Mechanism of Action of Guinea Pig Liver Transglutaminase

VIII. ACTIVE SITE STUDIES WITH "REPORTER" GROUP-Labeled HALOMETHYL KETONES

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SUMMARY

The reaction of \( \alpha \)-bromo-4-hydroxy-3-nitroacetophenone (BHNA) with transglutaminase in the presence of \( \text{CaCl}_2 \) (25 mM) produces a catalytically inactive labeled protein in which the phenacyl group is covalently attached to the active site —SH. The spectral properties of this group attached to the enzyme are consistent with that of the group in a hydrophobic region of the molecule. Addition of ethylene-diaminetetraacetate results in a shift in the spectrum toward shorter wavelengths, indicative of a more polar environment for this —SH in the absence of \( \text{Ca}^{2+} \). Attachment of the phenacyl group to positions in the enzyme other than the active —SH by reaction with BHNA in the absence of \( \text{Ca}^{2+} \) results in losses in transferase activity, but essentially no loss in esterase activity. The spectrum of this group bound to enzyme in the absence of \( \text{Ca}^{2+} \) is identical with that of the group in a hydrophobic region of the molecule. Addition of ethylene-diaminetetraacetate results in a shift of the spectrum toward shorter wave lengths, indicative of a more polar environment for this —SH in the absence of \( \text{Ca}^{2+} \). Attachment of the phenacyl group to positions in the enzyme other than the active —SH by reaction with BHNA in the absence of \( \text{Ca}^{2+} \) results in losses in transferase activity, but essentially no loss in esterase activity. The spectrum of the groups bound to enzyme in the absence of \( \text{Ca}^{2+} \) is identical with that of the phenacyl group in water. This spectrum is unchanged by subsequent addition of \( \text{Ca}^{2+} \).

In the catalytically inactive forms of transglutaminase, produced by the reaction of \( \alpha \)- and \( \beta \)-forms of methyl \( N \)-\( (2 \)-hydroxy-5-nitrophenylacyl)-2-amino-4-oxo-5-chloropentanoate (PACK) and \( \beta \)-chloro-4-(2-hydroxy-5-nitrophenylacyl)-amido-but-2-one (PBCK) with enzyme in the presence of \( \text{Ca}^{2+} \), the phenolic reporter group is attached covalently to the enzyme's active —SH. The rapid rate of inactivation by \( \beta \)-PACK compared to \( \alpha \)-PACK and PBCK implies that \( \beta \)-PACK, by virtue of its structural similarity to transglutaminase substrates, is properly oriented at the substrate-binding site of enzyme prior to the covalent reaction. The \( pK_a \) of the phenolic group in the acyl portion of each of these inactivators is shifted toward that of a weaker acid in the reporter-labeled enzyme proteins. The identical changes in \( pK_a \) with each inactivator suggest that the phenacyl side chain in each case is positioned in the same manner within the matrix of the calcium-activated enzyme derivative. Addition of EDTA results in a shift in \( pK_a \) of the phenolic group in each enzyme derivative back to that of the parent inactivator. This, together with the findings with BHNA, forms the basis for a suggestion that the active —SH is located at or near the surface in the inactivated enzyme, i.e., in the absence of \( \text{Ca}^{2+} \).

Active site titration procedures for transglutaminase are described. A rate assay procedure utilizing either BHNA or \( \beta \)-PACK was found to give results in excellent agreement with those of a direct spectrophotometric method carried out with the use of BHNA. The latter titration is based on the differences in the absorption spectrum of the phenacyl group bound to the enzyme's active —SH and that of this group attached to other positions on the enzyme molecule.

The sulfhydryl group of a single cysteine residue in transglutaminase has been identified as essential for the catalytic activities of the enzyme (1, 2). Although transglutaminase contains 17 or 18 free —SH groups (9), rapid selective alkylation of the essential —SH by iodoacetamide occurs between \( \text{pH} \) 6 and 7 in the presence of calcium ion, which is necessary for enzymatic activity (1, 2). A sequence of amino acids surrounding this cysteine has been reported (1). Identification of the same cysteine —SH as the site of acylation during the course of hydrolysis of \( \rho \)-nitrophenyl tri-methylacetate has supplied strong evidence for participation of this group in the intermediate formation of acyl enzyme through thioester linkage (3). Kinetic findings for hydrolysis and transfer of both active ester and amide substrates are in accord with the theory of acyl enzyme formation in the transglutaminase mechanism (4, 5).

The selective reactivity of the essential —SH, evidenced as the center of the active site of transglutaminase, together with the \( \text{Ca}^{2+} \) requirement for its reactivity, singles out this enzyme protein as an especially attractive model for study with the use of covalently attached environmentally sensitive groups. Several other features of transglutaminase specificity and catalysis stimulated and directed approaches to this study. These include a reversible conformational alteration in the enzyme protein induced by \( \text{Ca}^{2+} \) (6), the requirement that glutamine substrate be peptide or protein bound (7, 8), and evidence that glutamine substrate is the first to add to enzyme in the catalytic reaction (4, 5). Thus, the chemical agents employed for the study reported here were selected with a design to explore the environment in several regions of the enzymatic center of transglutaminase.

Early attempts to obtain knowledge of the area in the vicinity of the active site —SH of transglutaminase made use of the "reporter" group-containing agent, \( \alpha \)-bromoacetamido-4-nitrophenol (9). These proved unfruitful because of the tendency of the labeled protein derivative to precipitate rapidly from solution.

