Concerning the Mechanism of Ester Hydrolysis by Proteases

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SUMMARY

We have shown conclusively that during the hydrolysis of N-acylamino acid esters by proteases the substrate bond cleaved is the acyl-carbon-etheral oxygen bond. This was done by hydrolyzing the ethyl esters in H$_2^{18}$O-enriched water, isolating the ethanol formed, and determining the $^{18}O$/$^{16}O$ ratio. The amount of ethanol-$^{18}O$ found was that of natural abundance. This result supports what has hitherto been an assumption as to the site of bond cleavage. We discuss the implications of this for various "rack," or bond angle distortion, mechanisms which have been proposed.

A number of workers have addressed themselves to elucidating the mechanism of hydrolysis of N-acylated amino acid ester substrates by serine and sulfhydryl proteases. From this work, a general picture has emerged involving a tetrahedral intermediate (1, 2) formed during attack on the acyl carbon of the substrate by a nucleophilic center such as the active site serine hydroxyl in the serine proteases (3, 4). In this mechanism, the alkyl moiety of the substrate leaves as an alcohol, with bond cleavage between the acyl carbon and the ethereal oxygen. This corresponds to the site of cleavage during hydroxyl-catalyzed ester hydrolysis (5).

During base-catalyzed hydrolysis of lactones, a similar situation obtains, i.e. normally the acyl carbon-etheral oxygen bond is the one broken. However, in certain strained rings, such as $\beta$-lactones, the ring may be opened via $S_N$2 attack of neutral nucleophiles such as water on the alkyl carbon (6, 7). In this case, the alkyl-oxygen bond is cleaved as the $S_N$2 transition state breaks down into alcohol and acid. The oxygen of the resultant alcohol hydroxyl group is derived from the solvent, i.e. water.

One proposed general mechanism of enzyme action is the "rack" mechanism (8, 9) in which the susceptible substrate bonds are strained during the formation of the activated enzyme-substrate complex just prior to breakdown into products. If this strain were physical, i.e. involving actual bond angle distortion, then hydrolysis of esters by proteases might proceed by a mechanism similar to hydrolysis of $\beta$-lactones by water. The alcohol resulting from such a hydrolysis would derive its hydroxyl group from the solvent. A mechanistic scheme which involves such a possibility is shown in Scheme 1. While the details depicted (i.e. general base catalysis by imidazole, presence of a tetrahedral intermediate, and potentiation of the serine hydroxyl by hydrogen bonding to an imidazole nitrogen) are in general consistent with proposed mechanisms (1), the important feature is that cleavage of the alkyl carbon-oxygen bond may still yield the acylenzyme intermediate, and is consistent with all information which we could find pertaining to the mechanism of hydrolysis of amino acid esters by proteases. In order to test this possibility we hydrolyzed N-acylated amino acid esters with various enzymes, using as solvent water containing an excess of H$_2^{18}$O. Determination of the $^{18}O$ content of the resulting alcohol provided the test of the hypothesis.

Bender and Kemp (10) studied the reaction of chymotrypsin with methyl $\beta$-phenylpropionate labeled with $^{18}O$ in either the carbonyl or the alkoxyl oxygen. In the presence of 10 to 30% methanol extensive transesterification as well as hydrolysis occurred. After a period of reaction, reisolated ester was examined. If the starting substrate was labeled in the carbonyl oxygen the label was retained, while $^{18}O$ originally present in the alkoxyl oxygen was lost. Ethyl N-benzoyl-$\beta$-phenylalanine-carbonyl$^{18}O$ retained its label in a similar experiment. These results imply acyl oxygen fission during the enzyme catalyzed hydrolysis. However, they are also fully compatible with the reaction scheme presented above. Determination of the $^{18}O$ content of the freed alcohol remains the only unequivocal differentiation between the possibilities of acyl oxygen and alkyl oxygen fission.

Additionally, a recent communication has appeared (11) wherein quantum chemical calculations for the hydrolysis of acetylcholine have led to the prediction that the site of bond cleavage by cholinesterase is the alkyl oxygen bond. While we have not studied this particular enzyme, we have studied four...
was added, and the pH was kept constant by addition of 2.5 n.

The abbreviations used are: Bz-Arg-OEt, N-benzoyl-L-arginine ethyl ester; Ac-Tyr-OEt, N-acetyl-L-tyrosine ethyl ester.

The immediate significance of the results obtained is the direct experimental confirmation of what until now has been a quite reasonable assumption, i.e. that during enzyme-catalyzed hydrolysis of nonactivated esters of N-acylated amino acids, it is the acyl oxygen bond which is cleaved.

This finding, however, is also interesting with regard to what it contributes to a consideration of the rack mechanism of enzyme action. This mechanism is still rather nebulous and a variety of effects are included under this heading. In this paper we will briefly discuss two aspects of the question: strain in the enzyme-substrate complex resulting from bond angle distortion in the substrate; and strain in the enzyme-substrate complex which we will call electronic distortion, but not requiring changes in bond angles.

