Spackman, D. H. (1967), *Methods Enzymol. 11*, 3.
Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.
Stroud, R. M., Kay, L. M., and Dickerson, R. (1972), *Cold Spring Harbor Symp. Quant. Biol. 36*, 125.
Walsh, K. A., and Neurath, H. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 884.
Walsh, K. A., and Wilcox, P. E. (1970), *Methods Enzymol. 19*, 37.
Wofsy, L., and Singer, S. J. (1963), *Biochemistry 2*, 102.

# The Relation of the α-Amino Group of Trypsin to Enzyme Function and Zymogen Activation†

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ABSTRACT: Chemical modification of the α-amino group of ε-guanidinated trypsin by carbamylation, thiocarbamylation, or amidination renders the enzyme inactive toward specific ester and amide substrates. The carbamylated enzyme retains weak reactivity toward the pseudosubstrates diisopropyl phosphorofluoridate and *p*-nitrophenyl-*p'*-guanidinobenzoate. The modified enzyme resembles in this regard trypsinogen and guanidinated trypsinogen which react with these pseudosubstrates four to six orders of magnitude more slowly than trypsin. Since the common chemical characteristic of these weakly reactive enzyme derivatives is a blocked α-amino group of $Ile_7$, it follows that full enzymatic function requires that this group be free. It is proposed that during zymogen activation, *inter alia*, a latent enzymatic activity becomes very greatly enhanced rather than being generated *de novo*.

The conversion of trypsinogen to trypsin involves the obligatory cleavage of the peptide bond between $Lys_6$ and $Ile_7$ (Davie and Neurath, 1955; Desnuelle and Fabre, 1955) and results in the formation of a new α-amino group on $Ile_7$. The involvement of this newly formed α-amino group in enzyme function is suggested by its close proximity to the active site in the three-dimensional structures of trypsin and of homologous serine proteases (Stroud *et al.*, 1972; Blow *et al.*, 1969; Shotton and Watson, 1970). Deamination of the α-amino group of trypsin by nitrous acid (Scrimger and Hofmann, 1967) and similar modifications of chymotrypsin (Hess, 1971) cause inactivation of these enzymes.

In the present study the role of the α-amino group has been probed by determining the effects of chemical modification of this group on the activity of ε-guanidinated trypsin. This derivative is uniquely suited for this purpose since only the α-amino group of $Ile_7$ but none of the ε-amino groups is free (Robinson *et al.*, 1973). The amino group has been modified with various group-specific reagents and the activities of the products have been compared with those of the native enzyme and its parent zymogen. In the course of this study, catalytic activity of the zymogen as well as of the modified enzyme toward pseudosubstrates has been detected.

## Experimental Section

### *Materials and Methods*

Guanidinated trypsinogen and the acid protease of *Aspergillus oryzae* were prepared as described in the preceding paper (Robinson *et al.*, 1973). Trypsin was purified on a CHOM-Sepharose[1] affinity column (Robinson *et al.*, 1971) and dialyzed against 2 mM HCl.

*N*-α-Benzoyl-D,L-arginine-*p*-nitroanilide hydrochloride was obtained from Cyclo Chemical Co. Trifluoroacetic acid (Baker Chemical Co.) was distilled over chromic acid before use. Methyl isothiocyanate was purchased from Eastman Organic Chemical Co. and *N-tert*-butyloxycarbonyl-L-phenylalanine-*N*-hydroxysuccinimide ester from Fox Chemical Co.

Diisopropyl phosphorofluoridate (DFP) was obtained from Pierce Chemical Co. and [$^{14}$C]DFP from New England Nuclear. Guanidine hydrochloride was purchased from Heico Inc. (Ultra High Purity) and from Mann Research Laboratories (Mann Ultra Pure). Some lots of the Mann product contained ammonium ions which were removed as ammonia *in vacuo* after titration of saturated solutions to pH 10 with 8 M NaOH. The sources of other materials are listed in the preceding paper (Robinson *et al.*, 1973).

The methods of amino acid analysis, amino-terminal analysis, chromatography on SE-Sephadex and CHOM-Sepharose, active-site titrations with NPGB, and esterase

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[1] Abbreviations used are: BAEE, *N*-α-benzoyl-L-arginine ethyl ester hydrochloride; CHOM-Sepharose, chicken ovomucoid covalently coupled to Sepharose (Robinson *et al.*, 1971); DFP, diisopropyl phosphorofluoridate; DIP, diisopropylphosphoryl; Gdn·HCl, guanidine hydrochloride; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoic acid; *t*-Boc-Phe-OSu, *tert*-butyloxycarbonyl-L-phenylalanine-*N*-hydroxysuccinimide ester.

assays with BAEE have been described before (Robinson *et al.*, 1973).

Amidase activity was measured by the method of Erlanger *et al.* (1961) using 0.1 mM *N*-benzoyl-D,L-arginine-*p*-nitroanilide in 0.02 M $CaCl_2$–0.05 M Veronal (pH 8.5) at 25°. The absorbance change at 410 nm was followed in a Cary Model 16 dual-beam spectrophotometer.

*Preparation of ε-Guanidinated Trypsin.* ε-Guanidinated trypsin was prepared by activation of guanidinated trypsinogen (which had *not* been treated with hydroxylamine) and purified on a CHOM-Sepharose column (Robinson *et al.*, 1971, 1973). Guanidinated trypsinogen (10 mg/ml) was dissolved in 0.05 M sodium formate at pH 3.5 and activated with 5 units of acid protease/ml of solution. After 50–60% activation, the reaction was stopped by raising the pH to 6.00 in 0.02 M Mes, 0.05 M $CaCl_2$, and 0.5 M KCl, containing 1 mM benzamidine. This solution was applied to a CHOM-Sepharose column equilibrated with the same buffer. The column was washed with this buffer, then with the buffer lacking benzamidine, and finally with 0.1 M potassium formate–0.5 M KCl (pH 4.5). Active ε-guanidinated trypsin was eluted with 0.10 M formic acid–0.5 M KCl, and dialyzed against 2 mM HCl.

