The Application of Insolubilized α-Chymotrypsin to Kinetic Studies on the Effect of Aprotic Dipolar Organic Solvents*

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SUMMARY

Insolubilized α-chymotrypsin bound to porous glass gel has been prepared. The kinetic behavior of this fixed enzyme proved to be the same as that of the free enzyme. The effects of aprotic dipolar organic solvents on the kinetics of α-chymotrypsin-catalyzed hydrolysis were successfully studied using this insolubilized enzyme at concentrations up to 95% v/v dioxane, taking advantage of the inability of the enzyme to aggregate. This is a considerably higher organic solvent concentration than has been achieved in homogeneous solution.

The kinetic parameters of the insolubilized α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan p-nitrophenyl ester and N-benzoyl-L-tyrosine p-nitroanilide in aqueous organic solvent medium were determined. The specific rate constant of the acylation step, $k_a$, does not depend on organic solvent concentration. However, the apparent Michaelis constant, $K_m$, and deacylation rate constant, $k_d$, depend strongly on the organic solvent concentration.

The data are discussed in terms of microscopic reversibility proposed previously for the mechanism of α-chymotrypsin action.

It is of interest to investigate the effect of organic solvents on the kinetics of proteolytic enzymes. Many studies have been published on the effects of such solvents on α chymotrypsin catalyzed hydrolysis (1-8). It may be concluded generally that the value of $k_{cat}$ remains constant, whereas the value of $K_m$ increases with increasing solvent concentration (1, 5, 8). But these experiments were carried out in a small range of organic solvent concentration, except those with trypsin (9). α-Chymotrypsin is known to precipitate at a high concentration of organic solvent (~30% dioxane).

It is well established that enzyme derivatives covalently bound to a water-insoluble matrix can serve as easily recoverable reagents of considerably improved stability. The general significance and applicability of insolubilized enzymes have been reviewed (10-12).

It may be considered that the insolubilized enzymes should also be successfully applied to the investigation of the effect of dipolar aprotic solvents on enzyme kinetics. Even at high concentrations of organic solvent in which unbound enzyme undergoes precipitation preceded by aggregation, insolubilized enzymes may be studied without interference of the solvent. The reason for this is probably that enzyme molecules fixed covalently on the matrix surface may be prevented from interaction with each other.

A porous glass (13) was chosen as the support for the following reasons. (a) It is ionically neutral. (b) It can be used in either organic or aqueous solvent systems without affecting the size and shape of the support matrix. It is reported that the ionic properties of the support molecule influence the kinetics of the fixed enzyme (10). (c) Also, it is known that in the case of hydrophilic gels, like Sephadex, the shrinking and swelling caused by aqueous organic solvents changes the activity of the attached enzyme (14).

Chromogenic substrates were used for the reaction in aqueous organic solvent media because titrimetric measurements were not useful.

EXPERIMENTAL PROCEDURE

Materials—α-Chymotrypsin (CDI IIA), three times crystallized and lyophilized, was a product of Worthington. The porous glass support with primary amine groups (Biomaterial Supports, GAO-3940, 0.17 meq per g) was obtained from Corning Biological Products Group. N-Acetyl-L-tryptophan p-nitrophenyl ester (Lot H-4250) and N-acetyl-L-tryptophan methyl ester (Lot H-4224) were obtained from Cycle Chemical Co. and used without further purification. N-Benzoyl-L-tyrosine p-nitroanilide (Lot 120B-5340) was obtained from Sigma Chemical Co. Deuterium oxide, 99.8% D2O, was purchased from Stohler Isotope Chemicals. The pD of the deuterium oxide solutions was measured with a glass electrode and corrected by the addition of 0.40 pD unit. Dioxane (spectral grade) was obtained from International Chemical and Nuclear Corp. and used without further distillation.

Insoluble α-chymotrypsin was prepared by coupling α-chymotrypsin with diazotized porous glass which was converted from the commercial porous glass gel by p-nitrobenzoylation, reduction, and diazotization according to the usual procedure (15). In order to destroy any remaining diazonium groups, the coupled gel was treated with an excess of p-methoxyphenol solution.

Methods—The rates of steady state hydrolysis of N-acetyl-L-tryptophan methyl ester catalyzed by free α-chymotrypsin and insolubilized α-chymotrypsin were determined using a Radiometer Automatic Titrator II at 25° under a nitrogen stream (16) in aqueous solution only.

