Reactivity and Catalysis in Reactions of the Serine Hydroxyl Group and of O-Acyl Serines*

BRUCE M. ANDERSON, EUGENE H. CORDES, AND WILLIAM P. JENCKS

From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts

(Received for publication, July 1, 1960)

Certain acyl transfer and hydrolytic reactions catalyzed by chymotrypsin and related enzymes proceed with the formation of an isolable enzyme-substrate intermediate, in which the acyl portion of the substrate is covalently bound to the enzyme. A considerable body of evidence now exists which suggests that the site of enzyme acylation, as well as phosphorylation, is the hydroxyl group of a serine residue in the active site. The acylation and deacylation of this serine residue may be aided by the catalytic effect of the imidazole group of a histidine residue, which is held in a suitable position for catalysis by the tertiary structure of the protein, but is not adjacent to the serine in the peptide chain (1-9).

It has recently been shown that imidazole can catalyze certain acyl transfer reactions without the formation of acetylimidazole, by removing a proton from the attacking reagent in the transition state, i.e. by classical general base catalysis (10), and it has been suggested that imidazole may act in a similar manner in the enzymic acylation and deacylation of the serine hydroxyl group, i.e. by classical general base catalysis (10, 11, 12). The experiments reported here were carried out to obtain information on the nucleophilic properties of the enzyme-catalyzed reactions. Preliminary reports of portions of this work have been published (10, 14).

EXPERIMENTAL PROCEDURE

Materials—Acetylimidazole was prepared by the method of Boyer (15), stored over CaSO4 at -15°C, and dissolved in water immediately before use. p-Nitrophenyl acetate (m.p. 78-79°C) was prepared according to Chattaway (16), recrystallized from ethanol, and stored over CaSO4. Aqueous solutions were made up to 10-3 M concentration by prolonged stirring at room temperature and were stable for several days at 3°C. N-Acetylserinamide was prepared from serine methyl ester, according to Rothstein (17).

N-O-Diacetylserinamide was prepared from N-acetylserinamide by pyridine-catalyzed acetylation with acetic anhydride. Acetic anhydride (4.7 ml) was added dropwise to a solution of 5.0 g of N-acetylserinamide in 100 ml of pyridine. This solution was stirred for 2 hours at room temperature and the solvent evaporated under reduced pressure. The gummy residue was washed with ether and the washed residue was crystallized from ethanol. The product was then twice recrystallized from 95% ethanol and dried under vacuum over P2O5. Yield: 1.94 g; m.p. 157-159°C.

\[
\text{C}_4\text{H}_{12}\text{O}_4\text{N}_4^1
\]

Calculated: C 44.70 H 6.43 N 14.90

Found: C 44.75 H 6.49 N 14.80

Acetyl chymotrypsin was prepared according to Balls and Wood (6) from twice recrystallized α-chymotrypsin obtained from the Worthington Biochemical Corporation. Deuterium oxide, 99.8%, was prepared by the Atomic Energy Commission and was obtained through the courtesy of the Department of Chemistry, Harvard University. Water and deuterium oxide were glass-distilled before use.

Methods—Spectrophotometric measurements were carried out on a Zeiss PMQ II spectrophotometer equipped with a thermostated cell compartment. The alkaline and neutral hydrolysis and reaction with glycine of N,O-diacetylserinamide were followed by measuring the disappearance of ester, with a slight modification of the Hestrin (18) alkaline hydroxylamine-FeCl3 procedure. A 0.1-ml aliquot of the reaction mixture was added to 0.3 ml of a solution which was freshly prepared by adding 2 volumes of 3.5 N NaOH to 1 volume of 4 N NH2OH·HCl. The samples were allowed to stand for 10 minutes at room temperature and then 0.8 ml of a 10% FeCl3·6H2O solution containing 0.5 N HCl was added. The resulting ferric-hydroxamic acid complex was measured spectrophotometrically at 540 μm. For the alkaline hydrolysis of acetyl chymotrypsin and N,O-diacetylserinamide in 8 M urea, the formation of hydroxamic acid was carried out as described above, after which 0.2 ml of 50% trichloroacetic acid was added, and the denatured protein was removed by suction filtration with Celite filter aid. The filter was washed with 1.0 ml of 10% FeCl3·6H2O containing 0.1 N HCl, which was combined with the filtrate before measuring the absorption at 540 μm.

The neutral and alkaline reactions of hydroxylamine with N,O-diacetylserinamide and the alkaline hydroxylamine reaction with ethyl acetate were followed by adding 0.1-ml aliquots of the reaction mixture directly to 1.0 ml of 10% FeCl3·6H2O containing 0.1 N HCl and measuring the ferric complex at 540 μm. Incubation with concentrated alkaline hydroxylamine at the end of these reactions gave no further increase in hydroxamic acid formation.
Acetylimidazole reacted with N-acetylserinamide and acetyl chymotrypsin in 8 M urea, the 0.1 ml aliquots from the reaction mixture were first added to 0.2 ml of 50% trichloroacetic acid and then suction filtered with Celite filter aid. The filter was washed with 1.0 ml of 10% FeCl₃·6H₂O containing 0.1 N HCl, and the combined filtrate and washings were measured at 540 μm.

