4 μmol) in 10 ml of 0.25 M KCl/0.05 M $CaCl_2$. The inhibition reaction was stirred for 12 h at 25° C and pH 7, the pH being kept at 7 by periodic addition of 1 M KOH. All excess [^{14}C]*i*Pr*P*-F was removed by gel filtration on a 50 × 1.2 cm column of Sephadex G-25 equilibrated with 0.02 M formate (pH 3.8). DIP-Chymotrypsinogen was isolated as 7 mg of a lyophilized solid and tested separately for ^{14}C content and activatibility. Chymotrypsinogen A and DIP-chymotrypsinogen were activated by treating the zymogen (2.8 mg, 0.56 μmol, in 4.5 ml) with acetyl-trypsin (0.26 mg in 0.5 ml) for 2 h at 0° C in 0.1 M Tris (pH 7.6). The chymotryptic activity of the former reached 110% by the standard assay.

The reactions of chymotrypsin derivatives 3a and 4 with [^{14}C]*i*Pr*P*-F were run in an identical manner. The two assumed DIP-products were isolated and tested for ^{14}C content, employing ^{14}C-labeled DIP-chymotrypsinogen as a standard.

Results

Inhibition of chymotrypsin by Ac-Phe(OCN)-OEt. The aryl cyanate reagent Ac-Phe(OCN)-OEt (compound 1) was synthesized by reaction of Ac-Tyr-OEt with CNBr in 30% yield. In preliminary experiments Ac-Ph(OCN)-OEt was discovered to inactivate chymotrypsin rapidly and irreversibly. The inhibition reaction is pH dependent and proceeded with maximum velocity at pH 6—7, while the velocity dropped sharply outside pH 5—8.

For the inhibition reaction involving chymotrypsin (0.08 μM) and Ac-Phe-(OCN)-OEt (0.6 mM) at 25° C and pH 7.5, the kinetic constants $k_{2nd} = 4.4 \cdot 10^3\ M^{-1} \cdot min^{-1}$ and halflife = 1.6 min were obtained. These kinetic results represent the average values for three successive runs. No K_i value could be reliably obtained. The reaction is most likely a direct bimolecular process without an initial binding step. Alternatively, the reaction may proceed via a pre-equilibrium process but the enzyme-inhibitor complex must be relatively weak i.e. $K_i > 2$ mM.

Relatively mild reaction conditions were used for the preparative work to decrease the hydrolysis rate of the inhibitor's cyanate group to a carbamate and to minimize non-specific side reactions. Two modified enzyme derivatives were

obtained, compounds 3a and 3b, as the only major products, and were separated by CM-cellulose chromatography and purified by affinity chromatography. They were prepared in both unlabeled and ^{14}C-labeled form. Derivative 3a was consistently isolated as the predominant product and was eluted from the CM-cellulose column at a somewhat higher $[K^+]$ than compound 3b. By analogy with *O*-carbamyl-Ser-195 chymotrypsin A_α (compound 2a) and the disubstituted chymotrypsin A_α (compound 2b), this indicates that compound 3a could be less highly substituted than compound 3b.

An experiment was carried out to determine the stability of the enzyme derivatives in solution for a 24-h period at 25° C at various pH levels. Protein samples were assayed for changes in residual enzymatic activity after 24 h relative to initial activity. The initial activity corresponded to approx. 2% of normal chymotryptic activity. Both derivatives 3a and 3b showed little change in activity (less than 10%) upon remaining at pH 3.0 and 6.5 but not at pH 10.

Amino acid analysis. After chymotrypsin was reacted with Ac-Phe(OCN)-OEt an amino acid analysis was performed on the enzyme derivatives to ascertain their tyrosine content. Upon total acid hydrolysis, the Ac-Phe(OCN)-OEt moiety should liberate a tyrosine residue. If the entire inhibitor were attached to the derivative in some way then the presence of at least one extra tyrosine would be revealed, whereas if only a carbamyl group were attached upon hydrolysis then no extra tyrosine would appear. A complete amino acid analysis was performed in each case but the calculation for tyrosine was made by comparing the size of the phenylalanine and tyrosine peaks assuming that the phenylalanine peak corresponded to six residues. Chymotrypsin gave 4 (3.89) tyrosines per molecule as expected [9], while derivative 3a had 5 (4.98) and derivative 3b had an unusual 5.4 tyrosines. Derivative 2a had no additional tyrosine (3.86). Therefore derivative 3a must possess as one of its structural features an entire inhibitor molecule linked to one site on the enzyme.

