INACTIVATION OF CHYMOTRYPSIN BY DIPHENYLDIAZOMETHANE*

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α-Chymotrypsin is a protein composed of three peptide chains linked by disulfide bridges, with amino-terminal half-cystine, isoleucine, and alanine residues.\(^1\) The latter two end groups appear upon the enzymic conversion of chymotrypsinogen A (which only contains the amino-terminal half-cystine residue) to α-chymotrypsin. The specificity of α-chymotrypsin as an enzyme (and of other chymotrypsins derived from chymotrypsinogens A and B) is preferentially directed to the cleavage of substrates in which the CO group of sensitive amide or ester bonds is donated by an aromatic L-amino acid residue (tryptophan, phenylalanine, tyrosine); lower catalytic efficiency is exhibited toward bonds in which the CO group is donated by other L-amino acids bearing apolar side chains (methionine, leucine, etc.).\(^2\)

The available evidence strongly supports the view that the mechanism of chymotrypsin action on suitable amide and ester substrates [e.g., acetyl-L-tyrosinamide, acetyl-L-tyrosine ethyl ester (ATEE)]\(^3\) involves the formation of an acyl enzyme\(^4\) (e.g., acetyltyrosyl-chymotrypsin), and that the site of acylation is the β-hydroxyl group of a serine residue (no. 195 in the amino acid sequence of chymotrypsinogen A).\(^5\) Studies on the pH dependence of the catalytic action of chymotrypsin,\(^6\) and on the action of inhibitors such as tosyl-L-phenylalanyl chloromethane\(^7\) indicate the participation of a histidine residue (histidine 57), with the possible involvement of the other histidine residue (histidine 40), in the acylation and deacylation of the reactive serine hydroxyl group. These two histidine residues are held near each other by a disulfide bridge linking half-cystine residues 42 and 58.\(^8\)

The ready acylation of the reactive serine by p-nitrophenyl acetate\(^9\) or by cinnamoyl imidazole,\(^10\) and the relative stability of the acyl enzymes so formed, have permitted studies leading to notable progress in the description of the mechanism of chymotryptic catalysis.\(^11\) These studies have also brought out the striking difference in the rates of deacylation of acyl enzymes derived from reaction with a specific substrate (e.g., ATEE) and from reaction with an acylating agent such as p-nitrophenyl acetate; the replacement of the acetyl group of acetyl-chymotrypsin by an acetyl-L-tyrosyl group causes an enhancement of about 12,000-fold in the rate of deacylation. This difference can be interpreted most readily as evidence of the effect of specific binding of the acetyltyrosyl group at a “binding site” located in the vicinity of the “catalytic site” of the enzyme.

Although considerable information is available about the amino acid residues that participate in the action at the catalytic site (serine 195, histidine 57, possibly histidine 40), there is less knowledge about the nature of the amino acid side chains that are involved in the formation and maintenance of the specific binding site of chymotrypsin. Studies on the effect of oxidation\(^12\) or alkylation\(^13\) of α-chymotrypsin have indicated that chemical modification of one or both of the two methionine residues (180 and 192) alters the binding site to a greater extent than the catalytic site. Furthermore, evidence has been presented\(^14\) in favor of the view that the
α-amino group (in its protonated form) of the amino-terminal isoleucine residue of α-chymotrypsin is important in the maintenance of the conformation of the protein and in its enzymic activity.

In the present study, an effort was made to examine the contribution of carboxyl groups in α-chymotrypsin to the catalytic activity of the enzyme. Evidence suggesting the participation of carboxyl groups in the maintenance of enzymic activity has come from the examination of the pH dependence of the optical rotation of the protein,15 and from kinetic studies.16 Furthermore, esterification of chymotrypsinogen A by means of diazoacetamide gave preparations which, upon activation with trypsin, were converted to partially inactivated chymotrypsin.17