The reaction of N-acetylserinamide by p-nitrophenyl acetate was followed by measuring the rate of p-nitropheno-phenolate ion formation at 400 μm. The rate of p-nitrophenyl acetate hydrolysis was determined in identical reaction mixtures from which N-acetylserinamide had been omitted. The acetylation of N-acetylserinamide by acetylimidazole was measured by following the disappearance of acetylimidazole at 245 μm. The rate of acetylimidazole disappearance in 6.8 M ethanol was followed by the decrease in absorption at 245 μm, and the products of the reaction were analyzed by the Hestrin alkaline hydroxylamine method for ester determination (18).

Measurements for unsaturation were carried out by bromate titration (19). Infrared spectra were measured with KBr pellets, with a Perkin-Elmer model 21 spectrophotometer. Serine assays were carried out on a Beckman Spinco amino acid analyzer, according to the method of Moore and Stein (20, 21). Measurements of pH were made with a Radiometer pH meter, type PHM 4b with a G-200-B glass electrode. Hydroxide ion was estimated from the measured pH, taking Kₒ = 10⁻¹⁴. The apparent pH values of solutions containing alcohol were determined with the glass electrode and are reported for comparative purposes only. Kinetic experiments were carried out with one reactant in great excess so that pseudo first order kinetics was followed. Rate constants were obtained by plotting the extent of the reaction, zₑ - z₁, against time on semilogarithmic graph paper and by calculating the first order constants from the equation, k₁ = 0.693/₄₁. For the studies carried out in D₂O, all components of the reaction mixture were pre-equilibrated twice with 99.8% D₂O, evaporated to dryness, and redissolved in D₂O. Reactions at 100° were carried out by sealing each of 10 aliquots in small glass tubes, incubating in a boiling water bath, and removing aliquots for assay at appropriate time intervals.

<table>
<thead>
<tr>
<th>Acetylating reagent</th>
<th>k⁺</th>
<th>Reactivity NACT/HEO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylimidazole</td>
<td>1.2 × 10⁴</td>
<td>3500</td>
</tr>
<tr>
<td>p-Nitrophenyl acetate</td>
<td>2.2 × 10⁴</td>
<td>880</td>
</tr>
</tbody>
</table>

Nucleophilic Reactivity of the Serine Hydroxyl Group—N-Acetylserinamide, in which the amino and carboxyl groups of serine are protected in amide linkages, was selected as a model compound to evaluate the unaided nucleophilic reactivity of the serine hydroxyl group under conditions somewhat analogous to those for a serine residue in a peptide chain. To our surprise, dilute solutions of this compound were found to react at a readily measurable rate with acetylimidazole and p-nitrophenyl acetate, in spite of the enormously greater concentration of hydroxyl groups of the aqueous solvent. In imidazole buffers from pH 6.5 to 8.1, the rate of the reaction with 0.1 M N-acetylserinamide was found to be proportional to the concentration of hydroxide ion. This suggests that the serine hydroxyl anion is the reactive species. The products of the reaction were estimated by the Hestrin alkaline hydroxylamine method for ester determination (18). A reaction mixture which contained initially 12 μmoles of acetylimidazole in 0.2 M triethylamine buffer pH 9.0, was found to contain 8.4 μmoles of ester at 85% reaction. Under these conditions, the calculated amount of ester formation, based on the rate constants for the reaction with N-acetylserinamide and for the hydrolysis of acetylimidazole, is 8.5 μmoles. The reaction, therefore, represents an acetylation of the serine hydroxyl group, rather than a serine-catalyzed hydrolysis. It does not involve a base-catalyzed attack of an amide group, since no reaction could be detected with 8 M urea nor with 0.1 M N, O-diacylserinamide at pH 7.0 and 8.0, under conditions in which 0.1 M N-acetylserinamide caused a twofold increase in the observed rate of acetylimidazole disappearance.

In order to compare the reactivity of N-acetylserinamide with that of other hydroxyl-containing compounds, the rate constant for the base-catalyzed reaction was calculated according to the rate law

$$\text{rate} = k[\text{ROH}][\text{CH}_2\text{CN}][\text{OH}^-]$$  

(1)

The reaction of N-acetylserinamide with p-nitrophenyl acetate was also found to be rapid and base-catalyzed. The rate constants for these two reactions are given in Table I. The serine hydroxyl group is 3500 and 880 times more reactive than the hydroxyl group of water, expressed according to the same rate law, in the base-catalyzed reactions with acetylimidazole and p-nitrophenyl acetate, respectively.