Incorporation of ^{14}C. After chymotrypsin was reacted with Ac-Phe($O^{14}CN$)-OEt the enzyme derivatives were radioassayed for ^{14}C content to determine the stoichiometry of reaction. The ^{14}C-labeled cyanate carbon should remain connected to the enzyme whether the entire inhibitor is attached as above or hydrolyzed to a carbamyl group. The counting results are shown in Table I. The approximate stoichiometry of reaction was observed to be I : E = 2 : 1. Both derivatives 3a and 3b here incorporated slightly more than 2 mol of ^{14}C-labeled inhibitor per mol of enzyme, with the excess attributable to non-specific side reactions. Therefore derivative 3a, as well as derivative 3b, must have resulted

TABLE I

^{14}C INCORPORATION INTO CHYMOTRYPSIN DERIVATIVES

Reagent	DIP-chymotrypsinogen [a]	3a [b]	3b	4
[^{14}C] Ac-Phe(OCN)-OEt	—	2.08	2.39	1.13
[^{14}C] *i*-Pr*P*-F	0.92	0.20	—	0.18

[a] Control run.

[b] Values reported as the molar ratio of ^{14}C to protein, based on the specific activity on the reagent.

from reaction of the inhibitor at two different and separate sites on the enzyme. From the results it is apparent at this point that derivative 3a must possess an entire inhibitor molecule attached at one reaction site and a carbamyl group at the other site. These two sites are very likely but not necessarily the same in derivatives 3a and 3b.

Affinity chromatography. The binding properties of the enzyme derivatives upon a 4-phenylbutylamino Sepharose affinity column were examined to determine if any binding pocket residues were modified by reaction with Ac-Phe-(OCN)-OEt. *O*-Carbamyl-Ser-195 chymotrypsin A_α (derivative 2a) was prepared as a model in order to check the proper operation of the affinity chromatography. Chymotrypsinogen A, with its binding site blocked, passed thru the affinity column upon elution with 0.1 M Tris (pH 8.0), while *O*-carbamyl-Ser-195 chymotrypsin A_α, with its binding site still intact, was mostly bound to the affinity column until eluted with buffer of high ionic strength. The binding is due to chymotrypsin's disposition for a phenyl group in its binding pocket. Both derivatives 3a and 3b were retained on the affinity column upon elution with 0.1 M Tris (pH 8.0) and were not removable until eluted with buffer of high ionic strength. This is evidence that compounds 3a and 3b possess unaltered binding sites and normal binding capacities towards substrate-like aromatic groups. Minor proportions of all the chromatographed enzyme derivatives were unretained and probably consisted of denatured or fragmented protein.

Reaction with diiso[^{14}C]propylfluorophosphate. Knowing that the binding site appeared to be unaltered, it was suspected that one of the two reaction sites of chymotrypsin with Ac-Phe(OCN)-OEt might be at the active-site catalytic residue. After unlabeled derivative 3a was reacted with diiso[^{14}C]propylfluorophosphate, the resulting product was radioassayed for ^{14}C content to quantitate the incorporation of *i*Pr*P*-F. For serine proteases, *i*Pr*P*-F is a powerful inhibitor which reacts solely with the active-site serine residue [11,12] in both enzyme and zymogen. If derivative 3a were previously modified at active-site Ser-195 then it would not react further with *i*Pr*P*-F, whereas if derivative 3a were modified with its active site still intact then it probably would be inhibited by *i*Pr*P*-F. As shown in Table I the former case is true. Chymotrypsinogen A incorporated approx. 1 equiv. of *i*Pr*P*-F; it reacts very slowly with *i*Pr*P*-F, $k_{2nd} = 0.105\,M^{-1} \cdot min^{-1}$ at 25°C and pH 7 [11]. Under identical conditions, derivative 3a incorporated only 0.2 equiv. of *i*Pr*P*-F, and even this is probably due to non-specific interaction. The inescapable conclusion is that one of the two reaction sites in derivative 3a is at the active-site Ser-195.

