Alkyl Isocyanates as Active Site-directed Inactivators of Guinea Pig Liver Transglutaminase*

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Alkyl isocyanates are effective inactivators of guinea pig liver transglutaminase. Based on the specificity of the reaction, the protection against inactivation by glutamine substrate, and the essential nature of calcium for the inactivation reaction, it is concluded that these reagents act as amide substrate analogs and, thus, function in an active site-specific manner. Support for the contention that inactivation results from alkyl thiocarbamate ester formation through the single active site sulfhydryl group of the enzyme is (a) the loss of one free --SH group and the incorporation of 1 mol of reagent/m01 of enzyme in the reaction, (b) the similarity in chemical properties of the inactive enzyme derivative formed to those previously reported for another alkyl thiocarbamoylenczyme and an alkyl thiocarbamoylcysteine derivative, and (c) the finding that labeled peptides from digests of [methyl-14C]thiocarbamoyltransglutaminase and those from digests of iodoacetamide-inactivated enzyme occupy similar positions on peptide maps.

Transglutaminase was found to be inactivated neither by urethan analogs of its active ester substrates nor by urea analogs of its amide substrates. It is concluded on the basis of these findings that inactive carbamoylenzyme derivatives are formed only by direct addition of the transglutaminase active site —SH group to the isocyanate C—N double bond, and not, like several serine active site enzymes, by nucleophilic displacement with urethan analogs of substrate, or by nucleophilic displacement with urea analogs of substrate.

The transglutaminases catalyze hydrolysis and aminolysis of the carboxamide groups of peptide-bound glutamine residues (for review, see Ref. 2). The enzymes also catalyze these reactions with active esters, e.g. p-nitrophenyl esters (3, 4) and certain thioesters (5). In addition, some esters of aliphatic alcohols which are not active esters have been found to function as substrates for the guinea pig liver enzyme (6). Studies in this laboratory have provided evidence that all of these reactions proceed through acyl-enzyme intermediates formed between the essential —SH group in the Ca*+-activated enzymes and the acyl portions of substrates (for review, see Ref. 2). The kinetics of hydrolysis and transfer conform to a modified double displacement mechanism in which acyl-enzyme is partitioned between water and another nucleophile, e.g. a primary amine.

Recent investigations on the substrate specificity of guinea pig liver transglutaminase have led to the following active site model for this enzyme (7–9). Polypeptides containing a substrate glutamine residue bind in a single direction along the enzyme surface; the side chains of amino acids over a range of residues on each side of the substrate glutamine residue exert an influence on catalysis. The arrangement of the substrate peptide chain on the enzyme surface directs the specificity of the enzyme for glutamine residues of the L configuration; the α-hydrogen of an L-glutamine residue is on the side of this substrate that is directed away from the enzyme surface, whereas the α-hydrogen of a D-glutamine residue abuts the enzyme surface resulting in misalignment of the carboxamide side chain in its binding pocket and, thus, in a concomitant loss in catalytic efficiency. The hydrophobic binding pocket for glutamine side chain assumes dimensions of approximately 5 x 5 Å at some stage of catalysis. At the apex of this pocket is located the enzyme sulfhydryl group which participates in acyl-enzyme intermediate formation.

The use of alkyl isocyanates as active site-specific inactivators for serine proteases (10) and for the —SH enzyme, yeast alcohol dehydrogenase (11), has been introduced by Wold and co-workers. They have provided evidence that, following binding of these substrate analogs, inactivation occurs as a result of formation of stable N-alkyl urethans with the hydroxyl group of the active site serine in chymotrypsin and elastase (12). In the case of the dehydrogenase, analogous N-alkyl thiourethans result from reaction of isocyanate with three enzyme sulfhydryl groups (11).