*Preparation of α-Carbamyl-ε-Guanidinated Trypsin.* ε-Guanidinated trypsin (2 mg/ml) was denatured in 6 M Gdn·HCl (pH 3) for 2 hr at room temperature. An equal volume of 0.2 M Hepes buffer (pH 8) containing 6 M Gdn·HCl was then added and the pH was adjusted to 8. To this solution was added a 600-fold molar excess of KNCO in 0.01 M Hepes buffer containing 6 M Gdn·HCl (pH 8.0). After 90% inactivation, the solution was acidified to pH 2.3 and diluted to 2 M Gdn·HCl at pH 2.3. Slow removal of Gdn·HCl was found to be necessary in order to avoid precipitation of the protein. The excess reagent was first removed by dialyzing against 2 M Gdn·HCl in 0.1 M formic acid or desalting on Sephadex G-25 previously equilibrated with this solution. Gdn·HCl was then removed by dialyzing the protein successively against 1 M Gdn·HCl–0.1 M formic acid for 24 hr, 0.1 M formic acid for 24 hr, and finally two changes of 2 mM HCl. This procedure resulted in a soluble product.

Residual active ε-guanidinated trypsin was adsorbed on a CHOM-Sepharose column and the unretarded α-carbamyl ε-guanidinated trypsin collected by eluting with 0.1 M Tris, 0.50 M KCl, 0.05 M $CaCl_2$, and 1 mM benzamidine (pH 7.5). The solution was acidified to pH 2.3, dialyzed against 2 mM HCl, and concentrated by ultrafiltration.

*Preparation of DIP-Trypsin and DIP-ε-Guanidinated Trypsin.* DIP-trypsin and DIP-ε-guanidinated trypsin were prepared by inactivation of purified trypsin or ε-guanidinated trypsin (5 mg/ml) with 1 mM DFP in 0.1 M Tris containing 0.05 M $CaCl_2$ (pH 8.1) at room temperature. Less than 1% activity remained after 1 hr. The solutions of inactivated enzymes were acidified to pH 2.3 with formic acid and dialyzed against 2 mM HCl. Any residual active enzyme was removed by adsorption on a CHOM-Sepharose column in 0.1 M Tris–0.05 M $CaCl_2$ (pH 8.0). The unretarded DIP-proteins were acidified to pH 2.3 with formic acid, dialyzed against 2 mM HCl, and concentrated by ultrafiltration.

*Determination of Incorporation of Radioactivity into Proteins.* Incorporation of radioactivity was measured by adding approximately 0.05 mg of labeled protein in 1 ml of either water or 2 M Gdn·HCl to 10 ml of the following scintillant: 8 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene dissolved in 2 l. of toluene and mixed with 1 l. of Triton X-100. At least 2000 counts were recorded on a Packard Model 3003 Tri-Carb scintillation counter. The efficiency of counting (62.4%) was measured by adding a known amount of standard [$^{14}$C]toluene to each vial.

*Standardization of Radioactive Reagents.* POTASSIUM [$^{14}$C]-CYANATE. The specific radioactivity of this reagent ($3.90 \times 10^{11}$ dpm/mol) was determined by carbamylation of 20 mg of glycylphenylalanine using the method of Stark and Smyth (1963). The resulting carbamyl-dipeptide was purified by high-voltage paper electrophoresis at pH 6.5 (Naughton *et al.*, 1960), eluted with water, and cyclized by the method of Stark and Smyth (1963) to yield glycine hydantoin and free phenylalanine. The phenylalanine content was determined by amino acid analysis and the radioactivity was determined by counting an aliquot of the solution.

[$^{14}$*C*]DFP. The specific radioactivity of DFP was determined by reacting purified trypsin with $2 \times 10^{-3}$ M [$^{14}$C]DFP in 0.1 M Tris–0.05 M $CaCl_2$ at room temperature. More than 99% of the trypsin was inactivated in 10 min. After 1 hr, the solution was acidified to pH 2.3 with formic acid and desalted on a column of Sephadex G-25 equilibrated with 0.1 M formic acid. The concentration of protein and the radioactivity were measured separately. Since the purified trypsin contained 0.94 active site/mol (measured with NPGB), this value was used to calculate the specific radioactivity of [$^{14}$C]DFP ($4.03 \times 10^{11}$ dpm/mol). The same value was obtained when trypsin was treated for 100 hr with [$^{14}$C]DFP.

## Results

*Carbamylation with Potassium Cyanate.* In order to selectively modify with cyanate the α-amino group of ε-guanidinated trypsin, the reactivity of $Ser_{183}$ at the active site was first abolished by reversible denaturation of the protein in 6 M Gdn·HCl.[2] Approximately 70–75% reactivation occurred when Gdn·HCl was removed by dilution of the denatured enzyme with aqueous substrate solution (BAEE). Alternatively, when Gdn·HCl was removed by dialysis, 90–95% of the original activity was restored. Measurements of the extent of inactivation by cyanate were corrected for incomplete renaturation by expressing the activity as a percentage of a control lacking cyanate.

When ε-guanidinated trypsin was treated with a 600-fold molar excess of KNCO in 6 M Gdn·HCl, enzymatic activity toward BAEE and *N*-α-benzoyl-D,L-arginine-*p*-nitroanilide declined at the same rate according to pseudo-first-order kinetics. The product of carbamylation was converted to the hydantoin, isolated, hydrolyzed, and analyzed by the procedures of Stark and Smyth (1963). The rate of carbamylation of the amino-terminal isoleucine coincided with the rate of inactivation of the enzyme (Figure 1A). The progress of carbamylation was also followed in dialyzed aliquots of a reaction mixture containing KN$^{14}$CO. The incorporation of radioactivity increased in direct proportion to inactivation (Figure 1B).