The [^{14}C]*i*Pr*P*-F reagent was actually only 63% *i*Pr*P*-F as demonstrated by the following experiment. Chymotrypsinogen A was reacted with [^{14}C]*i*Pr*P*-F as usual and after the standard activation to chymotrypsin, it was found to be 92% inactivated by *i*Pr*P*-F. However, the same DIP-chymotrypsinogen exhibited only 58% incorporation of ^{14}C. Similar results were obtained beginning with chymotrypsin A and [^{14}C]*i*Pr*P*-F. Accordingly, the values for [^{14}C]*i*Pr*P*-F in Table I have been increased by a factor of 92/58 from the experimental values.

Reaction of N-carbamyl-Cys-1 chymotrypsin A_δ with Ac-Phe(OCN)-OEt. In turn it was suspected that the second reaction site with chymotrypsin in the Ac-Phe(OCN)-OEt reaction was at the N-terminal α-amino residue, analogous to the *p*-nitrophenyl cyanate reaction. Hence, chymotrypsin was first protected

with a N-terminal blocking group and then treated with Ac-Phe(OCN)-OEt. Reaction was expected only at Ser-195 since Cys-1 was now shielded. In order to maintain active-site Ser-195 intact, chymotrypsinogen A was modified by *p*-nitrophenyl cyanate to yield *N*-carbamyl-Cys-1 chymotrypsinogen A, a well-characterized material [8]. Upon rapid activation with acetyl-trypsin the zymogen was converted to *N*-carbamyl-Cys-1 chymotrypsin A_∂ [13]. The ∂-form differs little in reactivity at Ser-195 than the α-form used in the previous experiments. The reaction conditions with Ac-Phe(OCN)-OEt were virtually identical with those used in the preparative inhibition of chymotrypsin A_α, and 98% inhibition was achieved within 3 h. Only one main modified enzyme derivative was obtained, compound 4, and again it was prepared in both unlabeled and ^{14}C-labeled form. Its CM-cellulose column elution peak (0.083 M K^+) was very near to the position for derivative 3a (0.085 M K^+). Since one reaction site, Cys-1, on the starting enzyme was already modified, there were two other probable locations left at which reaction could have occurred, in the binding pocket or at Ser-195. Derivative 4 was mainly retained on the affinity column upon elution with 0.1 M Tris (pH 8.0), which implies that, as in derivative 3a, the binding site is unaltered.

An amino acid analysis of derivative 4 revealed that it contained 4 (3.98) tyrosines per molecule, meaning that no additional tyrosine moieties were introduced from the inhibitor. This points to a carbamyl group as a structural feature of the protein. When *N*-carbamyl-Cys-1 chymotrypsin A was reacted with Ac-Phe(O^{14}CN)-OEt, derivative 4 was found to incorporate approx. 1 mol of ^{14}C-labeled inhibitor per mol of enzyme (Table I). In this case the inhibitor had reacted with only one site on the enzyme. When unlabeled derivative 4 was subsequently reacted with [^{14}C]*i*Pr*P*-F, the product was found to incorporate only 18% as much *i*Pr*P*-F as chymotrypsinogen A did (Table I), much like derivative 3a. Therefore, the single reaction site in derivative 4 is clearly at the active-site Ser-195. Finally, the conclusion with respect to derivative 3a is that one of its reaction sites with the inhibitor is at the active-site Ser-195 and the other site is at the N-terminal Cys-1.

Discussion

It was recognized that the reaction of chymotrypsin A with Ac-Phe(OCN)-OEt could yield a variety of possible products and not just one derivative modified at the S_1 binding site. Modifications could occur at the following sites: Ser-195 hydroxyl group at the active site; Cys-1 α-amino group at the N-terminal; Ser-189 or -190 hydroxyl group in the binding pocket; or at other nucleophilic sites. The entire inhibitor could be attached to the enzyme thru the cyanate group via an iminocarbonate —OC(=NH)O—, or isoureido —NHC(=NH)O— linkage or this linkage may have subsequently hydrolyzed leaving a carbamyl group attached to the enzyme and releasing a tyrosine derivative.