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We report here the inactivation of guinea pig liver transglutaminase by a series of straight and branched chain aliphatic isocyanates. The data indicate that inactivation in each case results from formation of a N-alkyl thiourea at the single essential sulfhydryl group of the enzyme. That inactivation is a consequence of an active site-specific reaction is evidenced by the fact that the enzyme is inactivated most effectively by isocyanates which are structurally related to the glutamine side chain. Evidence is presented that the inactive N-alkylthio carbamoyltransglutaminases are formed by direct addition of the side chain. Evidence is presented that the inactive N-alkylthio carbamoyltransglutaminases are formed by direct addition of the side chain.

EXPERIMENTAL PROCEDURE

Materials—Transglutaminase was prepared from guinea pig liver by a published procedure (13). The enzyme exhibited 95 ± 5% of the reported specific activity when assayed by hydroxamate formation with the specific substrate, benzoylcarbonyl-L-glutaminylglycine (14). The extinction coefficient of $E_{280}$ was 15.8 and a molecular weight of 90,000 was used to determine enzyme concentration (14).

Methyl isocyanate, N-buty1 isocyanate, N-hexyl isocyanate, N-p-toluenesulfonylphenyl isocyanate, N-p-nitrophenyl chloroformate, methylamine hydrochloride, and benzyl alcohol were obtained from Aldrich; L-albizzin from K&K Laboratories, glycinet-buty1 ester from Cyclo; and Z-amino acid from Sigma. These materials showed physical constants in agreement with the literature values and were used without further purification with the exception of N-buty1 isocyanate, which was purified by distillation prior to use. [1-14C]Methylamine (50 Ci/mmol) and [1-14C]iodoacetamide (52 Ci/mmol) were products of Amersham/Searle.

Isobutyl isocyanate, N-pentyl isocyanate, and isopentyl isocyanate were prepared from isovaleryl chloride, N-caproyl chloride, and isocaproyl chloride, respectively, through intermediate formation of the acyl azides by the general procedure of Allen and Bell (15). The isocyanates were purified by distillation at atmospheric pressure under nitrogen. The observed boiling points were in agreement with the literature values.

p-Nitrophenyl-N-methylcarbamate and p-nitrophenyl-N,N,N-dimethylcarbamate were prepared by reaction of p-nitrophenyl chloroformate with the appropriate amine. The procedure was essentially that of Bender and Homer (16). p-Nitrophenyl-N,N,N-dimethylcarbamate was crystallized from benzene/pentane and recrystallized from aqueous ethanol (m.p. 90–92°).

$\text{C}_{5}\text{H}_{12}\text{N}_{2}\text{O}_4$ (311.2)

Calculated: C 51.2, H 5.2, N 13.2
Found: C 50.8, H 4.7, N 12.7

Alternatively, p-nitrophenyl-N-methylcarbamate was prepared in 90% yield by reaction of a 1:2 molar excess of methyl isocyanate with p-nitrophenol in refluxing benzene. After an 18-hour reaction period the benzene solution was washed with water and dried over Na$_2$SO$_4$. The exact concentrations of isocyanates were determined by the spectrophotometric titration method of Brown and Wold (10) using benzylamine in chloroform. A gas chromatographic procedure utilizing a column packing of 10% SP-400 on Supelcoport, 100/120 mesh (Supelco, Inc.), was used also to determine isocyanate concentration through quantitation of the N-alkyl-N-benzylurea derivatives formed with benzylamine.* This procedure was found to give values in good agreement with the spectral titration method and, in addition, supplied evidence that each of the isocyanates was essentially free of contaminants. This was derived from the single peak and characteristic retention time observed for each of the isocyanates.

$p$-Nitrophenyl-N-[1-14C]methylcarbamate was prepared in 50% yield through reaction of $p$-nitrophenyl chloroformate with an appropriately diluted sample of [1-14C]methylamine HCl in a stirred mixture of benzene and 1 eq of aqueous NaOH at 40–50°. The product was recrystallized from benzene (m.p. 158°).