Previous work in this laboratory has indicated that diazo compounds such as diphenyldiazomethane (DDM) may be valuable reagents for the specific esterification of carboxyl groups in catalytic proteins.18 DDM offers several advantages for studies of this type, in spite of its sparing solubility in water. Because of the considerable delocalization of the negative charge on the α-carbon of DDM (the initial site of protonation in esterification reactions19,20), greater selectivity may be expected than with diazokanes such as diazoacetamide. In the special case of chymotrypsin, which exhibits a preference for aromatic groups in its substrates, DDM may be expected to interact at the substrate-binding site and to effect esterification in the vicinity of that site; in this respect, DDM is formally analogous to diphenylcarbamyl chloride which has been used as a stoichiometric inhibitor of chymotrypsin.20

Materials and Methods.—α-Chymotrypsin (3X crystallized, lot CD1-6140-1) and trypsin (2X crystallized, lot TRL 6259) were obtained from the Worthington Biochemical Corporation, and chymotrypsinogen A (6 X crystallized, lot 136B) was obtained from Seravac Laboratories. Unlabeled DDM and C14-labeled DDM were prepared in the manner described previously.18 The specific radioactivity of the labeled DDM was found to be 12,480 cpm/μmole; for this determination, the benzophenone hydrazone from which the DDM was derived was converted to the corresponding azine, whose radioactivity was measured. The use of the azine in place of the hydrazone18 is recommended because of the slight volatility of the latter (Dr. G. R. Delpierre, personal communication). Acetyl-L-tyrosine ethyl ester41 (mp 78–80°; [α]D = +22.7 (c2 in methanol) and trans-cinnamoyl imidazole38 (mp 133–134°) were prepared in our laboratory. Recrystallized samples of p-nitrophenyl acetate (mp 78.5–79°) and of indole (mp 52–53°) were used. Dioxane was purified by heating under reflux for 48 hr, in the presence of sodium, followed by distillation; it was stored in the frozen state in a dark bottle.42

The enzymic activity toward acetyl-L-tyrosine ethyl ester (ATEEase activity) was determined at 25° titrimetrically, using a radiometer TTT1 and titrigraph SBR2c assembly. The assay mixture contained, in addition to the enzyme: 0.0072 M substrate, 0.1 M NaCl, and 0.0045 M phosphate buffer (pH 7.8). The titrant was 0.2 N NaOH, and only initial rates were recorded. One unit of ATEEase activity is defined as the amount of enzyme that causes the liberation of 1 μmole of H+ per minute under the above conditions. By assuming a molecular weight of 25,000, and by determining protein concentration spectrophotometrically at 280 μm (an absorbancy index of 0.50 was used), the specific ATEEase activity of the enzyme preparation employed in this work was calculated to be 5700 units/μmole.

For the stoichiometric determination of the catalytic sites in preparations of modified chymotrypsin, the "all-or-none" assay of Schonbaum et al.21 (method A, 335 μm) was used, with trans-cinnamoyl imidazole as the reagent. The unmodified α-chymotryptsin preparation employed in these studies gave, in replicate determinations, an average value of 0.88 catalytic sites per molecule of enzyme (assumed molecular weight, 25,000). In enzyme assays in which p-nitrophenyl acetate was used as the acylating agent, its concentration was 7.2 × 10−4 M, and the reaction was conducted at pH 7.4 (0.05 M phosphate) and at 25°. The liberation of p-nitrophenol was followed spectrophotometrically at 400 μm, and both the initial burst and the steady-state rates were
measured. The spectrophotometric measurements were performed with a Beckman DU spectrophotometer attached to a Gilford model 2000 multiple sample absorbance recorder.

In the experiments on the effect of DDM treatment on the ATEEase activity of chymotrypsin, the enzyme (ca. 10 mg) was dissolved in 5 ml of 0.001 M formic acid (pH 3.2), dioxane (ca. 4 ml) was added, and at zero time the requisite DDM in dioxane was introduced and the volume was adjusted to 10 ml with the formic acid solution. The final concentration of dioxane was 45% by volume. A control solution (no DDM) was prepared in a similar manner, and both flasks were kept at 25°C. At intervals, 1-ml samples were withdrawn and diluted to 10 ml with 0.001 N HCl, and aliquots of the diluted mixture were assayed for ATEEase activity in the manner described above. Because of the known effect of dioxane in lowering the specific activity of chymotrypsin, it should be noted that at least 90% of the original ATEEase activity was regenerated upon dilution with acid of the solution of chymotrypsin in 45% dioxane.

