THE INHIBITION OF TRYSIN

IV. REACTION WITH DIETHYL p-NITROPHENYL PHOSPHATE IN THE PRESENCE OF UREA*

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The inhibition of proteolytic enzymes by organophosphorus compounds has been extensively studied in recent years (1, 2). Such studies have shown that the reaction of trypsin with diisopropyl fluorophosphate (3) or diethyl p-nitrophenyl phosphate (DENP) (4) is accompanied by a loss in the proteolytic, esterase, and amidase activities of this enzyme. It is not certain, however, whether these inhibitors combine at the site responsible for the catalytic function of trypsin (4, 5).

In a previous paper in this series (6), it was shown that, although trypsin was inactivated by concentrations of urea ranging from 3 to 5 M, its enzymatic activity was retained in 6 to 8 M urea solution. Since most proteins are known to undergo rapid denaturation in high concentrations of urea, a more detailed examination has been made of the properties of trypsin in 8 M urea. During the course of such studies it was noted that, unlike native trypsin, the proteolytic activity of urea-treated trypsin was not inhibited by DENP. This observation prompted the present investigation, the object of which was to determine whether DENP reacts with trypsin at a site which differs from the site responsible for the catalytic function of the enzyme.

EXPERIMENTAL

Materials

The trypsin employed was a twice crystallized product containing 50 per cent MgSO₄ purchased from the Worthington Biochemical Corporation, Freehold, New Jersey. The enzyme was rendered free of salt by dialysis against 0.001 N HCl, followed by lyophilization. A fresh stock solution of trypsin was prepared prior to each experiment, and the concentration of trypsin protein (mg. per ml.) was determined spectrophotometrically at

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280 m\(\mu\) by a conversion factor of 0.586 (7). The desired concentration of trypsin was obtained by suitable dilution of the stock solution.

DENP was kindly furnished by Dr. William Summerson of the Chemical Corps Medical Laboratories, Army Chemical Center, Maryland. A stock solution of \(3 \times 10^{-2}\text{ M}\), prepared in isopropanol and stored at 4°, was diluted to the desired concentration with isopropanol just prior to its use.

Diethyl phosphoryl trypsin (DEPT) was prepared by treating 90 mg of trypsin in 5 ml of 0.05 M borate buffer, pH 7.6, containing 0.01 M CaCl\(_2\), with 2 ml of the stock DENP solution for 48 hours at room temperature. After removal of the unchanged DENP by dialysis, proteolytic activity was measured on casein as described below. Treatment with successive 2 ml portions of stock DENP solution was continued in this fashion until there was no further loss in activity. Three such treatments yielded a preparation which had from 0.5 to 1.0 per cent of the activity of a control enzyme solution which lacked DENP. DEPT was also prepared in the presence of urea by the same procedure with borate buffer containing 8.3 M urea. In this instance, however, precipitation of the protein occurred upon dialysis, which made it impossible to measure its activity. Three successive treatments with DENP were therefore assumed to yield completely changed DEPT. Both preparations of DEPT were lyophilized and stored in a dry state.

**Methods**

Proteolytic activity was determined by an adaptation of the method of Kunitz (8), either 2 per cent casein or 2 per cent denatured hemoglobin dissolved in 0.05 M borate buffer, pH 7.6, being used. Hemoglobin was denatured by using a buffer solution containing 6.7 M urea (9). To the enzyme solution in a volume of 1 ml was added 1 ml of the borate buffer, followed by the addition of 3 ml of substrate solution. After a digestion period of 10 minutes at 37°, the reaction was stopped with 5 ml of 5 per cent trichloroacetic acid, and the absorbance of the filtrate was read at 280 m\(\mu\).

Esterase activity was measured by the spectrophotometric method of Schwert and Takenaka (10) with \(\alpha-N\)-benzoyl-L-arginine methyl ester as the substrate.