Since the carbamylation of isoleucine, the uptake of $^{14}$C, and the loss of enzymatic activity all followed the same pseudo-first-order kinetics, it was concluded that 1 mol of [$^{14}$C]cyanate had been incorporated per mol of inactivated enzyme. Actually 1 mol of isoleucine was carbamylated (Figure 1A) but 1.56 mol of [$^{14}$C]cyanate was introduced per

[2] Denaturation prevents carbamylation of the serine residue at the active site of chymotrypsin (Shaw *et al.*, 1964; Robillard *et al.*, 1972). Denaturation of trypsin also exposes the α-amino group for chemical modification (Scrimger and Hofmann, 1967).

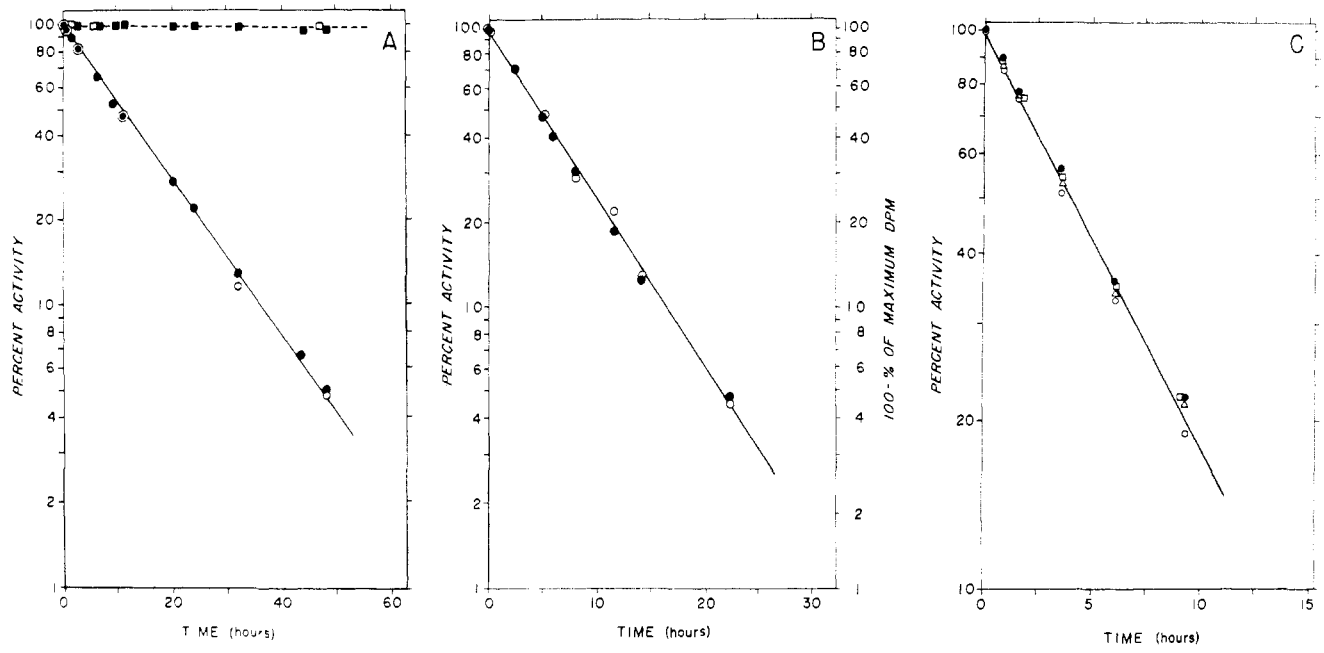


FIGURE 1: First-order plots of the inactivation (BAEE assay) of ε-guanidinated trypsin (1 mg/ml) by KNCO (1–2 mg/ml). (A) Comparison of the rate of inactivation and the rate of carbamylation of $Ile_7$. The data are expressed as percentages of active trypsin (solid symbols) and of maximum carbamylation (open symbols). Squares indicate controls lacking cyanate. (B) Comparison of the rate of inactivation (●) and the incorporation of radioactivity (○) from $KN^{14}CO$. (C) Comparison of the rate of inactivation of unfractionated ε-guanidinated trypsin (●) and that of the three species of ε-guanidinated trypsin (open symbols). The latter had each been purified on CHOM-Sepharose after acid protease activation of the corresponding guanidinated trypsinogens (Robinson *et al.*, 1973).

mol of protein (Figure 2), indicating that an additional 0.56 site had reacted with cyanate *at the same rate* as $Ile_7$. These additional sites are probably the fractional amino-terminal residues (0.3–0.5) besides isoleucine observed by Robinson *et al.* (1973) in ε-guanidinated trypsin.

In order to prove that inactivation of the enzyme was due only to carbamylation of $Ile_7$, the enzyme was first inhibited with $KN^{14}CO$ to the extent of 50% and then separated into active and inactive components by chromatography on a CHOM-Sepharose column. Analysis showed that 0.30 ± 0.05 mol of $^{14}C$/mol of protein had been incorporated into the active fraction, and 1.25 ± 0.1 mol into the inactive fraction. The difference (0.95 mol) corresponds to the modification of a single amino group in the inactivated enzyme. Evidently the additional incorporation of 0.3 mol/mol had no effect on the activity of the active fraction. Examination of the active and inactive proteins in the Beckman Sequencer confirmed that the only difference between them was the blockage of the amino-terminal isoleucine in the inactive fraction.