$\text{C}_{5}\text{H}_{12}\text{N}_{2}\text{O}_4$ (196.2)

Calculated: C 49.0, H 4.1, N 14.3
Found: C 49.4, H 4.2, N 14.4

$p$-Nitrophenyl-N-[1-14C]ethylcarbamate was prepared in 50% yield through reaction of $p$-nitrophenyl chloroformate with an appropriately diluted sample of [1-14C]methylamine HCl in a stirred mixture of benzene and 1 eq of aqueous NaOH at 40–50°. The product was recrystallized from benzene (m.p. 160°). The number of $-\text{SH}$ groups were calculated from the change in optical density of 0.1 M NaOH as measured spectrophotometrically at 400 nm.

1The abbreviation used is: Z-, benzoylcarbonyl-.

The Z-aminic acid was purified by the general procedure of Wolman et al. (17). Equimolar amounts of L-albizzin sodium salt (prepared from the amino acid and NaHCO$_3$ and benzyl-p-nitrophenyl carbonate (18) were heated at 100° for 30 min in dimethylformamide to give the Z-aminic acid. Neutralization and addition of dicyclohexyl carbodiimide yielded the crude product (oil). This material was purified by preparative thin layer chromatography on 2-mm plates of Brinkmann silica gel F$_254$ using chloroform/methanol/glacial acetic acid (76:24:1). The Z-aminic acid $p$-nitrophenyl ester ($R_t$ 0.45) was extracted with acetone and crystallized from aqueous ethanol in 26% yield (based on albizzin) (m.p. 143–145°).

$\text{C}_{9}\text{H}_{14}\text{N}_{2}\text{O}_4$ (402.4)

Calculated: C 53.7, H 4.5, N 13.9
Found: C 53.5, H 4.7, N 13.9

Z-L-Abizzinyglycine t-buty1 ester was obtained in 55% yield by reaction of the above Z-ester with glycine t-buty1 ester in pyridine (18 hours at 25°). After removal of solvent under vacuum, the product was crystallized from aqueous ethanol (m.p. 173°).

$\text{C}_{6}\text{H}_{14}\text{N}_{2}\text{O}_4$ (394.4)

Calculated: C 54.8, H 6.6, N 14.2
Found: C 55.0, H 6.9, N 14.6

This Z-dipeptide t-buty1 ester was converted to Z-L-albizzinyglycine by treatment with anhydrous trifluoroacetic acid at 0° for 2 hours. The bulk of the acid was removed at room temperature in a stream of nitrogen and the last traces of acid were removed under high vacuum. The crystalline product was obtained from aqueous ethanol in 56% yield (m.p. 122–124°).

$\text{C}_{10}\text{H}_{14}\text{N}_{2}\text{O}_4\cdot\text{H}_2\text{O}$ (366.3)

Calculated: C 47.2, H 5.6, N 15.7
Found: C 47.2, H 5.7, N 15.3

Stock solutions (approximately 1 M) of isocyanates and urethanes were prepared in anhydrous acetone and were stored at 5° over KOH pellets. The exact concentrations of isocyanates were determined by the spectrophotometric titration procedure of Brown and Wold (10) using benzylamine in chloroform. A gas chromatographic procedure utilizing a column packing of 10% SP-400 on Supelcoport, 100/120 mesh (Supelco, Inc.), was used also to determine isocyanate concentration through quantitation of the N-alkyl-N-benzylurea derivatives formed with benzylamine.* This procedure was found to give values in good agreement with the spectral titration method and, in addition, supplied evidence that each of the isocyanates was essentially free of contaminants. This was derived from the single peak and characteristic retention time observed for each of the isocyanates. The exact concentrations of the urethanes were determined by measurement at 400 nm of $p$-nitrophenol released in 0.1 M NaOH. Dilutions of stock solutions were made in acetone immediately prior to use. Stock solutions of peptide derivatives (about 0.2 M) were prepared by dissolving weighed amounts of the materials in the appropriate amount of 0.2 M NaOH. The pH of these solutions was adjusted to 7.0. The exact concentrations were determined on aliquots by amino acid analysis after acid hydrolysis.