R. L. Boothe and J. E. Folk, unpublished observation.
Mechanism of Transglutaminase. VIII

Transglutaminase was prepared from fresh guinea pig liver by a published procedure (10). The enzyme showed 95 ± 5% of the reported specific activity when assayed by hydroxamate formation with the specific substrate benzylxycarbonyl-L-glutamylglycine (2). Enzyme concentration was determined by the use of the $E_{15}$ of 15.8 and a molecular weight of 90,000 (2).

A sample of $\alpha$-bromo-4-hydroxy-3-nitroacetophenone was kindly supplied by Dr. E. T. Kaiser. BHNA was also prepared by the method of Sipos and Szabo (11).

The following compounds were synthesized: $\textit{n}$ and $\textit{l}$ forms of methyl $N(2$-hydroxy-5-nitrophenylacetyl)-2-amino-4-oxo-5-chloropentanoate, $1$-chloro-4-(2-hydroxy-5-nitrophenylacetyl)-$l$-glutamine, and $N(2$-hydroxy-5-nitrophenylacetyl)-4-amino-nicotinamide.

Other materials and reagents have been described in previous publications (2-5).

Methods

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The abbreviations used are: BHNA, $\alpha$-bromo-4-hydroxy-3-nitroacetophenone; $l$-PACK, methyl $N(2$-hydroxy-5-nitrophenylacetyl)-2-amino-4-oxo-5-chloropentanoate; $n$-PACK, methyl $N(2$-hydroxy-5-nitrophenylacetyl)-2-amino-4-oxo-5-chloropentanoate; PBCK, 1-chloro-4-(2-hydroxy-5-nitrophenylacetyl)amidobutan-2-one; PG, methyl $N(2$-hydroxy-5-nitrophenylacetyl)-$l$-glutamine; PABA, $N(2$-hydroxy-5-nitrophenylacetyl)-4-amino-nicotinamide; Z, benzylxycarbonyl; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid). The abbreviations, PACK and PBCK, were adopted to conform with the commonly used abbreviations for chloromethyl ketones.

Synthesis of PACK

Methyl $\alpha$-Amino-4-oxo-5-chloropentanoate-HCl—$\textit{Z}$- Benzyl $\textit{Z}$-2-amino-4-oxo-5-diazopentanoate (2.3 g), prepared according to Khedouri, Anderson, and Meister (12) was converted to the $\textit{Z}$-benzyl group was removed by heating this compound for 1 hour at 90° in 20 ml of 95% trifluoroacetic acid. The oil remaining upon removal of the trifluoroacetic acid under vacuum was dissolved in 50 ml of absolute methanol. The methanol solution was cooled to 0° and saturated with HCl gas. After standing for 3 hours at room temperature the solvent was removed under vacuum. The resulting oil crystallized readily upon the addition of ether and was recrystallized from methanol-ether. The yield was 46% (0.61 g) based on the diazopentanoate starting material; m.p. 154°, $[\alpha]_D^{20} +15.1$ (c, 1% in HzO).

$l$-PACK—To 216 mg of the above ester in 5 ml of H$_2$O were added 1 ml of $n$ NaOH and 179 mg of 5-nitro-2-1,4benzodiazepine (13) dissolved in 7.5 ml of acetonitrile. After stirring for 15 min an additional 1 ml of $n$ NaOH was added and stirring was continued for 5 min. The solvents were removed under vacuum, and to the residue were added 25 ml of ethyl acetate and 25 ml of 0.5 x HCl. The ethyl acetate layer was separated, washed successively with water, dilute NaHCO$_3$ water, dilute HCl, and water, and dried over Na$_2$SO$_4$. The residue obtained upon removal of solvent was warmed with 5 ml of water while ethanol was added dropwise until it dissolved. Upon slow cooling and scratching the product crystallized; yield 83 mg (22%), m.p. 152°. The compound showed single areas (yellow upon exposure to NH$_3$ vapor) in two thin layer chromatography systems on silica gel. $R_f$ 0.7 in benzene-pyridine-acetic acid (80:20:2); $R_f$ 0.9 in chloroform-methanol-acetic acid (95:5:1). The findings by mass spectral analysis were in accord with the theoretical molecular weight; the molecular ion showed the expected chlorine isotope cluster.

$C_4H_{11}O_4N_C_l$ (153.7)

Calculated: C 46.9, H 4.2, N 7.8

Found: C 46.7, H 4.3, N 8.2

Methyl $\textit{n}$-2-Amino-4-oxo-5-chloropentanoate-HCl—$\textit{Z}$- $\textit{Z}$-2-amino-4-oxo-5-diazopentanoate (m.p. 71°) the procedure described for the $l$ compound (12). The $n$-chloropentanoic acid methyl ester HCl was prepared as outlined above for the $l$ compound and showed satisfactory values for elemental composition; m.p. 154°, $[\alpha]_D^{20} -15.2$ (c, 0.8% in H$_2$O).