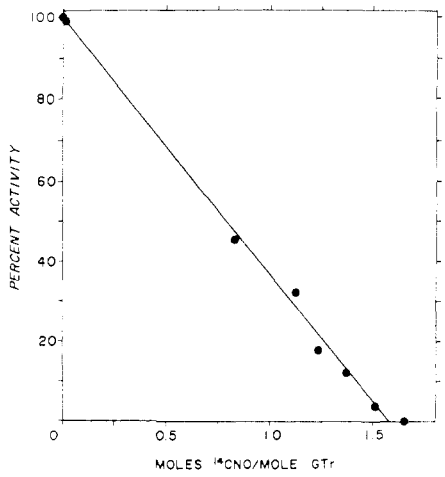


FIGURE 2: Correlation of the molar incorporation of [$^{14}$C]cyanate with the inactivation of ε-guanidinated trypsin.

The present preparation of ε-guanidinated trypsin contained three chromatographically different but fully active species (Robinson *et al.*, 1973). Their rates of inactivation were found to be identical with the rate of inactivation of the unfractionated enzyme (Figure 1C).

*Chain Shortening with Methyl Isothiocyanate.* Thiocarbamylation of the α-amino group of guanidinated trypsin by methyl isothiocyanate produced a derivative from which the terminal residue could be removed by treatment with anhydrous trifluoroacetic acid. Although such acid treatment denatures the protein, control experiments lacking methyl isothiocyanate indicated that 40–80% of the initial activity could be regained. The inactivation of ε-guanidinated trypsin by methyl isothiocyanate (700-fold molar excess of reagent in 6 M Gdn·HCl–0.25 M Hepes, pH 8.0) followed pseudo-first-order kinetics (Figure 3).

For removal of the terminal residue, a solution of the product (91% inactivated) was acidified to pH 2.5 with formic acid, lyophilized, and treated for 25 min at room temperature with 2 ml of anhydrous trifluoroacetic acid. The solution was then added to 5 ml of 6 M Gdn·HCl and adjusted to pH 3, and Gdn·HCl was gradually removed by dialysis. As a control, ε-guanidinated trypsin lacking methyl isothiocyanate was similarly treated. The control regained 35% activity but the product only 3.4% (9% of the control). Sequenator analysis of the product gave the sequence Val-Gly in 90% yield and Ile-Val in 10% yield. Thus removal of $Ile_7$ caused inactivation of ε-guanidinated trypsin.

*Chain Elongation with tert-Boc-L-Phenylalanine-N-hydroxysuccinimide Ester.* Reaction of ε-guanidinated trypsin with

TABLE I: Incorporation of [$^{14}$C]DFP into Trypsin, Trypsinogen, and Derivatives.

| Time of Reaction[a] (hr) | [$^{14}$C]DIP Incorporated (mol/mol of Protein) | | | |
|---|---|---|---|---|
| | Trypsin | Carb-Gu-Trypsin[b] | Tryp-sinogen | Gu-Tryp-sinogen |
| 1.0 | 0.94[c] | | | |
| 3.0 | 0.97 | 0.47 | 0.52 | 0.51 |
| 8.25 | | 0.90 | | |
| 18.0 | 1.09 | 1.20 | 1.07[d] | 1.10 |
| 40.0 | 1.10 | 1.33 | | 1.21 |

[a] Reaction conditions: 1 mg of protein/ml in 0.05 M $CaCl_2$, 0.10 M Tris (pH 8.0), and $2 \times 10^{-2}$ M [$^{14}$C]DFP, 25°. [b] Denotes α-carbamyl-ε-guanidinated trypsin. [c] $2 \times 10^{-3}$ M [$^{14}$C]DFP. [d] 16-hr reaction. Amino-terminal analysis yielded valine only.

*t*-Boc-Phe-OSu was explored as a possible means of investigating the effect of chain elongation on enzymatic activity. Treatment of the guanidinated enzyme (1.8 mg/ml in 6 M Gdn·HCl) for 5 min with a 60-fold molar excess of *t*-Boc-Phe-OSu in 2% $CH_3CN$–0.1 M Hepes (pH 8.3)) produced 88% inhibition. After acidification and gradual removal of excess reagent by gel filtration and dialysis (by the methods described for the preparation of the carbamylated enzyme derivative), 3.5 residues of phenylalanine/protein molecule were incorporated.[3] During sequenator analysis of the product the *t*-Boc group was removed in the acid cleavage step of the first turn. The second turn yielded phenylalanine and subsequent turns yielded isoleucine, valine, and glycine, respectively. In addition, the products of unmodified ε-guanidinated trypsin were detected at each step at a level of 10% which agrees with the residual activity of 12% of the modified enzyme. Apparently chain elongation is feasible, but reaction conditions need to be modified to exclude other reaction sites and to remove the *t*-Boc group quantitatively.

*Amidination.* ε-Guanidinated trypsin (1 mg/ml) was denatured in 6 M Gdn·HCl and amidinated according to the conditions described by Agarwal *et al.* (1971). A 100-fold molar excess of ethyl acetimidate in 0.2 M $NaHCO_3$ (pH 9.3) was added twice at 3-hr intervals. After acidifying to pH 3 and removing Gdn·HCl by dialysis, the enzyme retained only 12% of the original activity. Sequenator analysis indicated that 10% of the protein had not been amidinated. Thus amidination of $Ile_7$ appeared to inactivate the enzyme.

*Reaction with DFP.* The α-carbamyl derivative of ε-guanidinated trypsin reacted slowly with the pseudosubstrates DFP and NPGB but was unreactive toward the true substrates BAEE and benzoyl-D,L-arginine-*p*-nitroanilide. After 18-hr exposure to relatively high concentrations of [$^{14}$C]DFP (20 mM), 1.2 mol of DFP/mol of protein was incorporated (Table I). In parallel experiments with trypsin, 1 mol of DFP was incorporated during less than 10 min of exposure to 2 mM DFP. Unexpectedly, trypsinogen and guanidinated trypsinogen also incorporated approximately 1 mol of DFP at the same rate as

[3] Similar results were obtained in parallel experiments with guanidinated trypsinogen. Treatment of the product with 1 M hydroxylamine at pH 11 for 20 min removed all but one residue of phenylalanine/protein molecule.