Since the ionization constant for the formation of the hydroxyl anion of N-acetylserylamine is unknown, it might be argued that the observed high reactivity is due to an unusual acidity of the serine hydroxyl group, so that at a given pH, a greater concentration of N-acetylserinamide anion than of hydroxide anion would be present. No dissociation of the hydroxyl group could be detected by electrometric titration of N-acetylserinamide at pH 12. The nucleophilic reactivity of compounds of a given chemical class with acylating agents, such as p-nitrophenyl acetate and the conjugate acid of acetylimidazole, is known to increase with increasing basicity of the attacking group, with a Brønsted coefficient of approximately 0.8 (22, 23). If one assumes pKₐ values between 13 and 17 for the dissociation of the serine hydroxyl group, the calculated specific rate constants for reaction of the serine hydroxyl anion with acetylimidazole are still between 1000 and 6000 times larger than predicted from a Brønsted plot of slope 0.8 drawn through the
point for hydroxyl ion; similar results are obtained for the reaction with p-nitrophenyl acetate. The abnormal reactivity of N-acetylserinamide, therefore, cannot be ascribed to an unusual acidity of its hydroxyl group.

To determine whether a high nucleophilic reactivity compared to water is a general property of aliphatic hydroxyl groups or is a special property of the hydroxyl group of N-acetylserinamide, no reaction of acetylimidazole with ethanol could be detected. It was possible to compare the reactivities of water and ethanol, however, by measuring the rate of disappearance of acetylimidazole in 0.8 M ethanol and determining the relative amounts of acetate ion and ethyl acetate which were formed. Since the ratio of hydrolysis to alcoholysis, as determined by product formation, is equal to the ratio of the rate constants for the two processes, and since the over-all rate of acetylimidazole disappearance is the sum of these two rates, the rate constants for the individual reactions are readily obtained. The reaction of acetylimidazole with ethanol, like that with water (10), is catalyzed by imidazole (Fig. 1). The catalysis increases with increasing pH, suggesting that the catalytically active species of imidazole is the free base. The rates in the absence of imidazole catalysis were obtained by extrapolation to zero imidazole concentration and are compared in Table II. Although ethanol is 4.3 to 4.4 times more reactive than water, this difference is much less than the 3500-fold difference between the reactivities of N-acetylserinamide and water.

Although imidazole is a catalyst for the reactions of the hydroxyl groups of water and ethanol with acetylimidazole, no catalysis of the reaction of N-acetylserinamide with acetylimidazole could be detected in solutions up to 0.25 M in imidazole as the free base. The failure to observe imidazole catalysis of this reaction may be due not so much to the absence of imidazole catalysis as to its relatively slow rate, in comparison to the rapid hydroxide ion-catalyzed reaction; an imidazole-catalyzed contribution to the rate of the same magnitude as found with water and ethanol would not be detectable under the experimental conditions used.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>pHa</th>
<th>10^4 kba</th>
<th>klim</th>
<th>10^4 kbd</th>
<th>Reactivity Ethanol/H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcoholysis</td>
<td>6.8</td>
<td>2.65</td>
<td>0.148</td>
<td>3.10</td>
<td>4.3</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>6.8</td>
<td>2.00</td>
<td>0.106</td>
<td>0.715</td>
<td>1.23</td>
</tr>
<tr>
<td>Alcoholysis</td>
<td>7.4</td>
<td>4.7</td>
<td>0.144</td>
<td>5.47</td>
<td>4.4</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>7.4</td>
<td>3.45</td>
<td>0.109</td>
<td>1.23</td>
<td>4.4</td>
</tr>
</tbody>
</table>

- a The apparent pH as measured by the glass electrode.
- b kba = first order rate constant extrapolated to zero buffer concentration.
- c klim = kobs - kba
- d k2 = kba/[H2O] in the case of hydrolysis and k2 = kba/[ethanol] in the case of alcoholysis.
- * Reactivity ratio = (k2)ethanol/(k2)H2O

![Table II](http://www.jbc.org/)

**Fig. 1.** Reactions of acetylimidazole with water and ethanol in 0.8 M ethanol at constant pH, as a function of the concentration of imidazole buffer at 25°C and ionic strength 0.2.

**Fig. 2.** Imidazole catalysis of the hydrolysis of N,O-diacetylserinamide at pH 7.1 and 8.1 at 100°C and ionic strength 0.9 (pH determinations carried out at room temperature).