$n$-PACK—This compound was prepared as outlined for $l$-PACK. It showed satisfactory values for elemental composition and was judged homogeneous by thin layer chromatography; m.p. 151°.
Synthesis of PBCK

1-Chloro-4-Z-amidobutan-2-one—Z-3-aminobutyric acid (4.1 g) (Cyclo Chemical Company) was dissolved in 20 ml of thionyl chloride and heated at 40° for 30 min under anhydrous conditions. The unreacted thionyl chloride was removed under high vacuum, and to the residue was added excess diazonethane in ether. After standing overnight the mixture was reduced to an oil under vacuum. The residue was added excess diazomethane in ether. After unreacted thionyl chloride was removed under high vacuum, and heated at 40° for 30 min under anhydrous conditions. The oil obtained upon removal of the solvent formed crystals under pentane in the cold. After decolorization in ethanol and recrystallization from ethanol-pentane, 2 g (43%) of the compound were obtained; m.p. 39°.

C₁₂H₂₁O₇N₃Cl (300.7)
Calculated: C 47.9, H 4.4, N 9.3
Found: C 47.8, H 4.1, N 9.3

PBCK—The above chloroketone (256 mg) was decarbobenzoxylated by heating for 30 min at 90° in 2 ml of 95% trifluoroacetic acid. The acid was removed under vacuum and the resulting oil was washed three times with tritration with ether. This was dissolved in 2 ml of 0.5 N NaOH and added to a solution of 179 mg of 5-nitro-2-coumaranone in 10 ml of dioxane. After stirring for 15 min the solvents were removed under vacuum and the oil dissolved in ethyl acetate. The ethyl acetate solution was washed successively with dilute NaHCO₃, H₂O, dilute HCl, and water and dried with Na₂SO₄. After removal of ethyl acetate the compound was crystallized from water-ethanol; yield 90 mg (30%), m.p. 119-122°. The compound showed single areas in the two chromatography systems used for PACK. The findings by mass spectral analysis were in accord with the theoretical molecular weight; the molecular ion showed the expected chlorine isotope cluster.

C₁₂H₁₇O₅N₃Cl (302.3)
Calculated: C 47.9, H 4.4, N 9.3
Found: C 47.8, H 4.1, N 9.3

Synthesis of PG

Z-γ-glutamyl methyl ester (300 mg) (15) was decarbobenzoxylated by hydrogenation in a methanol-water mixture using Pd black catalyst. The solvents were removed under vacuum after removal of the catalyst by filtration. The resultant oil was dissolved in 1 ml of water and a solution of 179 mg of 5-nitro-2-coumaranone in 7.5 ml of dioxane was added. After stirring for 1 hour the solvents were removed under vacuum and the residue was dissolved in 20 ml of ethyl acetate. The ethyl acetate solution was washed with dilute HCl and water and dried over Na₂SO₄. The compound was crystallized by the addition of pentane; yield 114 mg (34%), m.p. 160°. A sample was recrystallized from water for analysis. The melting point was unchanged. This compound showed single areas in the two chromatography systems described above.

C₁₂H₁₇O₅N₃·3H₂O (348.3)
Calculated: C 48.3, H 5.2, N 12.1
Found: C 48.0, H 5.1, N 12.4

Synthesis of PABA

Z γ aminobutyramide—This material was prepared from Z-γ-aminobutyric acid (Cyclo Chemical Company) by the mixed anhydride procedure using triethylamine and isobutyl chloroacetate in chloroform and coupling with aqueous NH₄. The product crystallized readily from the chloroform layer and was recrystallized from ethyl acetate in 75% yield; m.p. 127°.

C₁₁H₁₅O₃N₃ (236.3)
Calculated: C 61.0, H 6.8, N 11.9
Found: C 60.6, H 6.7, N 11.7

PABA—Z-γ-aminobutyramide was decarbobenzoxylated by hydrogenation in methanol and coupled with 5-nitro-2-coumaranone as outlined above for the preparation of PG. The product was obtained in 40% yield after crystallization from absolute ethanol. It was recrystallized from water for analysis; m.p. 211-212°. It showed single areas in the two chromatography systems described above.

C₁₂H₁₅O₃N₄ (281.3)
Calculated: C 51.2, H 5.4, N 14.9
Found: C 50.9, H 5.7, N 15.1

Enzymatic Assays and Stock Solutions

The hydroxylamine incorporation assay was carried out as outlined previously (2) in 0.1 M Tris-acetate containing 30 mM Z-glutamylglycine, 1 mM EDTA, 5 mM CaCl2, and 0.1 M hydroxylamine, at pH 6.0 and 37°.

The esterase assay was carried out in 0.1 M Tris-HCl containing 0.5 mM p-nitrophenyl acetate, 30 μM EDTA, 10 mM CaCl2, and 5% n-propyl alcohol, at pH 7.0 and 25° (16). Rates of liberation of p-nitrophenol were measured at 400 μm, within the first 20 to 40 s of hydrolysis.

The transglutaminase-catalyzed incorporation of [14C]glycine ethyl ester in place of —NH₂ at the carboxamide groups of PG and PABA was measured by a paper strip ion exchange procedure similar in principle to that outlined by Sherman (17). Aliquots of incubation mixtures (10 to 30 μl) were applied to strips (1.5 × 14 cm) of cellulose phosphate ion exchange paper (P81; capacity, 18 μeq per cm²; basis weight 85 g per m² (Whatman)), enzymatic reactions were stopped by the immediate application of approximately 100 μl of absolute ethanol, and the strips were eluted with water in a manner similar to that outlined by Sherman (17). The 14C labeled amine, glycine ethyl ester, remained at the position of application of the reaction mixture, while PG, PABA, and the labeled products of the transfer reaction were washed toward the bottom of the strips. After drying, the lower 5 cm of the strips were removed, placed in counting vials containing 10 ml of Liquifluor-toluene counting fluid (New England Nuclear), and the radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. Experiments in which PG or PABA and the reaction products were eluted from the lower 5 cm of the paper strips with NaHCO₃ solution showed quantitative recovery of these materials as measured by absorbance at 410 μm.