The effect of DENP on the proteolytic and esterase activities of trypsin in the presence or absence of urea was studied as follows. To 600 \(\gamma\) of trypsin in 4 ml of 0.05 M borate buffer, pH 7.6, containing 8.3 M urea was added 1 ml of 6 \(\times 10^{-3}\text{ M}\) DENP in isopropanol. A similar system from which urea had been omitted contained 0.02 M CaCl\(_2\) to minimize self-digestion of the enzyme (11–13). 1 ml of isopropanol replaced the DENP solution in controls set up to measure losses in activity in the absence of
DENP. Enzyme-free controls were also necessary to correct for the spontaneous hydrolysis of DENP during the course of the reaction. The reaction was allowed to proceed at 25°, during which time the liberation of p-nitrophenol (PNP) was measured according to the method of Hartley and Kilby (14), and the proteolytic (casein substrate) and esterase activities on 0.1 ml. aliquots were determined as described above. The reaction was assumed to be complete when no further increase in PNP could be detected (usually after 48 hours, although final readings were recorded at the end of 72 hours). The maximal PNP released under these conditions in the presence or absence of urea was found to be 0.90 and 0.92 mole per mole of trypsin, respectively, assuming the latter to have a molecular weight of 20,000 (15). This corresponds rather closely to the theoretical value of 1.0 based on a stoichiometric reaction between DEPT and trypsin in a molar ratio of 1:1 (4).

Results

Reaction of DENP with Trypsin in Presence or Absence of Urea

The liberation of PNP and the changes in proteolytic activity of trypsin treated with DENP in the presence and absence of urea are recorded in Fig. 1. In the absence of urea, the rate at which PNP was liberated closely paralleled the loss in proteolytic activity, an observation similar to that reported earlier by Kilby and Youatt (4). In the presence of urea, a simi-
lar rate of release of PNP was obtained, but of particular interest is the fact that the proteolytic activity of the enzyme was essentially unaffected by DENP. The loss in activity that did occur could be accounted for by the loss in activity which was apparent in the control to which no DENP had been added. Although not included in Fig. 1, the changes in esterase activity were quite similar to those presented for the proteolytic activity of the enzyme.

To rule out the possibility that trypsin was merely catalyzing the hydrolysis of DENP, samples of DEPT prepared in the presence and absence of urea were analyzed for total phosphorus by the method of Sumner (16). DEPT prepared in buffer alone and in buffered urea solution was found to contain 0.150 and 0.129 per cent total P, respectively (moisture-free basis). The calculated P content of DEPT is 0.155 per cent. The release of PNP must therefore reflect a chemical reaction between trypsin and DENP both in the presence and in the absence of urea.

<table>
<thead>
<tr>
<th>Urea concentration</th>
<th>Per cent reactivation†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No CaCl₂</td>
</tr>
<tr>
<td>no. of moles/l.</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10.0</td>
</tr>
<tr>
<td>2</td>
<td>8.0</td>
</tr>
<tr>
<td>4</td>
<td>4.0</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Based on the extinction coefficient of DEPT at 280 mλ (E₁% cm⁻¹ = 15.4), a value which is about 10 per cent less than that reported for trypsin (17).
† Per cent of the activity of an equivalent level of trypsin as measured in the absence of urea or CaCl₂.

Reactivation of DEPT

The observation that trypsin in the presence of urea could react with a stoichiometric amount of DENP without losing its enzymatic activity suggested the possibility that urea might be capable of restoring the activity of DEPT which had been prepared in the absence of urea.

DEPT was dissolved in various concentrations of urea with and without...
CaCl₂ and its activity subsequently measured on urea-denatured hemoglobin. As shown in Table I, the greatest degree of reactivation is obtained when DEPT is dissolved in urea-free solution containing Ca ions. Increasing the concentration of urea caused a progressive decrease in the restoration of activity that was obtained. These data were contrary to what had been anticipated, and will be further considered in the discussion of these results.

![Graph showing the release of PNP and the loss in proteolytic activity accompanying the reaction of DENP with urea-treated trypsin in the presence and absence of cysteine. The ordinate on the left is the PNP released as a percentage of the theoretical maximum (broken curves). The ordinate on the right is the loss in proteolytic activity (on casein substrate) as a percentage of the initial activity (solid curves). ○, with cysteine; ●, without cysteine.]

**Fig. 2.** The release of PNP and the loss in proteolytic activity accompanying the reaction of DENP with urea-treated trypsin in the presence and absence of cysteine. Each system contained 600 γ of trypsin in 4 ml. of 0.05 M borate buffer, pH 7.6, containing 8.3 M urea and 0.01 M CaCl₂, in the presence or absence of 0.005 M L-cysteine. After adding 1 ml. of 6 × 10⁻³ M DENP in isopropanol, the release of PNP and proteolytic activity were determined as described under “Methods.” The ordinate on the left is the PNP released as a percentage of the theoretical maximum (broken curves). The ordinate on the right is the loss in proteolytic activity (on casein substrate) as a percentage of the initial activity (solid curves). ○, with cysteine; ●, without cysteine.