**Imidazole Catalysis of Reactions of N,O-Diacetylserinamide—**

N,O-Diacetylserinamide was chosen for studies on the susceptibility to catalysis and reactivity of a model for the acylated active site of chymotrypsin and related enzymes. At neutral pH, the hydrolysis of this compound proceeds very slowly at room temperature, but can be measured satisfactorily at 100°C. As shown in Fig. 2, this hydrolysis is catalyzed by increasing concentrations of imidazole buffers at a given pH. An increase in rate with increasing buffer concentration could be due to either the acidic or basic component of the buffer. The fact that the catalysis is greater at pH 8.1 than at 7.1 shows that the rate of the catalyzed reaction is proportional to the concentration of imidazole present as the free base, and not to the concentra-
catalyzed reaction. Imidazole catalysis of the hydrolysis of p-nitrophenyl acetate and phenyl acetate, which is known to proceed by nucleophilic catalysis with the intermediate formation of acetylimidazole (24-26), is not significantly decreased in deuterium oxide solution (Table IV).

Table III

<table>
<thead>
<tr>
<th>Reaction with glycine</th>
<th>Solvent</th>
<th>$k_{o} \times 10^{4}$</th>
<th>$k_{im} \times 10^{4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral hydrolysis of acetyl ester at pH 7.1 and ionic strength 1.0</td>
<td>H$_2$O</td>
<td>1.6</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>99.8% D$_2$O</td>
<td>1.6</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>k$<em>{H2O}$/k$</em>{D2O}$</td>
<td>1.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Neutral reaction with 0.3 M hydroxylamine at pH 9.7 and ionic strength 1.6</td>
<td>H$_2$O</td>
<td>2.8</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>99.8% D$_2$O</td>
<td>1.5</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>k$<em>{H2O}$/k$</em>{D2O}$</td>
<td>1.9</td>
<td>2.4</td>
</tr>
</tbody>
</table>

$\frac{k_{im}}{k_{o}} = \frac{k_{obs} - k_{o}}{[\text{imidazole}]_{\text{free base}}}$

$\frac{k_{H2O}}{k_{D2O}}$ ratio of 1.9 for the imidazole-catalyzed reaction and 1.9 for the uncatalyzed reaction. At pH 10.25, the reaction of N$_2$O-diacyctylserinamide with hydroxylamine is almost entirely hydroxide ion-catalyzed, and no increase in reaction rate was found on the addition of 0.5 M imidazole.

Other reactions of N$_2$O-diacyctylserinamide in water are also subject to general base catalysis. The rates of these reactions may be demonstrated by a greater than first order dependence of the rate on the concentration of the attacking nucleophile (27, 28). The reaction of reaction with glycine, shown in Fig. 4, increases considerably faster than the concentration of glycine and follows satisfactorily the solid line in the figure, which was calculated by assuming a reaction second order in respect to glycine; thus, this reaction proceeds exclusively through a general base-catalyzed pathway, with no detectable contribution of an uncatalyzed reaction to the observed rate. The reaction with hydroxylamine was also found to be dependent on more than the first power of the hydroxylamine concentration, thus indicating catalysis of this reaction by a second mole of hydroxylamine.

Under the conditions used for the reactions of N$_2$O-diacyctylserinamide with hydroxylamine and glycine, no detectable reaction was observed between ethyl acetate and these nitrogen bases.

Certain serine phosphates and related esters may decompose reaction. At pH 10.25, the reaction of N$_2$O-diacyctylserinamide with hydroxylamine is almost entirely hydroxide ion-catalyzed, and no increase in reaction rate was found on the addition of 0.5 M imidazole.

amline is very much faster than the neutral hydrolysis, and can readily be studied at 25°C. Imidazole catalysis of this reaction was studied by measuring the rate of hydroxamic acid formation from N-O-diacyctylserinamide and 0.3 M hydroxylamine in 0.10, 0.25, and 0.50 M half-neutralized imidazole buffers. Each rate determination was carried out in duplicate, and the reaction was studied in water and in deuterium oxide. The results were similar to those for the hydrolysis reaction, showing catalysis by imidazole, which is decreased in deuterium oxide. The rate constants for the imidazole-catalyzed and the uncatalyzed reaction, summarized in Table III, reveal a $k_{H2O}/k_{D2O}$ ratio of 2.4 for the imidazole-catalyzed reaction and 1.9 for the uncatalyzed reaction.

Deuterium isotope effects on imidazole catalysis of hydrolysis of phenyl acetate and p-nitrophenyl acetate at 25°C

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$k_{H2O}$</th>
<th>$k_{D2O}$</th>
<th>$\frac{k_{H2O}}{k_{D2O}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenyl acetate hydrolysis, ionic strength 0.35</td>
<td>0.48</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td>H$_2$O</td>
<td>99.8% D$_2$O</td>
<td>0.45</td>
<td>1.07</td>
</tr>
<tr>
<td>p-Nitrophenyl acetate hydrolysis, ionic strength 0.1</td>
<td>31.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>H$_2$O</td>
<td>99.8% D$_2$O</td>
<td>31.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$* k_{im} = k_{obs} - k_{o}$ where $k_{o}$ is extrapolated to zero imidazole concentration.