Transglutaminase-catalyzed incorporation of glycine ethyl ester into PG and PABA was confirmed by thin layer chromatography on silen gel using as solvent, benzene-pyridine-acetic acid (80:20:2). The substrates (PG, RF 0.18; PABA, RF 0.15) and the products (RF values of 0.47 and 0.40, respectively) were visualized by exposure of the chromatograms to NH₃ vapor.

Stock solutions of BHNA (1 mM) were prepared in 0.01 M Tris-HCl, pH 7.0; those of PACK and PBCK (1 to 2 mM) in H₂O; those of PG (0.1 mM) in water and PABA (0.09 mM) in H₂O at 50° with 1 eq of NaOH. The concentrations of halomethyl ketones

Found : C 48.0, H 5.1, N 12.4

Calculated : C 48.3, H 5.2, N 12.1

Calculated : C 47.9, H 4.4, N 9.3

Calculated : C 47.9, H 4.4, N 9.3

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found, however, that each of these chloromethyl ketones reacted with the -SH of GSH at the same rate under the conditions of transglutaminase by L-PACK, D-PACK, and PBCK. It was this probably also true for the halomethyl ketones listed in Table I, e.g., no inactivation by L-PACK was observed in the absence of Ca++. Since the dissociation constant for Ca++ is 6 to 8 x 10^-8 M (16, 19), one may assume that the rate constants given in Table I are listed the over-all bimolecular rate constants for inactivation of transglutaminase calculated using the expression

\[ k = \frac{1}{E_{0t}} \ln \left( \frac{E_{0t}}{E(t)} \right) \left[ \frac{I(O) - E_{0t}}{I(t) - E_{0t}} \right] \]

where \( E_{0t} \) is the total enzyme concentration, \( I(O) \) is the initial concentration of inactivator, and \( I(t) \) is the total active enzyme at time \( t \). The level of Ca++ (25 mM) used for these inactivation studies and for the other studies reported here was chosen because the enzyme is stable for periods of at least 30 min at pH 7 at this level of metal ion. The rate of inactivation of transglutaminase by iodoacetamide is a function of the Ca++ concentration (19). In the presence of Ca++, as in the case with PACK and PBCK, equivalent losses in transferase and esterase activities occurred. However, without Ca++, no pronounced loss in esterase activity was found with up to 3 moles of BHNA per mole of enzyme, whereas significant losses in hydroxylamine-incorporating activity were observed.

### Table I

**Rate constants for inactivation of transglutaminase**

| Inactivator       | Second order rate constant \( M^{-1} min^{-1} \times 10^4 \) |
|-------------------|----------------------------------------------------------|
| L-PACK            | 45.0                                                     |
| L-PACK without Ca++ | *                                                        |
| D-PACK            | 1.0                                                      |
| PBCK              | 10.0                                                     |
| Chloroacetamide   | 4.3                                                      |
| Iodoacetamide     | ~72.0*                                                   |

* No loss in activity observed after 30 min at a 25 \( \mu \)M level of L-PACK.

in stock solutions were verified by reaction at pH 7.0 with the sulfhydryl of GSH. The spectrophotometric DTNB method was used for -SH measurements (18).

### Spectral Measurements

Spectra were measured with a Cary model 11 recording spectrophotometer in 1-cm silica cuvettes at room temperature. Corrections were applied for small volume changes made by additions of concentrated solutions of EDTA, CaCl₂, etc. For spectrophotometric titrations, small portions of appropriate concentration of acid, base, or reagent were added and the solutions were rapidly mixed. In all cases the pH levels of solutions were determined before and after measurement of spectra.

### RESULTS

**Inactivation of Transglutaminase by Halomethyl Ketones**—In Table I are listed the over-all bimolecular rate constants for inactivation of transglutaminase calculated using the expression above.

There are pronounced differences in the rates of inactivation of transglutaminase by L-PACK, D-PACK, and PBCK. It was found, however, that each of these chloromethyl ketones reacted with the -SH of GSH at the same rate under the conditions of Table I (second order rate constant for each, 900 \( M^{-1} min^{-1} \)). The second order rate constant for reaction of chloroacetamide with GSH has been reported to be less than 0.3 \( M^{-1} min^{-1} \) at 30°, pH 7, and 0.1 ionic strength (20).

Inactivation of transglutaminase by BHNA at pH 7.0 and 25° in the presence of 25 mM Ca++ was found to be so rapid that no estimate of a rate constant could be obtained. At equimolar levels of enzyme and BHNA, activity was completely lost within the time required to commence the assays. Table II compares the changes in enzymatic activities of transglutaminase that occur as a result of reaction with BHNA in the presence and absence of Ca++. In the presence of Ca++, as in the case with PACK and PBCK, equivalent losses in transferase and esterase activities occurred. However, without Ca++, no pronounced loss in esterase activity was found with up to 3 moles of BHNA per mole of enzyme, whereas significant losses in hydroxylamine-incorporating activity were observed.