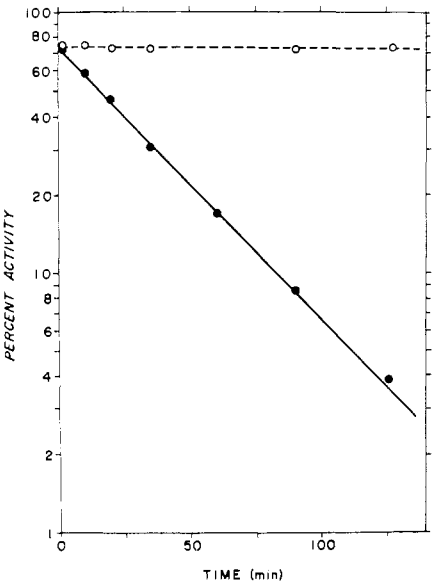


FIGURE 3: First-order rate plot of the inactivation of ε-guanidinated trypsin (1.8 mg/ml) by methyl isothiocyanate (1.5 mg/ml). The reagent was added to the reaction in 0.02 ml of $CH_3CN$; the same amount of $CH_3CN$ was added to the control. (●) BAEE activity during reaction; (○) BAEE activity of the control (lacking reagent).

α-carbamyl-ε-guanidinated trypsin. Sequenator analysis of DIP-trypsinogen (treated for 16 hr with DFP) demonstrated that the amino-terminal sequence of the zymogen was intact.

Preliminary experiments indicated that the uptake of [$^{14}$C]-DFP prevented subsequent activation of the zymogen. After incorporation of 0.5 and 1.1 equiv of reagent, 40 and 75% of activatability was lost. A more detailed report on the effects of DFP on the activation of trypsinogen (and chymotrypsinogen) is given elsewhere (Morgan *et al.*, 1972).

As judged by peptide mapping, [$^{14}$C]DFP was incorporated into the same site in trypsinogen as in trypsin. About 0.5 mg of each labeled protein was dried, oxidized as described by Hirs (1967), and digested with an equal weight of porcine trypsin for 12 hr at 37°. After paper electrophoresis of the two digests, the distributions of their radioactive peptides were virtually identical (Figure 4).

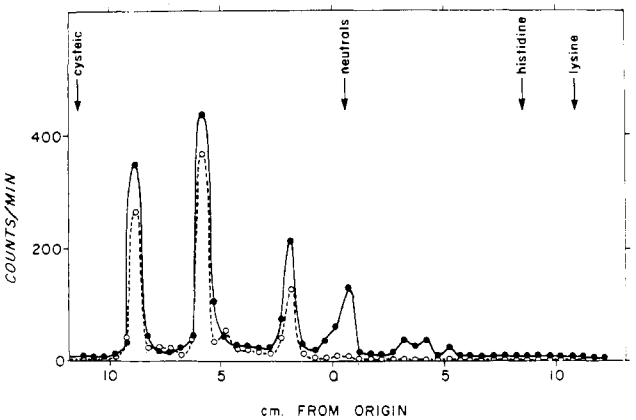


FIGURE 4: Comparison of tryptic digests of oxidized [$^{14}$C]DIP-trypsin (prepared by treatment with 2 mM DFP for 1 hr) and of oxidized [$^{14}$C]DIP-trypsinogen (prepared by treatment with 20 mM DFP for 16 hr) by paper electrophoresis (40 V/cm, pH 6.5, 60 min). The positions of reference amino acids are indicated by the arrows. (●) [$^{14}$C]DIP-trypsinogen and (○) [$^{14}$C]DIP-trypsin.

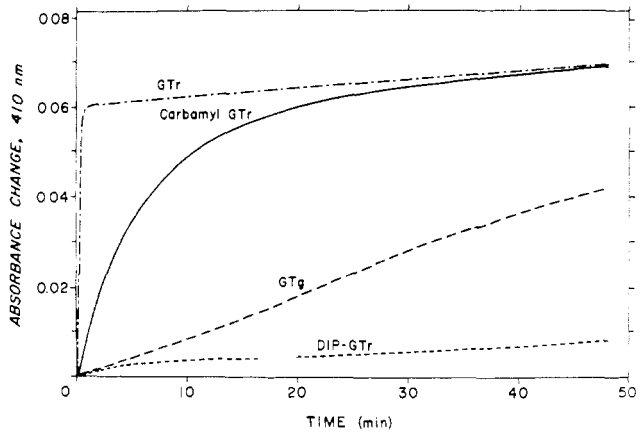


FIGURE 5: Comparison of the release of *p*-nitrophenol from NPGB ($1.4 \times 10^{-4}$ M) by ε-guanidinated trypsin (GTr), α-carbamyl-ε-guanidinated trypsin (carbamyl GTr), guanidinated trypsinogen (GTg), and DIP-guanidinated trypsin (DIP-GTr). The concentration of each protein was $3.9 \times 10^{-6}$ M.

*Reaction with NPGB.* The reactivities of α-carbamyl-ε-guanidinated trypsin, ε-guanidinated trypsin, guanidinated trypsinogen, and DIP-ε-guanidinated trypsin toward NPGB are shown in Figure 5. ε-Guanidinated trypsin reacted in less than 1 min to give a normal "burst" of nitrophenol (Chase and Shaw, 1969) whereas DIP-ε-guanidinated trypsin was essentially inactive. α-Carbamyl-ε-guanidinated trypsin and guanidinated trypsinogen each released approximately 1 equiv of nitrophenol at very slow rates, indicating specific acylation of the active site. α-Carbamyl-ε-guanidinated trypsin reacted faster than guanidinated trypsinogen and both reactions appeared to be pseudo-first order with respect to protein (Figure 6, insert).