*Influence of Cysteine*

Although low concentrations of thiol compounds have been reported to have little, if any, effect on trypsin activity (18, 19), urea-treated trypsin was found to be rapidly inactivated by 0.005 M cysteine. As shown in Fig. 2, not only was urea-treated trypsin completely devoid of proteolytic activity in the presence of 0.005 M cysteine, but its ability to react with DENP was likewise destroyed. The reaction of urea-treated trypsin in the absence of cysteine is presented for comparative purposes. In this instance it is again evident that much of the proteolytic activity of the enzyme has been retained in spite of its ability to react with DENP, as indicated by the release of nearly the theoretical amount of PNP.
Evidence for Denaturation of Trypsin by Urea

The enhanced susceptibility of urea-treated trypsin to cysteine inactivation suggested the unmasking of disulfide linkages which often accompanies denaturation of proteins (20). Further evidence, relating to the possibility that urea-treated trypsin possesses the characteristics of denatured protein, was therefore sought.

Optical Rotation Studies—Advantage was taken of the fact that the optical rotation of many proteins is known to increase upon denaturation (21, 22). The optical rotation of trypsin exposed to 8.3 M urea was compared with untreated trypsin and with trypsin which had been denatured by heat. The data presented in Table II demonstrate that trypsin dissolved in urea remained fully active, yet had an optical rotation almost 3 times as great as trypsin prepared in buffer alone. The rotation of heat-denatured trypsin was only slightly greater than that of the urea-treated trypsin.

Table II
Optical Rotation of Urea-Treated Trypsin

In each system was 1 per cent trypsin dissolved in 0.05 M borate buffer, pH 7.6, containing 0.02 M CaCl₂. The buffer also contained 8.3 M urea in the case of the urea-treated trypsin. Proteolytic activity was measured on casein substrate as described in the text.

<table>
<thead>
<tr>
<th>Time</th>
<th>Trypsin in buffer</th>
<th>Trypsin in urea</th>
<th>Heat-treated trypsin†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[a]D</td>
<td>Loss in activity†</td>
<td>[a]D</td>
</tr>
<tr>
<td></td>
<td>degrees</td>
<td>per cent</td>
<td>degrees</td>
</tr>
<tr>
<td>30 min</td>
<td>-45.4</td>
<td>0</td>
<td>-124.8</td>
</tr>
<tr>
<td>18 hrs</td>
<td>-47.0</td>
<td>3.0</td>
<td>-124.8</td>
</tr>
</tbody>
</table>

* All solutions were allowed to stand at room temperature (25.5°) for the time indicated.
† Autoclaved at 15 pounds pressure for 15 minutes. The precipitated material was redissolved in buffer solution containing urea.
‡ Compared to the initial activity at zero time.

Digestion by Tetrahymena Proteinase—From concurrent studies in this laboratory (23, 24), a proteinase, isolated from Tetrahymena pyriformis W, was available which was capable of attacking denatured proteins but unable to split native proteins. The susceptibility of urea-treated trypsin to attack by a crystalline preparation of this proteinase (24) was therefore studied. As can be seen from Table III, native trypsin was not attacked by the proteinase, and the slight loss in activity which ensued was the same in the presence or absence of the proteinase. The urea-treated trypsin,
on the other hand, was readily digested under the same conditions as those revealed by the absorption of the split products at 280 m\(\mu\). Unfortunately, the presence of cysteine which is required to activate the *Tetrahymena* proteinase (24) caused a complete loss in the activity of trypsin, even in the urea system lacking the proteinase. This made it impossible to assess the effect of digestion *per se* on the activity of trypsin. It is interesting to note that the activity of trypsin in the absence of urea was not affected by the amount of cysteine used to activate the proteinase.

**Table III**

*Digestion of Trypsin in Presence or Absence of Urea by Crystalline *Tetrahymena* Proteinase*

<table>
<thead>
<tr>
<th>System</th>
<th>Trypsin in buffer</th>
<th>Trypsin in 8.3 M urea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Loss in activity*</td>
<td>Absorbance at 280 m(\mu)</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Without proteinase</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>With proteinase</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

* Compared to the activity of systems in which the enzyme-cysteine solution was replaced by borate buffer.