Methods—Enzymatic assays by hydroxylamine incorporation were carried out as outlined previously (14) in 0.1 M Tris-acetate buffer containing 30 mM Z-L-glutaminylglycine, 1 mM EDTA, 5 mM CaCl$_2$, and 0.1 M hydroxylamine, at pH 6.0 and 37°. Enzyme assays were carried out in 0.1 M Tris-chloride buffer containing 0.5 mM p-nitrophenyl acetate, 50 μM EDTA, 10 mM CaCl$_2$ and 2% 2-propanol, at pH 7.0 and 25°. Rates of liberation of p-nitrophenol were measured at 400 nm within the first 20 to 40 s of hydrolysis.

Sulphhydril groups were estimated in sodium acetate/acetic acid buffer containing 0.5 mM 4,4′-dithiodipyridine (Altrich) at pH 4.0 by the general procedure of Grassetti and Murray (18). For non-isomers, this was derived from the single peak and characteristic retention time observed for each of the isocyanates.

*p-Nitrophenyl acetate was prepared by distillation prior to use. Stock solutions of peptide derivatives (about 0.2 M) were prepared by dissolving weighed amounts of the materials in the appropriate amount of 0.2 M NaOH. The pH of these solutions was adjusted to 7.0. The exact concentrations were determined on aliquots by amino acid analysis after acid hydrolysis.

Spectrophotometric measurements were made on a Cary model 118

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Inactivation of Transglutaminase by Methyl Isocyanate

Treatment of the enzyme with increasing levels of methyl isocyanate in the presence of Ca\(^{2+}\) at pH 5.7. It is evident from these data that loss in enzymatic activity is a stoichiometric function of the amount of isocyanate used and that total inactivation occurs as a result of addition of 1 mol of reagent/mol of enzyme. The assays of Fig. 1 were performed 2 min after addition of reagent. Longer incubation with reagent (up to 30 min) caused no further loss in activity. The very rapid nature of the inactivation reaction was evidenced from the fact that, at 1 eq of reagent to enzyme, total loss in activity, as measured by the continuous spectral esterase method, was observed within the time necessary to commence the assay (20 to 30 s).

Essentially, the same stoichiometry and rapid rate of inactivation with methyl isocyanate were observed at pH 7.0. At pH 7.5 and above, higher levels of reagent were required for inactivation. This is probably due to the rapid loss of reagent by hydrolysis, the rate of which may become comparable to, or exceed, that of enzyme inactivation. Brown and Wold (10) have reported a half-life of about 1 min for n-butyl isocyanate at pH 7.7 and 0°. We have observed a half-life of approximately 30 min for methyl isocyanate at pH 5.7 and 0° under the buffer conditions of Fig. 1. This half-life was estimated from the loss in degree of inactivation of enzyme added during the course of incubation.

Treatment of the enzyme with 1 eq of methyl isocyanate in the absence of Ca\(^{2+}\) at pH 5.7 resulted in a 25% reduction in esterase activity and approximately a 45% loss in hydroxylamine-incorporating activity. Complete loss in both enzymatic activities in the absence of Ca\(^{2+}\) was observed only after addition of 6 to 8 eq of reagents.

Under the conditions of Fig. 1, the transglutaminase substrate, Z-L-glutaminylglycine showed a pronounced protection against inactivation. At the level of 1 eq of isocyanate, addition of 20 and 40 mM substrate reduced the per cent inactivation to 78 and 52, respectively.