† Rates measured in half neutralized imidazole buffers, constant ionic strength maintained with KCl. Buffer concentrations were varied from 0.2 M to 0.7 M in phenyl acetate reactions and 0.02 M to 0.10 M in p-nitrophenyl acetate reactions.

‡ Imidazole buffers pre-equilibrated with D$_2$O and half-neutralized with DC1; constant ionic strength maintained with KCl in D$_2$O.
in alkaline solution by elimination of the acid anion to give an aminoacrylic acid derivative, rather than by hydrolysis (29, 30). To determine whether such an elimination reaction might take place in the reactions reported here, aliquots of reaction mixtures in which the base-catalyzed, neutral, or imidazole (0.9 M)-catalyzed decomposition of N,O-diacetylserinamide had occurred were analyzed by spectrophotometry at 241 m\(\mu\) and by bromate titration (19). Based on the molar extinction coefficient of 5300 for ac-N-carbobenzoxylaminoacrylic acid (30), it was estimated that less than 5\(\%\) of unsaturated product was formed; the same result was obtained by bromate titration. Samples of the reaction mixtures were subjected to acid hydrolysis and analyzed chromatographically for serine\(^2\) by the procedure of Moore and Stein (20, 21). After correction for a 25\(\%\) loss during hydrolysis in a control sample of N-acetylserinamide, the recoveries of serine were 90\(\%\) for the base-catalyzed reaction, 96\(\%\) for the uncatalyzed reaction, and 62\(\%\) for the imidazole-catalyzed reaction. It is possible that the lower yield for the imidazole-catalyzed reaction may be due to imidazole catalysis of serine decomposition during the acid hydrolysis. In any case, the imidazole-catalyzed reaction must proceed predominantly to give the expected products, since under the conditions of the experiment, 85\(\%\) of the total reaction was imidazole-catalyzed. These conclusions were confirmed by direct isolation of N-acetylserinamide from the lyophilized reaction mixture and crystallization from acetonitrile; a 60\(\%\) yield was obtained from the imidazole-catalyzed reaction and a 73\(\%\) yield from the base-catalyzed reaction. The melting points, mixed melting points, and infrared spectra of the products were identical with those of N-acetylserinamide.

**Comparison of Reaction Rates of N,O-Diacetylserinamide and Acetyl Chymotrypsin**—Dixon, Dreyer, and Neurath have shown that the high reactivity of the acetyl group, which is associated with the catalytic activity of the enzyme, is reversibly lost when acetyl chymotrypsin is dissolved in 8 M urea (4). The properties of the acetyl enzyme in 8 M urea should, therefore, reflect the properties of the acetyl enzyme bond in the absence of the rate-enhancing effects of other groups which contribute to the catalytic effectiveness of the active site. With this in mind, a comparison was made of the reactivity of acetyl chymotrypsin in 8 M urea with that of the model compound, N,O-diacetylserinamide.

**Fig. 4.** The rate of the reaction of N,O-diacetylserinamide with glycine, as a function of glycine concentration at pH 9.75, 25\(^\circ\), and ionic strength 1.1. Solid line calculated for a reaction second order in respect to glycine, from the rate law: rate = 0.036 (ester) (glycine)\(^2\).

**Fig. 5.** Logarithmic plots of the rates of hydrolysis of N,O-diacetylserinamide and acetyl chymotrypsin against pH in 0.1 M carbonate buffers at 25\(^\circ\) and ionic strength 0.3. *\(\Delta\)*, N,O-Diacetylserinamide hydrolysis in water; *\(\square\)*, N,O-diacetylserinamide hydrolysis in 8 M urea; *\(\bigcirc\)*, acetyl chymotrypsin hydrolysis in 8 M urea.

The rates of alkaline hydrolysis of acetyl chymotrypsin and of N,O-diacetylserinamide are compared in Fig. 5. The pseudo first order rate constant for each reaction was obtained at each pH value from five measurements of remaining acetyl ester, determined by the alkaline hydroxylamine method. The reactions followed pseudo first order kinetics in each case. At a given apparent pH value, the model compound, in water, was found to react faster than acetyl chymotrypsin in urea, but if the rates of hydrolysis of the model compound and of acetyl chymotrypsin are compared in 8 M urea, they may be seen to be almost identical.

The rates of the base-catalyzed reactions of acetyl chymotrypsin and N,O-diacetylserinamide with hydroxylamine are compared in Fig. 6. Again the model compound in water reacts... 