The justification for proposing certain of the above structures is based on the previous results of the reaction of chymotrypsin A with *p*-nitrophenyl cyanate [8]. Chymotrypsin A_α reacts with *p*-nitrophenyl cyanate to yield predominantly *O*-carbamyl-Ser-195 chymotrypsin A_α, modified by carbamylation at Ser-195. Chymotrypsin A_∂ reads with this inhibitor to yield exclusively *N*-(*p*-

nitrophenoxyiminomethyl)-Cys-1 *O*-carbamyl-Ser-195 chymotrypsin A_∂, modified by isourea formation at the α-amino group of Cys-1 in addition to carbamylation at Ser-195. Furthermore, the disubstituted chymotrypsin A_∂ could be hydrolyzed to *N*-carbamyl-Cys-1 *O*-carbamyl-Ser-195 chymotrypsin A_∂ upon treatment at pH 10, yielding an enzyme modified by carbamylation at both Cys-1 and Ser-195. The effect of these modifications is the irreversible inhibition of chymotryptic activity by carbamylation of the catalytic residue.

The evidence gathered is sufficient to characterize the two important enzyme derivatives 3a and 4 (Fig. 1). Chymotrypsin A_α reacts with Ac-Phe(OCN)-OEt to give derivative 3a which is found to be *N*-(*O*-tyrosyliminomethyl)-Cys-1 *O*-carbamyl-Ser-195 chymotrypsin A_α. An entire inhibitor moiety is linked to the amino group of N-terminal Cys-1 while a carbamyl group is attached to the active-site Ser-195. This assignment is consistent with the observations of the incorporation of 2 equiv. of ^{14}C label (when using Ac-Phe($O^{14}CN$)-OEt) and one extra tyrosine residue, and the non-reactivity toward diisopropylfluorophosphate. Reaction of *N*-carbamyl-Cys-1 chymotrypsin A_δ with Ac-Phe(OCN)-OEt yields only one derivative, *N*-carbamyl-Cys-1 *O*-carbamyl-Ser-195 chymotrypsin A_δ, derivative 4. This assignment is consistent with the observations of the incorporation of 1 equiv. of ^{14}C label and no extra tyrosine residue, and the non-reactivity towards *i*Pr*P*-F.

It is demonstrated clearly that active-site Ser-195 has undergone some kind of modification because neither derivative 3a nor 4 was catalytically active nor could be made to react with [^{14}C]*i*Pr*P*-F. It is conceivable that Ac-Phe(OCN)-OEt could modify a reactive residue in the active-site region other than Ser-195 and sterically block access of substrates to the binding region of the enzyme. This is the situation in chymotrypsinogen, where the active site is catalytically fully operational but the binding site is undeveloped [14,15]. Chymotrypsinogen A does react with *i*Pr*P*-F, though very slowly [11]. Thus derivatives 3a and 4 could probably be made to react with *i*Pr*P*-F at least as fast as the zymogen if Ser-195 were intact. However, neither derivatives 3a nor 4 was observed to react significantly with *i*Pr*P*-F which means that Ser-195 itself must have been modified by Ac-Phe(OCN)-OEt.

In connection with this, the binding pocket of chymotrypsin has not been modified because derivatives 3a, 3b, and 4 were all mainly retained on an af-

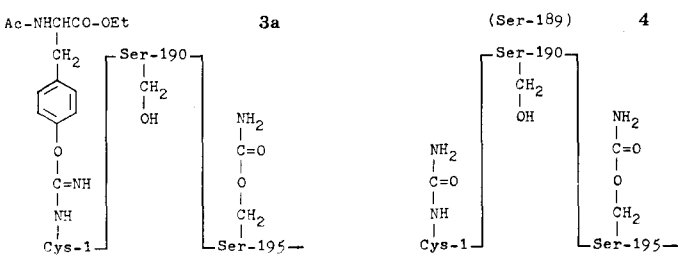

Fig. 1. Diagram of assigned structures of chymotrypsin A derivatives formed by modification with Ac-Phe(OCN)-OEt. 3a, a *N*-tyrosyliminomethyl-Cys-1 *O*-carbamyl-Ser-195 chymotrypsin A_α 4, *N*-carbamyl-Cys-1 *O*-carbamyl-Ser-195 chymotrypsin A_∂.