Specific Nature of Isocyanate Inactivation—In addition to methyl isocyanate, several other aliphatic isocyanates were found to inactivate transglutaminase (see later section, “Inactivation by Various Aliphatic Isocyanates”). The report of Twu and Wold (11) that the inactivation of yeast alcohol dehydrogenase by n-butyl isocyanate is a consequence of thio carbamate ester formation through essential —SH groups of the enzyme suggests that isocyanate inactivation of transglutaminase may also occur by way of a thio carbamyl reaction. If so, however, this must involve only a single essential enzyme —SH group. This contention is based on the present observation that transglutaminase is fully inactivated by treatment with one equivalent of methyl isocyanate (Fig. 1). It is given support by the earlier findings that alkylation, e.g. by iodoacetamide (14, 19), or trimethylacetylation (20) of a single essential —SH group in the enzyme leads to complete inactivation. Two samples of transglutaminase that had been fully inactivated by treatment with equimolar amounts of methyl isocyanate as outlined in Fig. 1 were examined for total —SH content by the use of the spectrophotometric dithio dipyridine procedure. Values of 17.0 and 17.4 —SH groups/molecule were found. Comparison with values of 18.3 and 18.7 —SH groups/molecule, obtained by the same procedure for samples of the untreated enzyme, supplies strong evidence that inactivation by methyl isocyanate results from reaction of this reagent with an —SH group in the enzyme.

Reaction of isocyanates with sulfhydryl compounds occurs by nucleophilic addition to the C—N double bond (Reaction 1). The thio carbamyl enzymes that would be formed by reaction of isocyanates with the single essential —SH group

\[
R\cdot SH + R'\cdot N=C=O \rightarrow R'\cdot N-C-S-R
\]  

(1)

of transglutaminase bear structural resemblance to the thioester acyl enzyme intermediates believed to occur during the course of normal catalysis (see introduction). It seems possible that inactive thio carbamoyl enzyme derivatives could be formed by nucleophilic displacement reactions with mono substituted urea analogs or urethan analogs of transglutaminase substrates according to Reactions 2 and 3, respectively.

\[
R\cdot SH + R'\cdot N-C-NH_2 \rightarrow R'\cdot N-C-S-R + NH_3
\]  

(2)

\[
R\cdot SH + R'\cdot N-C-O-Ar \rightarrow R'\cdot N-C-S-R + Ar-OH
\]  

(3)

The very rapid rate and stoichiometry of enzyme inactivation by methyl isocyanate and the long half-life of this reagent in water at pH 5.7 precludes N,N'-dimethyl urea as an enzyme inactivating agent. This disubstituted urea could arise via Reactions 4a and 4b.

\[
CH_3N=C=O + HOH \rightarrow CH_3NH_2 + CO_2
\]  

(4a)

\[
CH_3N=C=O + CH_3NH_2 \rightarrow CH_3\cdot NH\cdot CN\cdot NH\cdot CH_3
\]  

(4b)
N-Methylurea at levels between 1 and 10,000 mol/mol of enzyme was found to cause no enzyme inactivation at pH 5.7 or 7.0 (buffer conditions of Fig. 1). This urea is the structural analog of n-propionamide, a substrate for transglutaminase (2). Because the affinity of the enzyme for aliphatic amides is low and since that for the urea may be even lower, it was judged worthwhile to test the urea analog of the glutamine substrate, Z-L-glutaminylglycine. This analog, Z-L-albizzinylglycine did not inactivate the enzyme nor did it function as a substrate. That the albizzin derivative was bound to the active site of the enzyme is evident from the finding that it showed linear competitive inhibition against Z-L-glutaminylglycine (Fig. 2). The inhibitor constant, \( K_{in} \), estimated from the data of Fig. 2, was \( 67 \pm 5 \) mM, a value similar to the apparent inhibition of transglutaminase by methyl isocyanate (Reactions 5 and 4a). The present observations are consistent with an inactivation of transglutaminase by methyl isocyanate, generated by breakdown of the urethan.

\[
\text{CH}_3\text{NHCO} + \text{NO}_2 \rightarrow \text{CH}_3\text{N} = \text{C} = \text{O} + \text{H}_2\text{O} + \text{NO}_2.
\]

On the basis of kinetic studies, Christenson (21) suggested that alkaline hydrolysis of N,N-dialkyl urethanes proceeds by a different mechanism (Reaction 6) than that shown above for the N-monoalkyl urethan (Reaction 5).

\[
\text{R}_2\text{N} = \text{CO} + \text{OH}^+ \rightarrow \text{R}_2\text{N} = \text{C} = \text{O} + \text{R}^+ \text{OH}.
\]

In this case, the absence of the proton on the nitrogen atom precludes the unusual elimination reaction. It has been pointed out (16) that this dichotomy holds only for aryl esters. The hydrolysis of all aliphatic carbamate esters apparently follows Reaction 6.