---

**Fig. 6.** Logarithmic plots of the rates of reaction of hydroxylamine with N,O-diacetylserinamide and acetyl chymotrypsin against pH in 0.1 M carbonate buffers at 25\(^\circ\) and ionic strength 0.4.
TABLE V
Rate constants for base-catalyzed acetyl transfer reactions to water and hydroxylamine

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Temperature</th>
<th>Solvent</th>
<th>$k_a$</th>
<th>$M^{-2} \text{min}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline hydrolysis of acetyl ester linkage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl chymotrypsin</td>
<td>25</td>
<td>8 M urea</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>$N,O$-Diaceetylserinamide</td>
<td>25</td>
<td>8 M urea</td>
<td>16.2</td>
<td></td>
</tr>
<tr>
<td>$N,O$-Diaceetylserinamide</td>
<td>25</td>
<td>Water</td>
<td>48.8</td>
<td></td>
</tr>
<tr>
<td>Acetylimidazole</td>
<td>25</td>
<td>Water</td>
<td>19,000</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>25</td>
<td>Water</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>20</td>
<td>Water</td>
<td>57</td>
<td></td>
</tr>
</tbody>
</table>

Base-catalyzed hydroxylamine reactions

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Temperature</th>
<th>Solvent</th>
<th>$k_a$</th>
<th>$M^{-2} \text{min}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl chymotrypsin</td>
<td>25</td>
<td>8 M urea</td>
<td>1,320</td>
<td></td>
</tr>
<tr>
<td>$N,O$-Diaceetylserinamide</td>
<td>25</td>
<td>8 M urea</td>
<td>1,380</td>
<td></td>
</tr>
<tr>
<td>$N,O$-Diaceetylserinamide</td>
<td>25</td>
<td>Water</td>
<td>5,150</td>
<td></td>
</tr>
<tr>
<td>Acetylimidazole</td>
<td>25</td>
<td>Water</td>
<td>$f$</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>25</td>
<td>Water</td>
<td>152</td>
<td></td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>37</td>
<td>Water</td>
<td>8,330</td>
<td></td>
</tr>
</tbody>
</table>

$^a k_a = k_{\text{obs}}/[\text{OH}^-]$$^b$

$^b$ Reference (31).

$^c$ Reference (32).

$^d$ Reference (33).

$^e k = k_{\text{obs}}/[\text{OH}^-][\text{NH}_2\text{OH}]$$^f$

$^f$ Too fast to measure.

$^g$ Measured at pH 10.23, 10.39, and 10.48 in 0.125 M carbonate buffer, 0.4 M hydroxylamine, 0.01 M ethyl acetate, and ionic strength 0.0.

$^h$ Estimated from the data of Reference (18).

The neutral hydrolysis and reaction with hydroxylamine of the model compound, $N,O$-diaceetylserinamide, are catalyzed by imidazole. This catalysis is of interest because it represents catalysis of hydrolysis and acyl transfer reactions of a "low energy" ester and because it is decreased approximately one-half in deuterium oxide solution.

With highly reactive, "energy-rich" acylating agents, such as $p$-nitrophenyl acetate and acyl phosphates, imidazole catalyzes acyl transfer and hydrolysis by nucleophilic catalysis with the intermediate formation of acetylimidazole, which is itself an "energy-rich" compound (24-26, 31, 37). With "low energy" esters, acetylimidazole formation is much less likely because of the unfavorable equilibrium for its formation. It has been shown that acyl transfer reactions of acetylimidazole to water, amines, and sulfhydryl compounds, which cannot be catalyzed by imidazole through nucleophilic catalysis, are subject to classical general base catalysis, in which imidazole aids the removal of a proton from the attacking reagent in the transition state (10).

It was suggested that this type of catalysis might be favored in the case of substrates with a relatively poor leaving group, such as the imidazole catalysis of methyl oxalate hydrolysis reported by Brouwer et al. (26), since the potential attacking anion (e.g. OH-) formed in a general base catalyzed reaction could attack "low energy" as well as "high energy" substrates.

The fact that imidazole catalysis of $N,O$-diaceetylserinamide reactions is decreased in deuterium oxide solution strongly suggests that proton transfer takes place in the activated complexes of these reactions and that they are examples of classical general base catalysis by imidazole. No isotope effect was observed or would be expected for the imidazole-catalyzed reactions of $p$-nitrophenyl acetate and phenyl acetate, which proceed by nucleophilic catalysis with intermediate acetylimidazole formation.$^3$