### Table II

**Changes in enzymatic activities of transglutaminase upon reaction with BHNA**

| BHNA | CaCl₂ | Initial activity remaining |
|------|-------|---------------------------|
|      | mole/mole enzyme | mm | %  | %  |
| 1.0  | 0     | 25 | 48 | 50 |
| 2.0  | 0     | 25 | 15 | 96 |
| 3.0  | 0     | 25 | 4  | 92 |

Enzyme (14.4 \( \mu \)M) was incubated with the recorded levels of BHNA for 10 min in 0.2 mM Tris-HCl containing 0.33 mM EDTA and the indicated level of CaCl₂, pH 7.0, at 25°. The reactions in the absence of Ca++ were complete within 15 min as observed by no further changes in enzymatic activities or in spectra (see "Spectral Properties of 4-Hydroxy-3-nitrophenacyl Group in BHNA-modified transglutaminase").

Enzyme (23.6 \( \mu \)M) was incubated with inactivator (25 to 40 \( \mu \)M) in 0.2 mM Tris-HCl containing 25 mM CaCl₂ and 0.33 mM EDTA, pH 7.0, at 25°. Aliquots were assayed for hydroxylamine incorporation and esterase activity. These two assays showed essentially the same degree of loss in enzymatic activity. Controls, incubated as outlined except without inactivator, showed less than 5% loss in initial enzymatic activity after 30 min.

* Calculated from data of Reference 19 for rate of inactivation at 25 mM Ca++.
tryptophan by treatment with carboxypeptidase A followed by thin layer chromatography. The NH₂-terminal amino acid, glycine, was identified as its 1-dimethylaminonaphthalene-5-sulfonyl (dansyl) derivative following acid hydrolysis of the dansylated peptide. The acid hydrolysate was dansylated, and the derivatives were identified as those of glycine and glutamic acid by two-dimensional thin layer chromatography using chloroform-butyl alcohol-acetic acid (6:3:1) and chloroform-ethanol-acetic acid (38:4:3) on silica gel. A third dansyl derivative remained at the origin in both chromatographic systems, as did the dansylated product of the reaction of L-2-amino-4-oxo-5-chloropentanoic acid with cysteine (equimolar quantities of each at pH 8 for 1 hour).

Essentially the same isolation and identification procedure was carried out with a sample of transglutaminase that had been inactivated with BHNA in the presence of Ca+++. The findings were the same with the exception that a chromatogram of an acid hydrolysate of the chromophoric chymotryptic peptide showed a single yellow area (RF 0.63 in 1-butanol-acetic acid-H₂O (4:1:2)) identical with that formed by the reaction of BHNA with cysteine (equimolar quantities of each at pH 7 for 10 min). The dansyl derivatives of glycine and glutamic acid, together with a yellow derivative which remained at the origin in both chromatographic systems, as did the dansylated product of reaction of BHNA with cysteine, were found as components of the dansylated acid hydrolysate of this peptide. Thus, the peptide liberated by chymotrypsin A from L-PACK-inactivated transglutaminase appears to be identical in amino acid sequence with that obtained by chymotrypsin A digestion of the BHNA-inactivated enzyme. These peptides have an NH₂-terminal glycine, a COOH-terminal tryptophan, and contain glutamic acid or glutamine and a derivative of cysteine. These observations support the conclusions that both PACK and BHNA are incorporated into calcium-activated transglutaminase by alkylation of the —SH group of a single cysteine residue, that this —SH is the same in each case, and that this is the same essential —SH group that is alkylated by iodoacetamide under similar experimental conditions. A portion of the amino acid sequence surrounding the cysteine that is alkylated by iodoacetamide has been identified as Gly-Gln-Cys-Trp (2).

Substrate Properties of PG and PABA—Z-L-glutamine methyl ester has been found to be a substrate for transglutaminase (8). It was anticipated that PG, the glutamine analogue of L-PACK, would also act as a transglutaminase substrate. This proved to be the case. Using the SEQUEN computer program of Cleland (22) the following estimates for constants were obtained from reactions carried out in 0.2 m Tris-HCl containing 25 mM CaCl₂ and 0.33 mM EDTA, pH 7.0, at 25°C: Kₐ, the Michaelis constant for PG at saturating glycine ethyl ester, 19.0 ± 1.1 mM; Kₐb, the Michaelis constant for glycine ethyl ester at saturating PG, 0.18 ± 0.02 mM; Kₐb, the Michaelis constant for hydrolysis of PG, 20.7 ± 3.7 mM; Vₐ, the maximum velocity for transfer, 10.2 ± 0.3 μmoles per min (per μ mole of enzyme). These kinetic constants have been defined elsewhere in terms of rate constants (4).