For the activation of chymotrypsinogen, the conditions described by Eisenberg and Schwert for the formation of z-chymotrypsin were used with slight modification. The chymotrypsin derived from the zymogen preparation used in this work gave a value of 0.78 catalytic sites per molecule of enzyme, as determined from the initial burst in the cleavage of p-nitrophenyl acetate.

For the preparation of samples of partially active enzyme, after treatment of z-chymotrypsin with labeled DDM, the reaction mixtures (prepared as described above) were kept at 25°C for 4 hr; prior experiments had shown that this time period was sufficient for the completion of the reaction. The reaction mixture was dialyzed for 30 hr against 5 liters of 0.001 N HCl and filtered through Schleicher and Schuell analytic filter paper no. 507, and the clear filtrate was lyophilized. The product was dissolved in 4 ml of 0.001 N HCl, and the solution was filtered and placed on a Sephadex G-10 column (30 × 1.5 cm) previously equilibrated with 0.001 N HCl, which was also used for elution. The fractions containing the protein were pooled and lyophilized. Spectrophotometric examination of solutions of this product in 0.001 M formic acid (pH 3.2) indicated a recovery of about 65% of the protein initially used in the reaction with DDM. The unmodified enzyme, on being subjected to this procedure, lost about 5% of its original specific activity toward ATEE.

In the above procedure, dialysis removes the dioxane, the filtration removes the solid by-products (chiefly benzhydryl chloride and benzhydrol), and the gel filtration removes the benzhydryl formate. The gel filtration may also be expected to remove material reported to be present in some commercial preparations of z-chymotrypsin, and to inhibit binding of substrates and inhibitors. To determine the efficiency of the above procedure in the removal of radioactive contaminates, a dioxane solution (10 ml) containing DDM-C14 (3.4 μmoles) and formic acid was kept at 25°C for 4 hr, after which time chymotrypsin (10.7 mg) was added, and the mixture was subjected to the treatment described in the preceding paragraph. The radioactivity of the resulting protein was not significantly above the background level (ca. 14 cpm). In the determination of the radioactivity of labeled, modified chymotrypsin, samples of a given protein preparation were plated in triplicate, and each planchet was counted at least five times using a Nuclear-Chicago gas-flow windowless counter (model 186-A).

In the experiments with chymotrypsinogen, the DDM-treated zymogen was prepared in the same manner as described above for chymotrypsin. Upon adjustment of a dioxane-treated solution of the zymogen to pH 7.4, for activation by trypsin, it was found that a significant portion (ca. 40%) of the protein separated from the solution. Similar amounts of insoluble protein were produced after exposure to dioxane in the presence and absence of DDM. The extent of isotope incorporation was determined both for the total protein and the fraction soluble at pH 7.4; for the determination of the loss of potential chymotryptic activity, only the soluble fraction of the modified zymogen was used.

Results.—The data in Table 1 show that chymotrypsin is partially inactivated with respect to its ATEEase activity by DDM at pH 3.2 and 25°C, with 45 per cent dioxane as the solvent. The choice of these reaction conditions was dictated by the following considerations. (1) To achieve a homogeneous reaction mixture, in view of the sparing solubility of DDM in water, the addition of an organic solvent was necessary. Separate experiments showed that the dioxane concentration used was insufficient to cause significant irreversible denaturation of the en-
TABLE 1
INHIBITION OF ATEEASE ACTIVITY OF CHYMOTRYPSIN BY DDM-C14