The hydrolytic rates of N-acetyl-L-tryptophan p-nitrophenyl ester and N-benzoyl-L-tyrosine p-nitroanilide in aqueous organic solvent were determined spectrophotometrically by using a Gilford spectrophotometer at 340 nm and 405 nm, respectively (17, 18).
The reactions were carried out in a thermostated reaction vessel at 25° with stirring. The optical density change of this reaction medium during a certain period was recorded as a function of substrate or dioxane concentration. It was confirmed that the stirring speed used in these reactions was sufficient enough, that is, the velocities of these heterogeneous reactions were independent of the stirring speed used. The reaction rate was corrected for spontaneous hydrolysis and also corrected by using the corresponding $\Delta$ in each aqueous dioxane system.

RESULTS

Characterization of $\alpha$-Chymotrypsin Attached to Porous Glass—The amount of enzyme coupled with the gel was determined to be 0.106%, w/w, using a rate assay and a molecular weight of 24,800 for $\alpha$-chymotrypsin (19). The activity was measured at pH 8.0, 25°, using N-acetyl-l-tryptophan methyl ester as a substrate. It is reported that the diazoo coupling procedure leads to insoluble $\alpha$-chymotrypsin or trypsin of low activity (20) presumably because $\alpha$-chymotrypsin and trypsin are partially inactivated by low molecular weight diazonium salts (21); but this activity is not too low for the present purpose.

Solvent isotope effects of free and bound $\alpha$-chymotrypsin are summarized in Table I. The values $K_m$ and $k_{cat}$ in each aqueous dioxane medium were obtained using a Lineweaver-Burk plot. In the case of amide (anilide) substrates, the rate-limiting step is acylation. The observed $k_{cat}$ values essentially equal the acylation rate constant, $k_a$ (23, 24). For experimental reasons the initial substrate concentration used in these experiments increased with increasing dioxane concentration.

The Lineweaver-Burk plots of hydrolytic rates were normal. The $K_m$ increased with increasing dioxane content, whereas the value of $k_{cat}$ remained constant (Table II). The kinetics was carried out at the concentration of the substrate as high as possible because Axen et al. (25) reported that at low substrate concentration the reaction kinetics of the insolubilized enzyme deviate from ordinary Michaelis-Menten kinetics. The value of $K_m$ in 10% aqueous dioxane, $2.0 \times 10^{-6}$ M, is considerably smaller than that of free $\alpha$-chymotrypsin in 9.7% acetone solution, $3.4 \times 10^{-4}$ M, reported by Bundy (26).

In Table II the virtual independence of the acylation rate constant and the small dependence of the Michaelis constant on the presence of dioxane in the medium is seen. Although the accuracy of the data, especially at high dioxane concentration, is not completely satisfactory (see Table II) it is obvious that the value of $K_m$ depends somewhat on dioxane concentration whereas $k_{cat}$ is independent.

Effects of Dioxane on Insoluble $\alpha$-Chymotrypsin-catalyzed Hydrolysis of N-Acetyl-l-tryptophan p-Nitrophenyl Ester—The

![Graph](https://example.com/graph.png)

**Fig. 1.** pH activity curve of $\alpha$-chymotrypsin and porous glass $\alpha$-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl ester at 25°, 0.01 M Tris, 0.1 M KCl, 0.05 M CaCl$_2$, 3% acetonitrile. [S]$_1$ = $5 \times 10^{-4}$ M, $\alpha$-chymotrypsin concentration = $2.45 \times 10^{-4}$ M, porous glass-attached $\alpha$-chymotrypsin concentration = 8 mg/4 ml. ○, $\alpha$-chymotrypsin; ●, porous glass $\alpha$-chymotrypsin.