Transglutaminase displays an almost absolute stereospecificity toward the L isomer of a glutamine substrate (5). The inactive analogs have been designed to act in a substrate-like manner. As expected, the α form of PACK was the more effective inactivator (Table I). PCCK, in which the —COO⁻ group of PACK was replaced by hydrogen and, consequently, in which there is no asymmetric carbon atom, was a more efficient inactivator than α-PACK (Table I). It was of prime interest to determine if the carboxamide analogue of PBCK, PABA, would function as a transglutaminase substrate. Indeed, PABA did serve as a substrate, evidence that the α-carboxyl portion of the glutamine moiety is not an essential part of substrates for transglutaminase. The time-dependent accumulation of product in the PABA-glycine ethyl ester reaction was observed by thin layer chromatography. The limiting solubility of PABA precluded estimation of meaningful kinetic constants. However, at 7.4 mM PABA and 1.2 mM [³⁵S]glycine ethyl ester, the rate of transfer product formation was found to be 1.6 μmoles per min (per μ mole of enzyme) under the experimental conditions used with PG. The rate is in the range of those observed with PG.
Mechanism of Transglutaminase. VIII

Vol. 246, No. 21

FIG. 1 (left). Absorption spectra of BHNA and of the 4-hydroxy-3-nitrophenacyl group attached in the presence of Ca$^{++}$ to transglutaminase. Experimental conditions: 0.2 M Tris-HCl containing 0.33 mM EDTA and 25 mM CaCl$_2$, pH 7.02, 23°. Curve 1, 28.6 μM BHNA. Curve 2, 29 μM transglutaminase + 28.6 μM BHNA. Curve 3, 29 μM transglutaminase + 28.6 μM BHNA, after 5 min solution made 28 mM in EDTA. Spectra of enzyme + BHNA were measured against enzyme. Spectra were obtained and enzymatic assays were conducted approximately 5 min after preparing the solutions. The enzyme retained less than 25% of its initial hydroxylamine-incorporating and esterase activities (see Table II).

FIG. 2 (right). Absorption spectra of BHNA and of the 4-hydroxy-3-nitrophenacyl group attached in the absence of Ca$^{++}$ to transglutaminase; absorption spectra of the 4-hydroxy-3-nitrophenacyl group attached to GSH. The experimental conditions were those of Fig. 1 except without CaCl$_2$. Curve 1, 28.6 μM BHNA. Curve 2, 29 μM transglutaminase + 28.6 μM BHNA. The identical curve was obtained with 66 μM GSH + 28.6 μM BHNA. Curve 3, 66 μM GSH + 28.6 μM BHNA, 35% (v/v) dioxane. The spectrum of enzyme + BHNA was measured against enzyme. The spectral changes and changes in enzymatic activities were complete within 15 min after preparing the solutions. The enzyme retained 60% of its initial hydroxylamine-incorporating activity and 100% of its esterase activity (see Table II).

FIG. 3. Absorption spectra of L-PACK and of the acyl group in L-PACK-inactivated transglutaminase. The experimental conditions were those of Fig. 1. Curve 1, 34.6 μM L-PACK. Curve 2, 34.6 μM transglutaminase + 34.6 μM L-PACK. Spectra were obtained and enzymatic assays were conducted 30 min after preparing the solutions. The enzyme retained less than 2% of its initial hydroxylamine-incorporating and esterase activities.

Addition of Ca$^{++}$ and that displayed the typical 334 m$\mu$ peak absorbance (Curve 2, Fig. 1), were placed in matched cuvettes in the blank compartment and the sample compartment of the spectrophotometer. Portions of BHNA solution and equal volume portions of dilute Tris buffer, pH 7, were added to the solutions in the sample and blank compartments, respectively. A spectrum was obtained after each addition. These spectra, observed up to the level of 2.5 moles of BHNA per mole of enzyme, were typical of that found for enzyme labeled with BHNA in the absence of Ca$^{++}$ (Curve 2, Fig. 1). The solution in the sample compartment showed increasing slight turbidity after each addition of BHNA. Addition of more than 2.5 moles of reagent per mole of enzyme resulted in visible precipitation.

EDTA, added in excess of the Ca$^{++}$ to a solution of phenacyl-labeled enzyme that was prepared in the presence of Ca$^{++}$, caused an apparent shift in the spectrum toward shorter wave lengths (compare Curves 2 and 3, Fig. 1). The broadness of the spectrum with added EDTA (Curve 3, Fig. 1) compared to that observed without EDTA (Curve 2, Fig. 1) suggests contribution of more than a single component. The spectrum was shifted back to that observed without EDTA (Curve 2, Fig. 1) upon addition of CaCl$_2$ in large excess over EDTA.

Addition of the substrates, Z-l-glutaminylglycine or glycine ethyl ester, to solutions of transglutaminase that had been inactivated with BHNA in the presence of Ca$^{++}$ caused no change in the spectrum shown in Fig. 1, Curve 2.

Spectral Properties of Chromophoric Acyl Groups in PACK- and PBCK-inactivated Transglutaminase—Spectral titration of the phenolic group of L-PACK showed it to have a pK$_a$ of 6.7. Reaction of L-PACK with excess GSH did not change this value. The identical pK$_a$ values were found for the phenolic groups in n-PACK and PBCK. These compounds showed an absorption maximum at 318 to 320 m$\mu$ in the un-ionized state and one at 410 m$\mu$ in the ionized state with an isosbestic point at 353 m$\mu$. A portion of the absorption spectrum of L-PACK at pH 7 is shown in Fig. 3, Curve 1. The identical spectrum was observed with equivalent concentrations of n-PACK or PBCK, or with L-PACK after reaction with excess GSH. Decrease in absorbance of L-PACK at 410 m$\mu$ was observed to be an almost linear function of the concentration of dioxane in the solution. Titration of the
FIG. 4 (left). Correlation between the change in absorbance and loss in enzymatic activity during reaction of transglutaminase with L-PACK. The experimental conditions were those of Figs. 1 and 3. Aliquots of the reaction mixture were removed at the indicated times for assay by the hydroxylamine incorporation method. A total change in absorbance of 0.18 at 410 nm was observed (see Fig. 3).