<table>
<thead>
<tr>
<th>Reaction Components</th>
<th>ATEEase Activity (units/mole CT)</th>
<th>Loss of ATEEase Activity (%)</th>
<th>Specific radioactivity (cpm/mg CT)</th>
<th>Gm atom C14/mole CT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT (mM) DDM (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.044</td>
<td>0</td>
<td>5500</td>
<td>34</td>
<td>175</td>
</tr>
<tr>
<td>0.042</td>
<td>0.17</td>
<td>3625</td>
<td>55</td>
<td>175</td>
</tr>
<tr>
<td>0.042</td>
<td>0.33</td>
<td>3150</td>
<td>55</td>
<td>426</td>
</tr>
<tr>
<td>0.044</td>
<td>0.44</td>
<td>2480</td>
<td>88</td>
<td>34</td>
</tr>
<tr>
<td>0.048</td>
<td>0.95</td>
<td>2200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0425</td>
<td>3.40</td>
<td>660</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The enzyme (CT) was treated with DDM-C14 for 4 hr at 25° (pH 3.2, 45% dioxane), and the modified protein was isolated as described in Materials and Methods. This isolation procedure was also applied to the enzyme in the control experiment (no DDM).

TABLE 2
STOICHIOMETRY OF REACTION OF CHYMOTRYPSIN WITH DDM-C14

<table>
<thead>
<tr>
<th>Reaction components</th>
<th>Specific radioactivity (cpm/mg CT)</th>
<th>Gm atom C14/mole CT</th>
<th>C14 sites/mole CT</th>
<th>Loss of catalytic sites (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td></td>
<td></td>
<td></td>
<td>0.87</td>
</tr>
<tr>
<td>CT + DDM (0.17)</td>
<td>193</td>
<td>0.39</td>
<td>0.80</td>
<td>8</td>
</tr>
<tr>
<td>CT + DDM (0.17) + indole (170)</td>
<td>256</td>
<td>0.52</td>
<td>0.34</td>
<td>60</td>
</tr>
<tr>
<td>CT + DDM (0.34)</td>
<td>402</td>
<td>0.81</td>
<td>0.49</td>
<td>43</td>
</tr>
<tr>
<td>CT + DDM (0.34) + indole (170)</td>
<td>480</td>
<td>0.96</td>
<td>0.30</td>
<td>66</td>
</tr>
</tbody>
</table>

The concentration of chymotrypsin (CT) in the reaction mixture was 0.0425 mM in all cases; the concentration (mM) of the other components is given in parentheses. The reaction components were kept for 4 hr at 25° (pH 3.2, 45% dioxane). The enzyme in the control experiment (no DDM) was processed in the same manner as the samples treated with DDM-C14 (for details, see Materials and Methods). The catalytic sites were titrated with cinnamoyl imidazole.

zyme under the conditions of these studies. (2) The pH was chosen as a compromise between the effect of increasing the concentration of H3O+ on the destruction of DDM and the failure of the reagent to cause rapid inactivation of chymotrypsin at pH values higher than 4.

As will be seen from the data in Table 1, the partial inactivation of chymotrypsin by DDM was accompanied by the incorporation of radioactivity into the protein, when DDM-C14 was used. At a relatively low DDM/enzyme ratio (4:1) in the reaction mixture, the extent of incorporation (0.35 gm atom C14/mole CT) paralleled the extent of inactivation (34%). At higher DDM/enzyme ratios (8:1 or 10:1) the extent of incorporation was approximately twice the extent of the fractional loss of ATEEase activity. Such partial inactivation could be a consequence of the impairment, in some of the enzyme molecules, of (1) their catalytic sites, (2) their binding sites, or (3) both their catalytic and binding sites. To determine the extent to which the catalytic sites had been inactivated by treatment with DDM, the "all-or-none" assay of chymotrypsin, with cinnamoyl imidazole as the reagent,22 was applied. As will be seen from Table 2, under conditions (DDM/enzyme = 4:1) that led to parallel inactivation of ATEEase activity and incorporation of C14, there was negligible loss of reactivity toward cinnamoyl imidazole. On the other hand, at a higher DDM/enzyme ratio (8:1), there was a significant loss of catalytic sites titrated by means of cinnamoyl imidazole. From determinations of the initial rates of inactivation of chymotrypsin (ATEEase activity) at various initial concentrations of DDM (0.17–3.4 mM), a value of Km (app.) of ca. 1 mM was estimated for DDM. The magnitude of this value is similar to those found for specific substrates of chymotrypsin (e.g., acetyl-L-tyrosinamide) whose Km (app.) approximates their Kt value.11
These findings suggest that DDM, in its action on \( \alpha \)-chymotrypsin, initially attacks a carboxyl group that is in the vicinity of the binding site for ATEE, and that the resulting monobenzhydryl-chymotrypsin retains its capacity to be acetylated by cinnamoyl imidazole. From this point of view, the apolar groups of DDM may be considered to interact with the substrate-binding site of the enzyme, and the esterification of a nearby carboxyl may be thought to fix these groups irreversibly near the binding site, thus inhibiting the enzymic hydrolysis of specific substrates such as ATEE. The data further suggest that this modification of the enzyme favors the reaction of DDM with a second carboxyl group that is essential for the maintenance of the integrity of the catalytic site.