$^3$ Butler and Gold (38) have recently reported a deuterium isotope effect in the pyridine-catalyzed hydrolysis of acetic anhydride, which probably proceeds through nucleophilic catalysis; but it seems very likely that this reaction is complicated by reaction of the intermediate acetylpseudorium ion with acetate to reform acetic anhydride, so that the rate-limiting step is not the initial nucleophilic attack of pyridine. This interpretation is supported by the known facile reaction of the acetylimidazolium ion with acetate (31). In any case, a mechanism of this kind may be ruled out for the reactions reported here, since in the dilute solutions employed, the known rate constants for the reaction of serine with any presumed acetylimidazole intermediate would not permit any appreciable reformation of starting material to compete with hydrolysis by solvent.
General base catalysis by imidazole of acyl serine hydrolysis could occur by removal of a proton from an attacking water molecule (XH) in the transition state (I) or by addition of a proton from imidazolium ion to the leaving serine group, accompanied by attack of hydroxide ion (X−) on the acyl group (II). Mechanisms I and II are kinetically indistinguishable since their transition states have the same stoichiometric composition and

\[ \text{[Imidazole]} = K \text{[Imidazole H+][OH−]} \]  

(2)

from the equilibrium expressions for the dissociation of imidazolium and water. In both cases, the rate will appear to be proportional to the concentration of imidazole as the free base.

General base catalysis has been suggested as a possible role for imidazole in chymotrypsin and related enzymes (e.g. 1, 7, 10–13) and, with appropriate substitutions for XH and for R, and 11 may refer also to the reaction with hydroxylamine and to the enzymatic reaction. Many reactions of chymotrypsin are reversible and presumably involve catalysis of both the acylation and deacetylation of serine, which are similar processes except for direction. Mechanism I involves proton removal from XH by imidazole in one direction and proton addition to X− from imidazolium ion in the other, as the serine hydroxyl anion is expelled or attacks, respectively. Mechanism II involves removal of a proton by imidazole from the attacking serine hydroxyl group in one direction, and addition of a proton to the leaving serine oxygen atom by imidazolium in the other direction as X− leaves or attacks, respectively. The two mechanisms differ, therefore, not in whether imidazole acts as a general base by removing a proton or as a general acid by donating a proton (from imidazolium, after a pre-equilibrium removal of a proton from the other reactant, as in Equation 2), but rather in whether imidazole is acting to remove or add protons to XH and X− (Mechanism I) or to the serine oxygen atom (Mechanism II). It is important to realize that for any reversible reaction, acceptance of a mechanism involving proton abstraction by imidazole in one direction, requires acceptance of proton donation by imidazolium cation, acting as a general acid, in the other direction, since the transition state must be the same for catalysis in the two directions. In both the model and enzymatic reactions, there is no evidence available at the present time to favor one of these two mechanisms over the other, and both are, a priori, equally likely.

Several modifications of this scheme should be considered. It is possible that a tetrahedral addition intermediate with an appreciable lifetime is formed (11, 39). The transition states would then be similar to I or II, except that one of the bonds to the carbonyl group would be fully, instead of partially, formed. There is at present no evidence for such an intermediate in the model or enzymic reactions (40). Imidazole catalysis of reactions of acyl serines is clear cut, but is of a low order, and if such catalysis occurs in the enzymic reaction, it must be aided by such factors as other general acid-base catalysis on the enzyme surface and the use of weak bonding forces to bring the reactants into close proximity in order to realize the rate enhancements found in certain intramolecular compared to intermolecular reactions (3), as well as other, less well understood factors (23). The site of substrate protonation in specific and general acid-catalyzed reactions is not definitely known, and it is possible that general acid catalysis increases the polarization and reactivity of the carbonyl group. The maximal potentialities of general acid and base catalysis would be realized in a transition state such as III, in which one of the B groups represents imidazole and the others represent other general acids or bases (e.g., peptide linkages), whereas XII represents a protonated leaving or attacking group (e.g., water). Substrate specificity, which is different for the different enzymes, is presumably mediated by the nature and position of still other groups at the active site.

The acetyl ester group of N,O-diacetylserinamide has a reactivity towards hydroxide ion which is very similar to that of acetylcholine and is only slightly greater than that of ethyl acetate (Table V). The rate of the base-catalyzed reaction with hydroxylamine, which is also similar to that of acetylcholine, is some 34 times faster than the corresponding reaction with ethyl acetate. The neutral reactions of N,O-diacetylserinamide with hydroxylamine and glycine, which are both general base catalyzed, show a much larger increase relative to ethyl acetate, since no such reactions could be detected with ethyl acetate under the conditions of measurement. It thus appears that the reactivity of the acetyl serine ester group, and presumably the intrinsic reactivity of acetyl chymotrypsin, is anomalously high towards nitrogen-containing nucleophilic reagents, particularly in general base catalyzed reactions. This may be of significance in explaining the known ability of proteolytic enzymes to transfer acyl groups to nitrogen-containing nucleophilic reagents, in preference to hydrolysis by water.