FIG. 5 (right). Active site titration of transglutaminase by the rate assay method using the inactivator L-PACK. The esterase activity of enzyme inhibited by varying amounts of L-PACK (conditions of Fig. 1, 30-min incubation with inactivator) is plotted relative to that of enzyme incubated without inactivator. The enzyme concentration was 4.7 × 10⁻⁶ M. Essentially the same results were obtained by using the hydroxylamine incorporation assay.

Fig. 4 shows that the reduction in absorbance at 410 nm that results from reaction of L-PACK with transglutaminase occurs concomitantly with the loss in catalytic activity of the enzyme. Essentially the same quantitative changes in absorbance with relation to loss in enzymatic activities were observed during the early stages of inactivation by n-PACK and PBCK. That no change in absorbance at 353 nm, the isoelectric point, occurred during enzyme inactivation by these agents further suggests that only ionization of the phenolic group is effected by attachment of the acyl portion of the inactivators to enzyme.

Evidence that removal of Ca²⁺ from L-PACK-inactivated enzyme solutions resulted in a shift in the pKₐ of the phenolic group back to that of L-PACK (pKₐ of 6.7) was obtained by spectral titration of this group in the enzyme after addition of EDTA in excess of the Ca²⁺. Raising the Ca²⁺ level above that of EDTA again shifted the pKₐ of this group to that of a weaker acid (pKₐ of 7.1 to 7.2).

Addition of the substrates, L-glutamylglycine or glycine, to solutions of enzyme that had been inactivated with L-PACK at pH 7.0 caused no change in the observed spectrum.

Active Site Titrations of Transglutaminase—Titration of the active site of transglutaminase was carried out by the use of BHNA and L-PACK. With L-PACK, titration was accomplished by measuring the degree of inactivation using rate assays employing both the hydroxylamine incorporation and esterase procedures. The results of such a titration are shown in Fig. 5. The same procedure, using BHNA in place of L-PACK, gave identical results.

A second approach using BHNA involved a direct spectrophotometric titration. This procedure utilizes the differences in the absorption spectrum of the phenacyl group bound to the en-
zyme's active site and that of this group attached at other positions in the enzyme (see "Spectral Properties of 4-Hydroxy-3-nitrophenacyl Group in BHNA-modified Transglutaminase"). The results of a direct spectrophotometric titration are shown in Fig. 6. Findings obtained by this procedure were in excellent agreement with those obtained using the rate assay method of Fig. 5.

Further evidence that L-PACK and BHNA titrate the same essential —SH group of transglutaminase is as follows. Reaction of enzyme, first inactivated by 1 mole of L-PACK per mole, with BHNA resulted in the appearance of a spectrum typical of reaction of groups in the enzyme other than the active site one (Curve 2, Fig. 2).

**DISCUSSION**

The findings reported here were forthcoming from an effort to define certain environmental features of the active center of transglutaminase. This effort, although limited by the insoluble nature of the enzyme derivatives at levels of pH below neutrality, was enhanced by the functional role of Ca++. There is strong evidence that, in the presence of Ca++, reaction with each of the halomethyl ketones results in formation of a catalytically inactive enzyme derivative in which reporter group is covalently bound through the same essential group in the enzyme, presumably the active site —SH.

The observed shift in the spectrum of the 4-hydroxy-3-nitrophenacyl group upon attachment at the active center of transglutaminase (reaction with BHNA in the presence of Ca++, Curve 2, Fig. 1) is congruent with orientation of this group in a hydrophobic region of the molecule. The tendency of this spectrum to shift to shorter wave lengths with the addition of EDTA (Curve 3, Fig. 1) suggests a more polar environment for this —SH group in the catalytically inactive form of the enzyme, i.e., in the absence of Ca++. Both hydrophobic (24) and polar (9) regions have been identified at the active center of chymotrypsin A. The specificity pattern of chymotrypsin A, i.e., preference for aromatic derivatives over short chain aliphatic ones, suggests that hydrophobic bonding plays an important role in substrate attraction. This is the basis for speculations that the hydrophobic portion of the active center is the substrate recognition site, while the catalytic site requires a high water concentration for mediation of the hydrolytic process (9, 24). Transglutaminase catalyzes hydrolysis with glutamine substrates (7, 8) and with active esters (16). There is substantial kinetic evidence for the formation of a common intermediate acyl enzyme in the hydrolysis and transfer reactions (4, 5). That transfer of acyl group from intermediate acyl enzyme to water (hydrolysis) is slower than transfer of this group to amine (4, 5) is a reflection of the rate-limiting nature of the deacylation to water. This could conceivably be a consequence of limiting water concentration at the active center. Because the enzyme does, indeed, catalyze hydrolysis, one may assume, as in the case of chymotrypsin A, that there exists in the active center a combination of hydrophobic and hydrophilic regions.