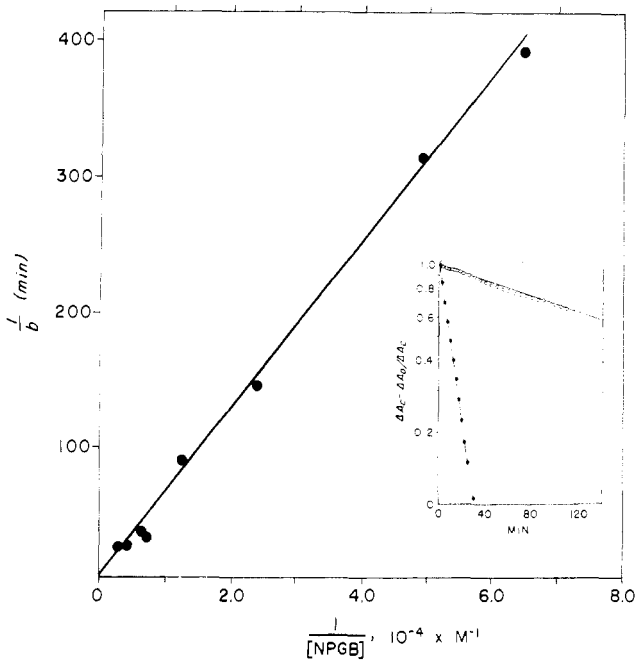


FIGURE 6: Evaluation of reaction rate constants in the reaction of NPGB with α-carbamyl-ε-guanidinated trypsin ($3.9 \times 10^{-6}$ M) at 25°. The first-order rate constants, *b*, were determined at various NPGB concentrations from the slope of first-order rate plots. *The insert* illustrates typical first-order plots for the modified enzyme (closed circles) and for guanidinated trypsinogen (open circles) in $2.5 \times 10^{-4}$ M NPGB. The per cent unreacted protein is plotted along the ordinate (assuming 1 mol of *p*-nitrophenol released per mol of protein). $\Delta A_0$ is the observed absorbance change at 410 nm after subtracting the absorbance due to nonspecific hydrolysis in DFP-treated ε-guanidinated trypsin controls; $\Delta A_c$ is the calculated absorbance change, assuming stoichiometric (1:1) acylation.

In order to estimate the apparent dissociation constant ($K_s$) and the maximal acylation rate ($k_2$), pseudo-first-order rate constants were determined at various concentrations of NPGB. These constants are defined by eq 1, where *b* is the

$$\frac{1}{b} = \frac{1}{k_2} + \frac{K_s}{k_2[S]_0} \qquad (1)$$

first-order rate constant for the presteady-state reaction at a given pseudosubstrate concentration, $[S]_0$ (Chase and Shaw, 1969). This equation predicts a linear relationship between $1/b$ and $1/[S]_0$ with intercepts of $-1/K_s$ and $1/k_2$. It was difficult to measure these constants for α-carbamyl-ε-guanidinated trypsin because the reactions proceeded very slowly (Figure 6). Minimum estimates (from the intercepts) indicated that $K_s$ is greater than $10^{-3}$ M and $k_2$ greater than 0.2 $min^{-1}$. These may be compared with the corresponding values for trypsin, *i.e.*, $K_s = 0.61 \times 10^{-6}$ M and $k_2 = 117$ $min^{-1}$. Since $K_s$ is a measure of the affinity of the enzyme for NPGB, it follows that trypsin binds NPGB at least 1600 times more strongly than α-carbamyl-ε-guanidinated trypsin. The bimolecular rate constant for the reaction of NPGB with the enzyme can be accurately determined since it is given by the ratio $k_2/K_s$, the reciprocal of the slope in Figure 6. A value of 160 $min^{-1}$ $M^{-1}$ was obtained which may be compared with $1.9 \times 10^6$ $min^{-1}$ $M^{-1}$ for trypsin (Chase and Shaw, 1969). Thus α-carbamyl-ε-guanidinated trypsin is $10^6$ times less reactive toward NPGB than trypsin.

## Discussion

The present investigation provides chemical evidence for the involvement of the α-amino group on $Ile_7$ of trypsin in enzymatic function. When all ε-amino groups are guanidinated and the α-amino group is free, the enzyme is fully active. When the α-amino group is modified by carbamylation, amidination, or thiocarbamylation, the enzyme is inactive. The degree of inactivation by $^{14}C$-labeled cyanate is proportional to the incorporation of the radioactive label. These conclusions are consistent with the reported inactivation of trypsin and the homologous enzyme chymotrypsin by deamination (Scrimger and Hofmann, 1967; Dixon and Hofmann, 1970), by acylation (Chevallier *et al.*, 1969; Oppenheimer *et al.*, 1966; Ghélis *et al.*, 1970) or by deprotonation (Hess, 1971) of their terminal α-amino groups.

A structural feature common to the homologous mammalian serine proteases trypsin, chymotrypsin, and elastase (Stroud *et al.*, 1972; Blow *et al.*, 1969; Shotton and Watson, 1970) is the formation of an ion pair between the α-amino group of $Ile_7$ and the carboxylate group of $Asp_{182}$ of the active site (the residues are numbered according to the amino acid sequence of trypsin). If the maintenance of this ion pair is essential for catalytic function, modification of the α-amino group, or its translocation, should cause enzyme inactivation. Thus when the α-amino group is blocked by carbamylation or shifted to $Val_8$ (by thiocarbamylation and treatment with trifluoroacetic acid) or when its positive charge is only slightly shifted by amidination, the resulting trypsin derivative is inactive. Although the product of chain elongation (addition of

phenylalanine) has not been sufficiently characterized, it may be expected to be inactive also.