The demonstration that acetyl chymotrypsin and N,O-diacetylserinamide in 8 M urea underwent alkaline hydrolysis at almost identical rates and react with hydroxylamine at almost the same rates provides strong quantitative evidence that the acetyl group of acetyl chymotrypsin is bound in an ester linkage to the hydroxyl group of a serine residue. Since the loss of the high enzymic reactivity of acetyl chymotrypsin in 8 M urea is fully reversible (4), any irreversible migration of the acetyl group from some other active site during urea denaturation is ruled out. A reversible migration seems extremely unlikely under the mild conditions of urea treatment and would seem to be thermodynamically highly improbable, if it involved an imidazole residue, because of the "energy-rich" nature of acetylimidazole; furthermore, the demonstration that protons are taken up rather than given off during chymotrypsin acylation at slightly acidic pH appears to rule out imidazole as the initial site of acylation (6, 8, 9). Although it is difficult to devise an experimental test which could unequivocally rule out a transient acyl-
tion of imidazole, too fast to be kinetically detectable or to be of kinetic significance, there is at present no evidence for any such acylation, and we regard the role of a serine residue as the site of acylation by reactive substrates as firmly established. It is not known whether the hydrolysis of "low-energy" substrates also proceeds through an acyl enzyme intermediate.

It is well known that serine and serine peptides are many orders of magnitude less reactive towards acylating agents than the serine hydroxyl group in the active site of chymotrypsin and related enzymes. For example, only one out of the many serine hydroxyl groups of chymotrypsin undergoes rapid acylation or phosphorylation, and qualitative observations have shown that serine-containing peptides do not react nearly as rapidly with p-nitrophenyl acetate as does chymotrypsin (e.g. (41)). It was, therefore, surprising to find that the hydroxyl group of N-acetyls erinamide is approximately three orders of magnitude more reactive in base-catalyzed attack on two acylating agents, acetylimidazole and p-nitrophenyl acetate, than the hydroxyl groups of water and ethanol. Although the reason for this high reactivity cannot be established on the basis of the available evidence, it is possible that this reaction proceeds with a bifunctional, concerted attack in which the serine oxygen anion is the nucleophilic reagent and the hydrogen atoms of one or both amide groups, which carry a partial positive charge, provide general acid catalysis to further polarize and increase the reactivity of the carbonyl group of the substrate. Molecular models of N-acetyls erinamide show that the conformation shown is a possible preformation, orientation; essentially the same conformation exists in an a-helix. A similar mechanism has been suggested as a possible explanation for the abnormal reactivity of the oxygen atom of hydroxylamine towards activated acyl groups (23, 42).

It might be expected that serine-containing peptides would show a similar enhanced reactivity. Although we have not studied such peptides, it is of interest that the rapid intramolecular reaction of the carbobenzoxyaspartylserinamide benzyl ester recently studied by Bernhard, Berger, Sela, and Katchalski (43) appears to require both the hydroxyl and the amide groups of the serine portion for rapid hydrolysis.

It is not intended to make a direct comparison between the abnormally high reactivity of the serine hydroxyl group and the much higher order of reactivity found in enzymic reactions. It seems reasonable, however, to suggest that this high reactivity will contribute to the still higher reactivity of the enzyme and provide an initial advantage of several orders of magnitude over "normal" reactants. Serine hydroxyl, sulfhydryl, and imidazole groups are frequently found in the active sites of enzymes, and it appears that the serine hydroxyl group, like thiol and imidazole, is an abnormally efficient nucleophilic reagent and is particularly well fitted for a role in enzymic catalysis.

**Summary**

1. The nucleophilic reactivity of the hydroxyl group of N-acetyls erinamide, a model for a portion of the active site of chymotrypsin and related enzymes, toward base-catalyzed acylation by acetylimidazole and p-nitrophenyl acetate is abnormally high by about three orders of magnitude.

2. The neutral hydrolysis and the reaction with hydroxylamine of N,O-di acetylserinamide, a model for the acylated active site of chymotrypsin, are catalyzed by imidazole. This catalysis is decreased twofold in deuterium oxide solution. This suggests that a proton bond is stretched in the activated complex of the catalyzed reaction and that the reaction proceeds by general base catalysis, rather than through the intermediate formation of acetylimidazole.

3. The rates of the base-catalyzed hydrolysis and reaction with hydroxylamine of N,O-di acetylserinamide and of acetyl chymotrypsin in 8 M urea are very similar. This provides quantitative support for the role of a serine residue as the primary site of acylation of chymotrypsin by reactive substrates.

4. N,O-Diacetylserinamide reacts normally with hydroxylamine, but reacts anomalously rapidly in general base catalyzed reactions with nitrogen bases.

5. The implications of these findings with respect to possible mechanisms of catalysis by chymotrypsin and related enzymes are discussed.

**References**


Reactivity and Catalysis in Reactions of the Serine Hydroxyl Group and of O-Acyl Serines

Bruce M. Anderson, Eugene H. Cordes and William P. Jencks


Access the most updated version of this article at http://www.jbc.org/content/236/2/455.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/236/2/455.citation.full.html#ref-list-1