Attachment of the 4-hydroxy-3-nitrophenacyl group to positions in transglutaminase other than the active site —SH (reaction with BHNA in the absence of Ca++, Curve 2, Fig. 2) has a pronounced influence on the hydroxylamine incorporation activity of the enzyme, but does not affect its esterase activity (Table II). The spectral property of the chromophoric groups bound in these positions on the enzyme is characteristic of this group in a hydrophilic environment both in the presence and absence of Ca++ (Curve 2, Fig. 2). Structural analogies between BHNA and DTNB, e.g., the nitro group ortho to a negatively charged group and approximately the same distance from the reactive portion, suggest that the striking similarity in the effects of these two agents on the catalytic activities of transglutaminase (compare Tables II and III) results from reaction with the same enzyme groups in each case. With 1 mole of DTNB per mole of enzyme in the absence of Ca++, a single intramolecular disulfide bond is formed in the enzyme (25). Inhibition studies suggest that this molecular change causes a loss in binding properties for glutamine substrate (25). If one or both of these —SH groups participate directly in glutamine substrate binding, it seems possible, on the basis of the spectral finding and the similarity in effects of DTNB and BHNA, that a portion of the binding site of the enzyme is polar in nature.

In enzyme reactions, where intermediate acyl derivatives are formed from enzyme and the acyl portion of substrate, the specificity of the enzyme may be directed toward a single configuration of the acyl portion of substrate, e.g., chymotrypsin’s specific action on peptide bonds in which the carboxyl part is contributed by amino acids of the L form and the almost absolute specificity of transglutaminase toward L-glutamine peptide (5). The question arises as to whether this is important only for proper noncovalent binding of substrate or whether the acyl portion of intermediate acyl enzyme remains or becomes aligned in a spatial arrangement especially suited for efficient deacylation. The chloromethyl ketones, n- and L-PACK, analogues of the transglutaminase substrates, were conceived with the intent of forming stable pseudo acyl enzymes in which similarities or differences in the orientation of the acyl group could be visualized. In this regard, it was anticipated that each of the inactivators, PBCK and the n and L forms of PACK, would react selectively with the enzyme’s active —SH by virtue of their halomethyl ketone feature. The total loss in enzyme activities upon reaction of each of the inactivators at the level of 1 mole per mole of enzyme is strong evidence for this. It seems evident from the more rapid rate of

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**Table III**

Changes in enzymatic activities of transglutaminase upon reaction with DTNB in absence of Ca++

| DTNB moles/mole enzyme | Hydroxylamine incorporation (%) | Esterase (%) |
|------------------------|--------------------------------|-------------|
| 0.2                    | 85                             | 100         |
| 0.4                    | 70                             | 100         |
| 0.5                    | 38                             | 100         |
| 0.8                    | 20                             | 99          |
| 1.0                    | 16                             | 97          |
| 1.2                    | 12                             | 97          |

* Data from Reference 25.
enzyme inactivation by L-PACK (Table I), that this isomer is acting in a substrate-like manner, i.e., is complexing with enzyme through the substrate-binding site. That L-PACK is a more efficient inactivator than PBCK supports this conclusion, as does the fact that each of these chloromethyl ketones reacts at the same rate with GSH. The identical change in the pH of the phenolic group in each of these inactivators upon covalent attachment to enzyme, as reflected in the spectral changes (example Fig. 3), may be evidence for the same positioning in the acyl enzyme of the side chain attached to the glutamine residue. To draw any firm conclusion from these findings would be presumptuous since the inactive pseudo acyl enzymes may not manifest the properties of intermediate acyl enzyme formed during catalysis.

The shift in the pH of the phenolic group back to that of a stronger acid as a result of addition of EDTA to the PACK- or PBCK-inactivated enzymes is compatible with evidence from the BHNA studies that the active site —SH is in a more polar environment in the absence of Ca++ than in the presence of this activating metal ion. The enzyme protein would appear to have no influence on the ionization of this phenolic group in the absence of Ca++ since under this condition the pH of the group is the same as that in the parent inactivators and in their reaction products with GSH. We speculate on this basis that the active site —SH is on or close to the enzyme surface in the unactivated enzyme.

PABA acts as a transglutaminase substrate. This was suggested by the finding that PBCK functioned as a more efficient inactivator of the enzyme than 3-PACK (Table I). Z-L-glutaminyglycine and Z-L-glutamine are substrates for transglutaminase, whereas L-glutamine, L-glutaminyglycine, and n-valeramide are neither substrates nor inhibitors (8, 26). It has been concluded from these findings that a peptide bond involving the α-amino group of glutamine is essential, while one at the carboxyl residue of glutamine is not. The present data show that the carboxyl of glutamine may be replaced by hydrogen. This is in accord with a suggestion that the single peptide bond through the amino group of glutamine participates directly in the binding of substrate to enzyme.

The nitro-substituted lactone, 5-nitro-2-coumaranone (13), proved invaluable in the preparation of the inactivators, PACK and PBCK, and the analogue substrates, PG and PABA, used in a portion of this work. Synthesis in each case was accomplished by lactone ring opening of this internal active ester mediated by nucleophilic attack by the amino group of the esterified amino acid. The potential use of this or similar reagents, e.g., 5-nitro-3H-1,2-benzoxathiole-2,2-dioxide (27), as a general method for the attachment of a chromophoric grouping in a proximity to a nucleophile would seem to be worthy of mention.

The active site titrations described here for transglutaminase are an addition to the rapidly growing number of such procedures for accurate quantification of enzymes. Both procedures described for transglutaminase are simple and rapid with obvious advantages of spectrophotometric measurements at wave lengths in the visual range.

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