**Table I**

<table>
<thead>
<tr>
<th>Water Concentration %</th>
<th>$K_m$</th>
<th>$k_{cat}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>(1.1 ± 0.1) x $10^{-4}$</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>90</td>
<td>(2.0 ± 0.3) x $10^{-4}$</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td>85</td>
<td>(5.0 ± 0.8) x $10^{-4}$</td>
<td>0.33 ± 0.07</td>
</tr>
<tr>
<td>80</td>
<td>(5.0 ± 1.0) x $10^{-4}$</td>
<td>0.28 ± 0.06</td>
</tr>
<tr>
<td>75</td>
<td>(7.7 ± 2.0) x $10^{-4}$</td>
<td>0.28 ± 0.10</td>
</tr>
<tr>
<td>70</td>
<td>(5.0 ± 0.6) x $10^{-4}$</td>
<td>0.25 ± 0.15</td>
</tr>
<tr>
<td>60</td>
<td>(9.1 ± 5.0) x $10^{-4}$</td>
<td>0.28 ± 0.20</td>
</tr>
<tr>
<td>50</td>
<td>(2.0 ± 1.5) x $10^{-3}$</td>
<td>0.28 ± 0.25</td>
</tr>
<tr>
<td>40</td>
<td>(2.5 ± 2.0) x $10^{-3}$</td>
<td>0.40 ± 0.35</td>
</tr>
<tr>
<td>30</td>
<td>(3.0 ± 2.5) x $10^{-3}$</td>
<td>0.40 ± 0.35</td>
</tr>
<tr>
<td>20</td>
<td>$10^{-2}$ ~ $10^{-3}$</td>
<td>0.40 ± 0.35</td>
</tr>
<tr>
<td>10</td>
<td>$10^{-3}$ ~ $10^{-4}$</td>
<td>0.40 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>$10^{-4}$ ~ $10^{-5}$</td>
<td>0.4 ± 0.4</td>
</tr>
</tbody>
</table>

* The errors are twice the standard deviation.

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*Substrate concentration = $5 \times 10^{-2}$ M; $\alpha$-chymotrypsin concentration = $2.45 \times 10^{-4}$ M; porous glass $\alpha$-chymotrypsin concentration: 8 mg/4 ml.
The effects of dioxane concentration on the porus glass α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan p-nitrophenyl ester in 0.05 M acetate, pH 5.8 buffer-dioxane mixtures at 25°.

<table>
<thead>
<tr>
<th>Water concentration</th>
<th>$V \times 10^7$</th>
<th>$k_3 \times 10$</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>[S] = 1.04 $\times 10^{-4}$ M</td>
<td>[S] = 3.12 $\times 10^{-2}$ M</td>
</tr>
<tr>
<td>100</td>
<td>6.7 ± 0.4*</td>
<td>26.5 ± 1.6*</td>
</tr>
<tr>
<td>95</td>
<td>6.7 ± 0.4</td>
<td>26.2 ± 1.6</td>
</tr>
<tr>
<td>90</td>
<td>6.7 ± 0.4</td>
<td>26.2 ± 1.6</td>
</tr>
<tr>
<td>85</td>
<td>5.6 ± 0.5</td>
<td>22.0 ± 2.0</td>
</tr>
<tr>
<td>80</td>
<td>5.1 ± 0.5</td>
<td>20.2 ± 2.0</td>
</tr>
<tr>
<td>70</td>
<td>2.6 ± 0.4</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>60</td>
<td>0.9 ± 0.1</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>50</td>
<td>0.7 ± 0.2</td>
<td>2.7 ± 0.7</td>
</tr>
<tr>
<td>40</td>
<td>0.12 ± 0.03</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>30</td>
<td>0.08 ± 0.03</td>
<td>3.1 ± 1.2</td>
</tr>
<tr>
<td>20</td>
<td>0.02 ± 0.01</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>15</td>
<td>0.01 ± 0.01</td>
<td>0.04 ± 0.04</td>
</tr>
<tr>
<td>10</td>
<td>0.03 ± 0.03</td>
<td>0.12 ± 0.12</td>
</tr>
</tbody>
</table>

* The errors are twice the standard deviation.

Kinetic data obtained with the ester substrate, N-acetyl-L-tryptophan p-nitrophenyl ester, are shown in Table III and Fig. 2. The observed rate constant is equal to the deacylation rate constant $k_3$ in the case of esters of specific substrates (23). It is shown that the insoluble α-chymotrypsin-catalyzed hydrolysis is independent of the substrate concentration used in this reaction. Therefore, the observed dioxane concentration dependency of the hydrolytic rate is not due to the change of $K_m$ but rather due to the change of $k_3$ itself. The apparent $K_m$ values of such substrates as p-nitrophenyl esters of specific substrates are quite small and it is reasonable that the value does not exceed $10^{-4}$ or $10^{-3}$ M, even in 75% dioxane solution.

The stability and conformation of insolubilized α-chymotrypsin in organic solvents should be considered. The activity of the enzyme toward N-benzoyl-L-tyrosine p-nitroanilide and N-acetyl-L-tryptophan p-nitrophenyl ester did not change even in 75% dioxane, conditions which were much more severe than those used in the kinetic measurements. The rate constants of insolubilized chymotrypsin toward the anilide and nitrophenyl ester substrates in dioxane are not different from those of native (soluble) chymotrypsin. Furthermore, there is very little effect of dioxane on deacylation kinetics. These experiments strongly suggest the stability and conformation of insolubilized chymotrypsin is not different from that of the native enzyme.