![Figure 2](http://www.jbc.org/) Inhibition of transglutaminase by Z-L-albizzinylglycine with Z-L-glutaminylglycine as the varied substrate (A) at 0.1 M hydroxylamine. Conditions were as outlined under "Methods," except with varied substrate and at pH 7.0 and 30°. The inhibitor concentrations were 0, 15, and 30 mM for Lines a, b, and c, respectively. Initial velocities are given in micromoles of hydroxamate formed per min (per pmole of enzyme). The constants given in the text were obtained by fits to the equation, \( v = \frac{VA}{Kl + (1/K_{in}) + A} \), using methods described earlier (2).
incubation for 18 hours at 25°, the solution was gel filtered as outlined above and portions of the protein peak were analyzed for protein concentration, radioactivity, and enzymatic activity; the salt fraction was tested for radioactivity. The protein was found to contain only 30% of the initial radioactivity (0.29 mol of 14C-labeled reagent/mol); the remainder of the radioactivity was accounted for in the salt fraction. The enzymatic activity was partially restored, showing 35% of that of the unmodified enzyme.

Peptide maps were prepared as outlined previously (22) from enzymatic digests of a sample of the 14C-methyl isocyanate-labeled enzyme and from a sample of enzyme that was fully inactivated by the incorporation of 1 eq of 14C-labeled iodoacetamide (14, 19). Digestion of these labeled enzyme samples was carried out as outlined earlier (19), except that approximately 1:1 molar ratios of digestive enzymes to labeled substrates were used and digestion times were limited to 1 hour for trypsin followed by 2 hours for chymotrypsin. The shorter digestion times were employed in order to minimize losses in radioactivity from the isocyanate-labeled enzyme. Autoradiograms of the peptide maps prepared from each of the labeled enzyme samples showed two areas of radioactivity in positions identical to those observed previously for 18- to 20-hour trypsin-chymotrypsin digests of the iodoacetamide-inactivated enzyme (Rf values of 0.50 and 0.95 in 1-butanol, acetic acid, water and in the area of the neutral amino acids in pH 3.5 electrophoresis) (23). Each of the areas in both peptide maps gave positive reactions for tryptophan residues by the use of p-dimethylaminobenzaldehyde in HCl. An additional radioactivity from the isocyanate-labeled enzyme. Autoradiograms of the peptide maps prepared from each of the labeled enzyme samples showed two areas of radioactivity in positions identical to those observed previously for 18- to 20-hour trypsin-chymotrypsin digests of the iodoacetamide-inactivated enzyme (Rf values of 0.50 and 0.95 in 1-butanol, acetic acid, water and in the area of the neutral amino acids in pH 3.5 electrophoresis) (23). Each of the areas in both peptide maps gave positive reactions for tryptophan residues by the use of p-dimethylaminobenzaldehyde in HCl. An additional radioactivity was observed on the peptide map from the isocyanate-inactivated enzyme. This area showed no reaction for trypsinophan. These two peptides from iodoacetamide-inactivated enzyme have been identified as Gly-Gln-Cys(S-carbamidomethyl)-Trp and Tyr-Gly-Gln-Cys(S-carbamidomethyl)-Trp (19). It is likely that two of these found in the digest of iodoacetate inactivated enzyme are the N-methylcarbamoyl counterparts. The carbamidomethyl and methylcarbamoyl group are structurally similar. One would expect S-carbamidomethyl and N-methylcarbamoyl peptides of the same amino acid sequence to occupy the same or very similar positions on peptide maps. The additional radioactive material found with the isocyanate inactivated enzyme was not identified. However, it occupied a position on the peptide map very different from that of methylvamine which would be expected as a product of alkaline hydrolysis of the enzyme methylthiocarbamate group.