Although $\alpha$-carbamyl-$\epsilon$-guanidinated trypsin is inactive toward specific substrates, it reacts slowly with the pseudosubstrates DFP and NPGB. The rates of reaction are approximately four (Morgan *et al.*, 1972) to six orders of magnitude below those of native and $\epsilon$-guanidinated trypsin. This difference in reactivity can be ascribed in part to at least a 1000-fold decrease in binding (of NPGB) when the $\alpha$-amino group is carbamylated. Similar changes in reactivity toward DFP and *p*-nitrophenyl acetate have been observed by Ghélis *et al.* (1970) when, in the homologous enzyme $\delta$-chymotrypsin, the $\alpha$-amino group of $Ile_{16}$ was acetylated. Thus in both serine proteases, blocking of the $\alpha$-amino group abolishes catalytic activity toward specific substrates and reduces—but does not abolish—reactivity toward pseudosubstrates.

Trypsinogen and its guanidinated derivative also react with the pseudosubstrates DFP and NPGB at rates comparable to those of $\alpha$-carbamyl-$\epsilon$-guanidinated trypsin. These similarities in reactivity between the zymogen and the enzyme derivative can be related to the fact that in both proteins the $\alpha$-amino group of $Ile_7$ is substituted by the activation peptide Val-$Asp_4$-Lys in trypsinogen (or by Val-$Asp_4$-HAr in guanidinated trypsinogen) and by a carbamyl group in the guanidinated trypsin derivative.

The present investigation suggests that in terms of function, a zymogen differs from the corresponding enzyme in degree rather than in kind and that during zymogen activation a latent catalytic power becomes amplified. This interpretation differs from previously held views that during activation, enzyme function is generated *de novo*. These conclusions are in accord with recent observations of the spontaneous activation of trypsinogen (Kay and Kassell, 1971) and pepsinogen (Bustin and Conway-Jacobs, 1971) and of intrinsic peptidase and esterase activities of procarboxypeptidases (Lacko and Neurath, 1970; Reeck and Neurath, 1972; Uren *et al.*, 1972; J. R. Uren and H. Neurath, in preparation).

The chemical basis of the latent activity of trypsinogen and the manner in which it is altered during the conversion of trypsinogen to trypsin are a matter of conjecture. A combination of three possibilities may account for the repressed catalytic power of the zymogen: (1) the substrate binding site is distorted, (2) the reactivity of the nucleophile of the active site ($Ser_{183}$) is incompletely developed prior to activation, and (3) the geometric relationships between the substrate binding site and the components of the catalytic site are different in the zymogen than in the enzyme. These possibilities will now be examined for trypsinogen and the homologous enzyme chymotrypsinogen A.

(1) A weaker binding of pseudosubstrates and inhibitors by trypsinogen than by trypsin is indicated by the present kinetic experiments with NPGB and by the binding studies of Glazer (1965, 1967) involving the inhibitors proflavin and thionine. Analogously, the "virtual" substrates (Vaslow and Doherty, 1953) and inhibitory substrate analogs (Deranleau and Neurath, 1966; Weiner and Koshland, 1965) are less tightly bound by chymotrypsinogen than by chymotrypsin. These observations are consistent with X-ray analyses (Freer *et al.*, 1970; Blow *et al.*, 1969) and electron paramagnetic resonance probes (Kosman, 1972) which indicate that in chymotrypsinogen, prior to activation, the substrate binding pocket is partially obstructed by $Met_{192}$.

(2) It is difficult to determine the intrinsic reactivity of the nucleophile of the active site ($Ser_{183}$) toward pseudosubstrates independent of the contributions of binding. Since it is now established[4] that in trypsinogen $Ser_{183}$ is uniquely phosphorylated by DFP, its reactivity toward this reagent (and, analogously, toward NPGB) must be greater than that of all other serine residues in the zymogen. Zymogen activation further enhances this reactivity by several orders of magnitude. The unique character of the nucleophile is also indicated by X-ray analysis which shows that in the homologous zymogen, chymotrypsinogen A, $Ser_{195}$ is a component of the same charge relay system as in chymotrypsin (Freer *et al.*, 1970; Blow *et al.*, 1969).

(3) The possibility that the lower reactivity of the zymogen is due to a distortion of the geometrical relationships between the binding and the bond-breaking components of the active site could be tested by designing "distorted" substrates. The small tolerance to variations in structure of the substrate is indicated by studies of substrate homologs of *p*-toluenesulfonyl-L-arginine methyl ester in which the side chain was lengthened or shortened by one carbon atom (Baird *et al.*, 1965). In either case, the Michaelis constant was increased and the maximum velocity decreased as compared to the parent substrate. While no systematic investigations of the modification of the substrate binding site of trypsin have yet been reported, Smith and Shaw (1969) have shown that peptide bond cleavage between $Lys_{176}$ and $Asp_{177}$ abolishes reactivity toward specific substrates and greatly reduces reactivity toward the pseudosubstrates DFP and NPGB. These observations are of significance since $Asp_{177}$ is the anionic component of the substrate binding pocket of trypsin. Modification of $Met_{192}$ of the substrate binding site of the homologous enzyme chymotrypsin also reduces the affinity of the enzyme toward specific substrates (Koshland *et al.*, 1962).

Regardless of the exact explanation, the latent reactivity of zymogens toward pseudosubstrates and its amplification during activation provide additional experimental parameters for the study of the mechanism of zymogen activation.

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## References

Agarwal, S. P., Martin, C. J., Blair, T. T., and Marini, M. A. (1971), *Biochem. Biophys. Res. Commun. 43*, 510.

Baird, J. B., Curragh, E. F., and Elmore, D. T. (1965), *Biochem. J. 96*, 733.

Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), *Nature (London) 221*, 337.

Bustin, M., and Conway-Jacobs, A. (1971), *J. Biol. Chem. 246*, 615.

Chase, T., and Shaw, E. (1969), *Biochemistry 8*, 2212.

Chevallier, J., Yon, J., and Labouesse, J. (1969), *Biochim. Biophys. Acta 181*, 73.

Davie, E. W., and Neurath, H. (1955), *J. Biol. Chem. 212*, 515.