To test this hypothesis, an experiment was conducted in which DDM was allowed to act on chymotrypsin (ratio of DDM/CT = 4:1) in the presence of indole, an effective competitive inhibitor of the enzyme.\(^\text{28}\) A large excess of indole was used because of the probability that the \( K_i \) for this inhibitor is greater in 45 per cent dioxane than in water.\(^\text{29}\) Indole does not react with DDM under the conditions of these experiments. It will be seen in Table 2 that, in the presence of 170 mM indole, the inactivation of chymotrypsin by DDM was greatly promoted, with the loss of about 60 per cent of the catalytic sites for acylation by cinnamoyl imidazole. An examination of the activity of the resulting modified chymotrypsin toward ATEE showed that it had retained 34 per cent of this activity. The maximal ATEEEase activity to be expected, in view of the result with cinnamoyl imidazole, was 40 per cent of that of unmodified chymotrypsin, indicating that a large proportion of the residual enzyme active toward cinnamoyl imidazole was also active toward ATEE. This finding suggests that the competitive inhibitor had blocked the attack of DDM at the carboxyl group preferentially esterified in the absence of indole.

It will be noted from Table 2 that the extent of isotope incorporation in the presence of 0.17 mM DDM and 170 mM indole (0.52 gm atom C\(^{14}\)/mole CT) paralleled the extent of inactivation toward cinnamoyl imidazole (60%). This result may be taken to indicate that each inactive chymotrypsin molecule contained a single benzhydryl group, and is in agreement with the conclusion drawn above that the presence of indole blocked the access of DDM to the carboxyl group preferentially esterified in the absence of the competitive inhibitor.

When chymotrypsinogen A was treated with DDM-C\(^{14}\) at a molar ratio of reagent to zymogen of 8:1, the extent of isotope incorporation was found to be 0.85 gm atom C\(^{14}\)/mole protein, a value similar to that found previously for chymotrypsin (Table 1). Because of the partial denaturation of the zymogen by dioxane, only the soluble fraction (ca. 60%) of the DDM-treated protein could be tested for its potential enzymic activity. This fraction contained 0.65 gm atom C\(^{14}\)/mole protein. For the determination of the catalytic sites in the chymotrypsin formed upon tryptic activation of the modified zymogen, p-nitrophenyl acetate was used in place of cinnamoyl imidazole. It was found that the values for the extent of the initial release of p-nitrophenol and for the rate of the succeeding steady-state reaction were not significantly different from the corresponding values for the enzyme derived from the unmodified zymogen. On the other hand, the chymotrypsin derived from the DDM-treated zymogen was found to exhibit only 40 per cent of the ATEEEase activity observed with the enzyme derived from unmodified chymotrypsinogen.
Discussion.—The results presented in this communication give added support to the view that a carboxyl group plays a significant role in the catalytic activity of \( \alpha \)-chymotrypsin. An attractive possibility is the ion pairing of the COO\(^-\) form of this group with the protonated \( \alpha \)-amino group of the amino-terminal isoleucine residue. According to this hypothesis, the activation of chymotrypsinogen A, with the initial tryptic cleavage of the bond linking arginine 15 and isoleucine 16, leads to the stabilization of the catalytic site of \( \alpha \)-chymotrypsin by the formation of the ion pair postulated above. It is noteworthy that the cleavage of chymotrypsinogen A by chymotrypsin, with the scission of the bond between leucine 13 and serine 14, gives rise to an inactive neochymotrypsinogen. Other sites of cleavage of the zymogen by chymotrypsin have been found, but the available evidence strongly indicates that the tryptic release of the amino group of isoleucine 16 is essential for the appearance of catalytic activity.\(^{29}\)