**Inactivation by Various Aliphatic Isocyanates**—It is apparent from experiments reported above that the rate of inactivation of transglutaminase by methyl isocyanate is too rapid to measure by the use of conventional sampling techniques and available enzyme assays. Each of the alkyl isocyanates tested, with the exception of isopropyl isocyanate, also was found to inactivate the enzyme very rapidly. Therefore, no attempt was made to determine rate constants for inactivation. Under the conditions of Fig. 1 and at the level of 1 mol/mol of enzyme, ethyl isocyanate, propyl isocyanate, butyl isocyanate, isobutyl isocyanate, pentyl isocyanate, and isopentyl isocyanate each reduced the level of catalytic activity to less than 25% of the initial value within 3 min after addition. Isopropyl isocyanate, on the other hand, effected less than 10% inactivation after 10 min.

The reaction of several of the isocyanates with the —SH group of reduced glutathione was examined. The results are given in Fig. 3. Here the —SH and isocyanate concentrations are 40-fold higher than those in the enzyme inactivation mixtures. The slow rates of reaction of the isocyanates with the glutathione —SH group compared to the rapid inactivation of enzyme by all except isopropyl isocyanate are in agreement with active site-directed enzyme inactivation. Although the rate of reaction of isopropyl isocyanate with the glutathione —SH group is only about one-half to two-thirds of the other isocyanates, this does not account for its very much lower degree of enzyme inactivation.

**DISCUSSION**

The findings reported here supply evidence that alkyl isocyanates inactivate guinea pig liver transglutaminase as a consequence of N-alkyl thiocarbamoyl ester formation with the single active site sulfhydryl group of the enzyme. The evidence for this active-site-specific reaction is briefly as follows. (a) Treatment of the enzyme in the presence of Ca++ with 1 eq of methyl isocyanate results in rapid total loss in catalytic activity and an accompanying loss in one —SH group in the enzyme. With p-nitrophenyl [14C]methylcarbamate as a source of labeled methyl isocyanate, the incorporation of 1 mol of reagent/mol of enzyme was observed. (b) The substrate, Z-lysylglycine, effectively protects against methyl isocyanate inactivation. (c) As in the case of other reagents that inactivate transglutaminase by reaction with its active site —SH group, e.g. iodoacetamide (14, 19) and α-bromo-4-hydroxy-3-nitroacetophenone (24), isocyanate inactivation is less specific in the absence of the catalytically essential metal ion, Ca++. (d) The loss of label and partial recovery of enzymatic activity that occurs upon alkaline treatment of the 14C-labeled inactive enzyme is characteristic only of the N-alkylcarbamoylcyesteine derivative (10). Butylcarbamoyl derivatives of lysine, tryosine, and serine have been shown to be completely stable to incubation at pH 10.5 and 37°.4 (e) The

*S. C. Q. Chin, unpublished observation cited in Ref. 10.*
locations on a peptide map of two radioactive peptides from the digest of labeled methylthiocarbamoyl enzyme were found to correspond in position to those from a digest of the labeled iodoacetamide-inactivated enzyme. Two peptides were found in each case, probably as a result of incomplete tryptic digestion (19). The \(-\text{SH}\) group of this cysteine residue has been identified as the one that is acylated during enzymatic hydrolysis of the active ester substrate, \(p\)-nitrophenyl trimethylacetate (20).