Deranleau, D. A., and Neurath, H. (1966), *Biochemistry 5*, 1413.

Desnuelle, P., and Fabre, C. (1955), *Biochim. Biophys. Acta 18*, 49.

Dixon, J. W., and Hofmann, T. (1970), *Can. J. Biochem. 48*, 671.

[4] This has been confirmed by isolation of the $^{14}C$-labeled peptide (Morgan *et al.*, 1972).

Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961), *Arch. Biochem. Biophys. 95*, 271.
Freer, S. T., Kraut, J., Robertus, J. D., Wright, H. T., and Xuong, Ng. H. (1970), *Biochemistry 9*, 1997.
Ghélis, C., Garel, J., and Labouesse, J. (1970), *Biochemistry 9*, 3902.
Glazer, A. N. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 171.
Glazer, A. N. (1967), *J. Biol. Chem. 242*, 3326.
Hess, G. P. (1971), *Enzymes 3*, 213.
Hirs, C. H. W. (1967), *Methods Enzymol. 11*, 197.
Kay, J., and Kassell, B. (1971), *J. Biol. Chem. 246*, 6661.
Koshland, D. E., Jr., Strumeyer, D. H., and Ray, W. J., Jr. (1962), *Brookhaven Symp. Biol. 15*, 101.
Kosman, D. J. (1972), *J. Mol. Biol. 67*, 247.
Lacko, A. G., and Neurath, H. (1970), *Biochemistry 9*, 4680.
Morgan, P. H., Robinson, N. C., Walsh, K. A., and Neurath, H. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 3312.
Naughton, M. A., Sanger, F., Hartley, B. S., and Shaw, D. C. (1960), *Biochem. J. 77*, 149.
Oppenheimer, H. L., Labouesse, B., and Hess, G. P. (1966), *J. Biol. Chem. 241*, 2720.
Reeck, G. R., and Neurath, H. (1972), *Biochemistry 11*, 3947.
Robillard, G. T., Powers, J. C., and Wilcox, P. E. (1972), *Biochemistry 11*, 1773.
Robinson, N. C., Neurath, H., and Walsh, K. A. (1973), *Biochemistry 12*, 414.
Robinson, N. C., Tye, R. W., Neurath, H., and Walsh, K. A. (1971), *Biochemistry 10*, 2743.
Scrimger, S., and Hofmann, T. (1967), *J. Biol. Chem. 242*, 2528.
Shaw, D. C., Stein, W. H., and Moore, S. (1964), *J. Biol. Chem. 239*, PC 671.
Shotton, D. M., and Watson, H. C. (1970), *Nature (London) 225*, 811.
Smith, R. L., and Shaw, E. (1969), *J. Biol. Chem. 244*, 4704.
Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.
Stroud, R. M., Kay, L. M., and Dickerson, R. E. (1972), *Cold Spring Harbor Symp. Quant. Biol. 36*, 125.
Uren, J. R., Neurath, H., and Walsh, K. A. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 916 Abstr.
Vaslow, F., and Doherty, D. G. (1953), *J. Amer. Chem. Soc. 75*, 928.
Weiner, H., and Koshland, D. E., Jr. (1965), *J. Biol. Chem. 240*, PC 2764.

# Mössbauer Investigations of Chloroperoxidase and Its Halide Complexes†

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ABSTRACT: The heme protein chloroperoxidase which is isolated from the mold *Caldariomyces fumago* has been investigated by Mössbauer spectroscopy. The heme iron of native chloroperoxidase is in a low-spin ferric state at low temperatures and undergoes a temperature-dependent spin transition to high-spin ferric around 200°K. The low-temperature Mössbauer spectra were simulated assuming that the heme iron resides in a ligand field potential of orthorhombic symmetry. The low-temperature Mössbauer spectra of the chloroperoxidase–Cl complex are quite similar to those of the native enzyme, suggesting that chloride does not bind as an axial ligand to the heme iron. The complexes of chloroperoxidase with iodide and fluoride are high-spin ferric at all temperatures. Both complexes reveal unusually large rhombic distortions at the heme iron. The ferrous form of chloroperoxidase is a high-spin species with an optical absorption spectrum and Mössbauer parameters which are almost identical with those of the reduced form of cytochrome P-450$_{cam}$. Like the cytochromes of the P-450 type, reduced chloroperoxidase forms a stable complex with carbon monoxide characterized by a Soret band at unusually long wavelength (443 nm). The heme iron of this complex is found to be in a low-spin ferrous state. This investigation suggests close structural similarities between the active sites of chloroperoxidase and P-450$_{cam}$.

Chloroperoxidase is a heme protein (mol wt ~ 42,000) which has been isolated from the mold *Caldariomyces fumago* (Morris and Hager, 1966). It catalyzes the chlorination reactions involved in the biosynthesis of caldariomycin (2,2-dichloro-1,3-cyclopentenedione). In the presence of hydrogen peroxide and a suitable halogen donor ($I^-$, $Br^-$, or $Cl^-$, but not $F^-$), the enzyme catalyzes the peroxidative formation of a carbon–halogen bond with a suitable halogen acceptor. In addition to the halogenation reaction, chloroperoxidase also catalyzes the peroxidative oxidation of classical peroxidase substrates such as pyrogallol and guaiacol (Thomas *et al.*, 1970). Moreover, it decomposes hydrogen peroxide to give molecular oxygen in a catalase-type reaction.

Chloroperoxidase is quite similar in many of its properties to other protoheme peroxidases such as horseradish peroxidase, Japanese radish peroxidase, and cytochrome *c* peroxidase. All are isolated as monomeric proteins having molecular weights in the range of 35,000–50,000 and exhibit similar optical spectra for the native and reduced forms, and for their cyanide and azide complexes. Although the physical properties of these peroxidases are quite similar, chloroperoxidase exhibits a basic catalytic difference in its ability to

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