**DISCUSSION**

The loss in enzymatic activity which accompanies the reaction of native trypsin with DENP would suggest that the site at which DENP reacts is normally so close to the catalytic site that the introduction of the diethyl phosphoryl moiety interferes with the hydrolytic function of the enzyme. That the site of reaction with DENP is not identical with the catalytic site, however, is strongly suggested by the observation that, in the presence of urea, the enzyme is capable of reacting with DENP without losing its ability to split peptide and ester bonds. The effect of urea may be visualized as an unfolding of the trypsin molecule due to the rupture of hydrogen bonds as postulated for other proteins (25). In so doing, these two sites may become spatially dissociated so that each becomes free to participate in its own peculiar function; *i.e.*, the liberation of PNP from DENP and the hydrolysis of peptide and ester linkages.
A further consequence of the structural change induced by urea appears to be the unmasking of disulfide linkages which are known to be present in trypsin (19). In the native molecule these disulfide linkages are not readily reduced (19), but, in the presence of urea, cysteine causes complete inactivation of trypsin with respect to its enzymatic activity as well as its reactivity towards DENP. It is suggested that the disulfide linkages provide restricting cross-linkages which protect the molecule from further unfolding in the presence of urea. Rupture of these linkages by reduction with cysteine may lead to a loss in activity in much the same manner as the inactivation of insulin by thiol compounds (26). Thus far, attempts to reactivate cysteine-inactivated trypsin have not proved successful; indeed, it is quite possible that the derangement of the molecule caused by the rupture of disulfide linkages may be an irreversible process (25).

Additional evidence that urea-treated trypsin represents an unfolded, expanded molecule characteristic of denatured protein is provided by (a) the marked increase in levorotation and (b) the enhanced susceptibility to digestion by the Tetrahymena proteinase which is known to attack denatured but not native proteins. While this work was in progress, Anfinsen et al. (27) reported that the enzymatic activity of ribonuclease was also retained under conditions in which the molecule exhibited the characteristics of denatured protein.

Although DEPT was essentially inactive when measured on casein substrate, 10 to 20 per cent of the original activity was obtained when urea-denatured hemoglobin was employed as the substrate. It seems likely that the urea contained in the hemoglobin substrate solution is responsible for this difference between the two substrates, the urea acting to unmask the catalytic site of the enzyme in accordance with the views expressed above. On the other hand, exposing DEPT to increasing concentrations of urea up to 8 M prior to the addition of the substrate reduced the extent of reactivation observed upon the addition of the urea-denatured hemoglobin. It may be that, in the absence of substrate, the unfolding of the DEPT molecule caused by urea has irreversibly exceeded the point required for catalytic activity. The presence of substrate may restrict the complete unfolding of the molecule so that its catalytic function is at least partially restored. This stabilizing effect of the substrate appears to be enhanced by Ca ions. The protective influence of substrate and Ca ions on the inactivation of trypsin by lower concentrations of urea, 3 M to 5 M, has already been pointed out (6).

**SUMMARY**

In the absence of urea, the reaction of diethyl p-nitrophenyl phosphate (DENP) with trypsin, as measured by the liberation of p-nitrophenol, was
accompanied by corresponding losses in proteolytic and esterase activities. In the presence of 8.3 M urea, the reaction between DENP and trypsin proceeded without concomitant losses in enzymatic activity.

Although ineffective against trypsin in the absence of urea, cysteine added to trypsin in the presence of urea completely destroyed its enzymatic activity as well as its reactivity towards DENP.

Although enzymatically inert towards casein, diethyl phosphoryl trypsin (DEPT) displayed 10 to 20 per cent of its original activity when urea-denatured hemoglobin was provided as the substrate. Exposure of DEPT to urea prior to the addition of the latter substrate tended to reduce the amount of reactivation that could be obtained.

An increased optical rotation and an enhanced susceptibility to digestion by the *Tetrahymena* proteinase support the view that urea-treated trypsin is in a denatured state.

**Addendum**—Subsequent to the acceptance of this paper for publication, several reports (28, 29) have appeared which indicate that trypsin treated with 8 M urea is actually enzymatically inactive and denatured but rapidly regains its activity when assayed by dilution under the conditions described under “Methods.” The activity measurements recorded here reflect, therefore, the ability of the denatured enzyme to revert to its native, active form.

**BIBLIOGRAPHY**

THE INHIBITION OF TRYPSIN: IV. REACTION WITH DIETHYL p-NITROPHENYL PHOSPHATE IN THE PRESENCE OF UREA
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