Of all of the straight and branched chain isocyanates tested, only isopropyl isocyanate failed to inactivate transglutaminase effectively. Comparison of the structures and inhibitor properties of the aliphatic isocyanates with the structures and substrate properties of the aliphatic amides (7) and \(Z\)-methylglutaminylglycine isomers (8) offers an explanation for this finding. The spatial dimensions of the glutamine side chain binding pocket of transglutaminase (see introduction) precludes the catalytically productive binding of methyl branched-chain amides and methyl glutamine peptide derivatives in which the methyl branch is in a position \(\alpha\) or \(\beta\) to the carboxamide group. The structural analogy of isopropyl isocyanate (Structure I) to \(\beta\)-methylbutyramide (Structure II) and the \(\beta\)-methylglutamine residue (Structure III) is apparent. A methyl group in the \(\gamma\) position to the carboxamide in the branched chain amides, e.g., \(\gamma\)-methylvaleramide (Structure IV), and in the \(L\) isomer of the \(\alpha\)-methylglutamine residues (Structure V) does not prevent productive binding. In these cases, the methyl group may be situated outside of the confines of the glutamine side chain binding pocket. The effective inactivation of transglutaminase by isobutyl isocyanate (Structure VI) may be explained on the basis of its structural similarity to these substrates. Thus, we conclude that inactivation by alkyl isocyanates is active site-specific by virtue of a unique noncovalent complex formed between enzyme and reagent, that this complex is similar to that formed between enzyme and amide substrates, and that its formation brings the active site sulfhydryl group and the isocyanate group into proximity for facilitated reaction.

Because \(\beta\)-methylbutyramide exhibits competitive inhibition against a glutamine substrate for transglutaminase, it has been suggested that this amide binds at the glutamine side chain site of the enzyme (7). The failure of enzyme to utilize the \(\alpha\) - and \(\beta\)-branched amides as substrates may reflect prevention or distortion of a substrate-induced conformational alteration in the active center that is essential for enzyme acylation. That this is the more likely case than that these branched chain amides block the entrance to a binding pocket of stable dimensions derives from the fact that certain amides with branched \(N\)-substitutions, e.g. \(Z\)-\(\alpha\)-l-glutamyl(\(\gamma\)-l-alanine ethyl ester)glycine (3), serve as substrates and that products with branched \(N\)-substitutions readily dissociate from the enzyme (3). The fact that isopropyl isocyanate does not rapidly inactivate transglutaminase, whereas straight chain isocyanates do, suggests that a conformational change in the enzyme is also essential for isocyanate inactivation and that this involves proper positioning of the active site sulfhydryl group and the isocyanate group for facilitated reaction.

\(p\)-Nitrophenyl \(N\)-methylcarbamate, which is the urethan analog of the transglutaminase substrate, \(p\)-nitrophenyl propionate, is not an inactivator for the enzyme. It was concluded that the inactivation observed in the presence of this urethan was caused by methyl isocyanate formed as a result of spontaneous breakdown of the compound (Reaction 5). This conclusion is supported by the finding that neither inactivation of transglutaminase nor release of \(p\)-nitrophenol occurred with \(p\)-nitrophenyl \(N\)-\(N\)-dimethylcarbamate. This compound, the urethan analog of the transglutaminase substrate, \(p\)-nitrophenyl isobutrate, is not spontaneously hydrolyzed to an isocyanate (Reaction 6). The enzyme was also not inactivated by \(N\)-methylurea or by \(Z\)-\(L\)-albizninylglycine, the monosubstituted urea analog of the glutamine substrate, \(Z\)-\(L\)-glutaminylglycine. It follows, therefore, that formation of inactive alkylthiocarbamoyl transglutaminases occurs only by way of nucleophilic addition of enzyme active site \(-\text{SH}\) to the isocyanate double bond (Reaction 1) and not through nucleophilic displacement with active ester or urea analogs of substrates (Reactions 3 and 2, respectively).

In contrast to this finding, several serine active site enzymes, notably acetylcholine esterases (25), trypsin (26), and chymotrypsin (26) form inactive carbamoyl derivatives through nucleophilic displacement with urethan analogs of certain of their substrates. Whether cysteine active site enzymes, other than guinea pig liver transglutaminase, can form thiocarbamoyl derivatives by this route or whether formation of these derivatives by \(-\text{SH}\) active site enzymes is limited to the addition reaction with isocyanates is not known at present.

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