Experiments designed to establish the nature of the amino acid residue bearing the crucial carboxyl group, and its position in the linear sequence of chymotrypsinogen A, are in progress. Previous studies with model compounds have shown that DDM reacts most rapidly with the \( \beta \)-carboxyl group of the aspartyl residue and the \( \gamma \)-carboxyl group of the glutamyl residue, the former having a somewhat greater reactivity.\(^{18}\) As the rate of esterification by DDM of a series of carboxylic acids increases with increased acidity, it might be expected that the sensitive carboxyl group in \( \alpha \)-chymotrypsin belongs to an aspartyl residue; this expectation must be tempered, however, by the likelihood that the the pK\(^\prime\) values of individual side-chain carboxyl groups of the protein may be different depending on the microenvironment in which they are located in the native enzyme. Also, steric factors may be expected to play a large role.

The action of DDM in reducing the apparent catalytic activity of \( \alpha \)-chymotrypsin toward ATEE, without significant loss of the sites titrated with cinnamoyl imidazole or \( p \)-nitrophenyl acetate, is similar in principle to the effect of reagents that modify the methionine residues of the enzyme.\(^{12,13,20}\) The most evident kinetic effect of such modification was found to be an increase in the values of \( K_m \) (app.) for the hydrolysis of specific substrates, but no significant loss of catalytic sites titratable with cinnamoyl imidazole was noted. Clearly, the much lower \( K_m \) (app.) values for cinnamoyl imidazole and \( p \)-nitrophenyl acetate must be taken into account in the interpretation of the results of such studies, including our own. In particular, it must be noted that the data presented in this communication on the titration of catalytic sites refer to the extent of reaction with cinnamoyl imidazole or \( p \)-nitrophenyl acetate, and not the rates of these reactions.

It appears reasonable to interpret the data on the inactivation of chymotrypsin by DDM as indicating the initial attack of the reagent on a carboxyl group that is near the binding site, but not essential for the maintenance of the catalytic activity of the enzyme. The irreversible attachment of the benzhydryl group may be considered to cause a conformational change in the protein so as to make an essential carboxyl group more readily accessible to attack by the reagent. This view is supported by the result of the experiment in the presence of indole, where the preferential attack is at a carboxyl group necessary for catalytic activity. This finding is analogous to earlier observations such as the enhanced rate of inactivation of trypsin by iodoacetamide (through alkylation of a histidine residue) in the presence
of methyl guanidine. The effect of competitive inhibitors such as indole (in the case of chymotrypsin) or n-butyl ammonium ion (in the case of trypsin) on the rate of hydrolysis of substrates lacking a specific amino acid residue (e.g., L-tyrosyl or L-lysyl) has also been explained in terms of a conformational change in consequence of binding of the inhibitor at the specific site of the enzyme.

The finding that treatment of chymotrypsinogen with DDM at a molar ratio of reagent to zymogen of 8:1 did not cause a significant loss of potential catalytic sites suggests that the conformational change postulated above in the case of chymotrypsin, does not occur so readily in the zymogen. As with chymotrypsin, however, the initial attack of DDM appears to be at a carboxyl group near the potential substrate-binding site. It has been shown that chymotrypsinogen A binds chymotrypsin substrates (e.g., acetyl-L-tryptophanamide), although more weakly than does chymotrypsin itself.

Summary.—Data are presented in favor of the conclusion that diphenyldiazomethane inactivates α-chymotrypsin with initial attack at a carboxyl group near the substrate-binding site of the enzyme, followed by substitution of a second carboxyl group essential for the maintenance of the catalytic activity.

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3 The following abbreviations are used: acetyl-L-tyrosine ethyl ester, ATEE; diphenyldiazomethane, DDM; chymotrypsin, CT.
18 Delpierre, G. R., and J. S. Fruton, these PROCEEDINGS, 54, 1161 (1965).
34 Fairclough, G. F., and J. S. Fruton, unpublished experiments.