finity chromatography column. This 4-phenylbutylamino Sepharose column is known to bind chymotrypsin-like proteases, but not zymogens or chymotrypsin inhibited by Tos-PheCH$_2$Cl or *p*-nitrobenzenesulfonyl fluoride [10]. However, chymotrypsin with a carbamyl group on Ser-195 is bound to a 4-phenylbutylamino column. The fact that the column also binds derivatives 3a, 3b and 4 means that their binding sites are unobstructed as in the parent enzyme. Hence the loss of chymotryptic activity cannot be due to the inhibitor causing alteration of the enzyme's binding capacity.

It is also demonstrated that N-terminal Cys-1 has been modified by comparing the reactions of native and *N*-carbamyl-Cys-1 chymotrypsins A with the inhibitor. The observation is that derivative 4, derived from the N-terminal-blocked chymotrypsin A, incorporated 1.0 less equiv. of Ac-Phe(OCN)-OEt than derivative 3a, derived from unblocked chymotrypsin A, according to ^{14}C labeling results. This identifies Cys-1 as the second reaction site on derivative 3a besides Ser-195. The information is also sufficient to classify the types of reaction at these two sites. Apparently in both derivatives 3a and 4 the initially formed iminocarbonate intermediate at Ser-195 is spontaneously hydrolyzed at pH 6.5 to the carbamate since an amino acid analysis of derivative 4 revealed no extra tyrosine present. In contrast, in derivative 3a the initially formed isoureido intermediate at Cys-1 is stable at pH 6.5 and resistant to hydrolysis since in derivative 3a there was found to be one extra tyrosine present. Although derivative 3b was not characterized as thoroughly as derivatives 3a or 4, it is thought to be a *N*-tyrosyliminomethyl-Cys-1 *O*-carbamyl-Ser-195 chymotrypsin A_α derivative similar to derivative 3a with partial reaction at a third undetermined site which interacts more slowly than the first two with the inhibitor.

The surprising selectivity of aryl cyanates for certain reaction sites on chymotrypsin A is a notable feature with respect to their utility and value as protein reagents. The interactions between enzyme and Ac-Phe(OCN)-OEt and *p*-nitrophenyl cyanate are confined mostly to two reaction sites: active-site Ser-195 and N-terminal Cys-1. It is credible that the reagents would interact with the enzyme at Ser-195 since this residue is highly nucleophilic from being specially activated by the charge relay system. However, it is harder to explain why the reagents react with the enzyme at Cys-1 so specifically and not elsewhere, e.g. at lysine ϵ-amino groups or tyrosine phenolic groups. Perhaps the orientation of Cys-1 or its surrounding environment renders this site more reactive. By virtue of this limited selectivity combined with their moderate reactivity, aryl cyanate reagents should be useful modifiers and inhibitors of other proteins.

Since chymotrypsin A underwent modification by Ac-Phe(OCN)-OEt not within the binding pocket but rather at active-site Ser-195, the remaining question is why the reaction did not proceed according to plan. The reason seems to lie in a combination of high reactivity of the enzyme active site and the low reactivity of residues in the binding site. The reactive Ser-195 may attack the inhibitor as it diffuses toward the binding site, or in and out of the binding pocket, and form an irreversible adduct before any reaction has an opportunity to occur in the binding pocket with the much less reactive Ser-189 or Ser-190. Thus, it is necessary to protect the reactive active-site residues during selective

enzyme modification procedures when the intended sites of alteration are residues other than the catalytic ones.

In conclusion the synthesis of a new enzyme with altered substrate specificity must await the development of improved reagents in order to selectively modify substrate binding regions while leaving catalytic residues intact. The potential value of such a modified enzyme with a tailored activity makes the goal well worth pursuit.

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