**DISCUSSION**

The different effects of aprotic organic solvents on the individual enzymatic reaction steps, such as formation of the Michaelis complex, acylation, and deacylation, is the most important information coming from this study. Previously reported results (1, 5, 27) can be explained by our finding that $K_m$ and $k_3$ are dependent on organic solvent content, but $k_3$ is independent.

The report that $K_m$ increased 2.5-fold, while $k_{cat}$ remained constant in the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tyrosine amide from water to 5.15 M methanol (1), is consistent with our observations, because the $k_{cat}$ of an amide substrate is equal to $k_3$. Also, in the case of N-acetyl-L-tryptophan methyl ester the increase of $K_m$ and small decrease of $k_3$ (8) in 14% acetonitrile does not contradict our findings. If this experiment was carried out in higher concentration of acetonitrile, $k_3$ would be found to change strongly. Applewhite et al. (5) reported the $k_{cat}$ value of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate does not change in aqueous dioxane. This may indicate that in the presence of dioxane the rate-determining step of such a specific substrate is acylation, not deacylation, in spite of its being an ester substrate (27).

Dioxane changes the pH as measured by the glass electrode. The apparent pH change of acetate buffer by addition of dioxane is larger than that of Tris buffer as shown in Tables II and III. It is a difficult problem to obtain a satisfactory pH scale in organic solvent-water mixtures. Marshall and Grunwald have noted that the glass electrode responds to changes in hydrogen ion activity in the same way in aqueous dioxane as in water (23). This indicates that the pH reading in aqueous dioxane has the same meaning as the measurement in water. The change in pH of Tris buffer in aqueous dioxane is small enough to neglect its effect on the rate constant, but the change for acetate buffer is not. The curve in Fig. 2 (see Table III) as a result would be expected to decrease even more steeply because the pH rate profile of $k_3$ ascends with increasing pH (23).

Finally, the variations of $K_m$, $k_3$, and $k_3$ with dioxane content should be considered. Although the rate constants of acylation and deacylation were obtained at different pH values there is no doubt that the mechanism does not differ with pH (29-31). High concentrations of dioxane have little effect on the dissociation of monoeionic acids and appear to have only a small effect on the pH dependence of chymotrypsin attached to porous glass. The large decrease in the steady state hydrolysis rate of N-acetyl-L-tryptophan p-nitrophenyl ester cannot result...
from decreased ionization of the active site histidine as in contrast to the results presented in Table II, such a decrease should also be accompanied by a comparable decrease in activity toward the amide substrate N-benzoyl-L-tyrosine-p-nitroanilide. The mechanism of the α-chymotrypsin-catalyzed hydrolysis reaction was proposed to involve base catalysis (32) based in part on deuterium isotope effects on acylation and deacylation steps. This mechanism is based on the concept of microscopic reversibility (31), but the dependence on water concentration in deacylation is for the first time explicitly demanded by the present experiments (Scheme 1).

Assuming that the proportions of the base and acid forms of the active site imidazole at a given pH do not change with increasing concentrations of dioxane, the acylation rate would be expected to be independent of water concentration. The effect of solvent polarity cannot be completely evaluated. Such effects are expected to be relatively minor, however, as previous results indicate acylation and deacylation reactions proceed via neutral transition states (32).

The effect of dioxane concentration on the deacylation rate constant can be explained by Scheme 1. The deacylation rate should be dependent on the water concentration. The components of the transition state of deacylation must include the acylserine ester, imidazole, and a molecule of water. A water molecule and imidazole are involved in the rate-determining proton transfer reaction. The first order dependence on the nucleophile water is expected by analogy to the kinetics of methanolysis reported previously (33). As shown in Fig. 2, it is hard to show that the reaction is linear in water concentration since the reaction is also dependent on secondary effects of the aqueous organic solvent such as that on the structure of the solvent. Taking into account microscopic reversibility in respect to acylation and deacylation, the assumption that the solvent polarity affects only the deacylation step can be ruled out.

Dioxane as employed does not cause irreversible denaturation of the porous glass-bound enzyme but the question can arise whether this organic solvent causes reversible denaturation, or a conformational change of the enzyme, or both. This also appears unlikely for, again in contrast to the experimental findings, such effects should be reflected by changes in both acylation and deacylation rates. The actual decrease in the availability of water with increasing dioxane appears to best account for both the small effect on acylation and the large effect on deacylation.

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