Bond angle distortion, or strain, has been inferred from the observations of Hofstee (12) that the rate of hydrolysis of acyl phenyl esters increases as the chain length of the acyl portion increases. This is taken to mean that increased interaction of the acyl chain with the enzyme provides additional energy which is used to induce increasing amounts of strain in the ester bond. In opposition to this is the finding of Iserel and Glazer (13) that the methyl ester of N-acetyltirosine is hydrolyzed much faster than the ethyl ester by subtilisin. If interaction of the substrate with the enzyme produces distortion of the sensitive bond, it would appear necessary that substituents on both sides of the bond should interact with the enzyme, and serve as "handles" for the distortion. One would expect that increased chain length of the alkyl moiety would increase this interaction provided, of course, that the larger alkyl group

It is apparent that the ethanol derived from all the enzymatic hydrolyses has an \(^{18}O\) content equal to the natural abundance within experimental error. This fact demonstrates that none of the oxygen in the ethanolic hydroxyl group was derived from the solvent water, and so the bond which was broken during hydrolysis must have been the acyl carbon-ether oxygen bond.

discussion

**Table I**

<table>
<thead>
<tr>
<th>System</th>
<th>C(^{18}O):C(^{16}O) \times 10(^{4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chymotrypsin + Ac-Tyr-OEt(^a)</td>
<td>0.14</td>
</tr>
<tr>
<td>Chymotrypsin + Ac-Tyr-OEt(^b)</td>
<td>0.20</td>
</tr>
<tr>
<td>Subtilisin + Ac-Tyr-OEt(^d)</td>
<td>0.17</td>
</tr>
<tr>
<td>Trypsin + Bz-Arg-OEt(^*)</td>
<td>0.11</td>
</tr>
<tr>
<td>Papain + Bz-Arg-OEt(^c)</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Solvent water

\[ \text{H}_{2}^{18}O: \text{H}_{2}^{16}O \times 10^{4} \] = 0.93 ± 0.15

\(^{a}\) All reaction systems were at ambient temperature, 20 ml of total volume, and contained 1 millimole of the specified ester.

\(^{b}\) Enzyme, 5 mg; the solvent was 5% in dimethylformamide, pH maintained at 6.

\(^{c}\) Chymotrypsin, 1 mg; the solvent was 5% in p-dioxane, pH maintained at 7.6.

\(^{d}\) Enzyme, 1 mg; the solvent was 5% in dimethylformamide, pH maintained at 8.

\(^{*}\) Enzyme, 5 mg; pH maintained at 8.

\(^{c}\) Enzyme, 10 mg; pH maintained at 6.

**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Bond angle</th>
<th>(k_{OH}) (^{b})</th>
<th>(k_{\alpha}) (^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>116(^{76})</td>
<td>6.5(^{a})</td>
<td>9.2 \times 10^{-9} (^{a})</td>
</tr>
<tr>
<td>γ-Butyrolactone</td>
<td>108(^{86})</td>
<td>70(^{a})</td>
<td>10^{-7} to 10^{-11} /</td>
</tr>
<tr>
<td>β-Butyrolactone</td>
<td>94(^{76})</td>
<td>45(^{a})</td>
<td>0.85 \times 10^{-2} (^{a})</td>
</tr>
<tr>
<td>β-Propiolactone</td>
<td>94(^{76})</td>
<td>130(^{a})</td>
<td>3.3 \times 10^{-2} (^{a})</td>
</tr>
</tbody>
</table>

\(^{a}\) Rate constants were determined at 25°, at comparable ionic strengths.

\(^{b}\) Reference 9, p. 513.

\(^{c}\) Reference 5.

\(^{d}\) Reference 8.

\(^{e}\) Reference 7.

\(^{f}\) Reference 10.

\(^{g}\) Reference 11.

\(^{h}\) Reference 14.

\(^{i}\) Reference 15.

\(^{j}\) Reference 16.

\(^{k}\) Reference 17.

\(^{l}\) Reference 18.

\(^{m}\) Reference 19.

\(^{n}\) Reference 20.

\(^{o}\) Reference 21.

\(^{p}\) Reference 22.

\(^{q}\) Reference 23.

\(^{r}\) Reference 24.

\(^{s}\) Reference 25.

\(^{t}\) Reference 26.

\(^{u}\) Reference 27.

\(^{v}\) Reference 28.

\(^{w}\) Reference 29.

\(^{x}\) Reference 30.

\(^{y}\) Reference 31.

\(^{z}\) Reference 32.

\(^{aa}\) Reference 33.

\(^{ab}\) Reference 34.

\(^{ac}\) Reference 35.

\(^{ad}\) Reference 36.

\(^{ae}\) Reference 37.

\(^{af}\) Reference 38.

\(^{ag}\) Reference 39.

\(^{ah}\) Reference 40.

\(^{ai}\) Reference 41.

\(^{aj}\) Reference 42.

\(^{ak}\) Reference 43.

\(^{al}\) Reference 44.

\(^{am}\) Reference 45.

\(^{an}\) Reference 46.

\(^{ao}\) Reference 47.

\(^{ap}\) Reference 48.

\(^{aq}\) Reference 49.

\(^{ar}\) Reference 50.

\(^{as}\) Reference 51.

\(^{at}\) Reference 52.

\(^{au}\) Reference 53.

\(^{av}\) Reference 54.

\(^{aw}\) Reference 55.

\(^{ax}\) Reference 56.

\(^{ay}\) Reference 57.

\(^{az}\) Reference 58.

\(^{aa}\) Reference 59.

\(^{ab}\) Reference 60.

\(^{ac}\) Reference 61.

\(^{ad}\) Reference 62.

\(^{ae}\) Reference 63.

\(^{af}\) Reference 64.

\(^{ag}\) Reference 65.

\(^{ah}\) Reference 66.

\(^{ai}\) Reference 67.

\(^{aj}\) Reference 68.

\(^{ak}\) Reference 69.

\(^{al}\) Reference 70.

\(^{am}\) Reference 71.

\(^{an}\) Reference 72.

\(^{ao}\) Reference 73.

\(^{ap}\) Reference 74.

\(^{aq}\) Reference 75.

\(^{ar}\) Reference 76.

\(^{as}\) Reference 77.

\(^{at}\) Reference 78.

\(^{au}\) Reference 79.

\(^{av}\) Reference 80.

\(^{aw}\) Reference 81.

\(^{ax}\) Reference 82.

\(^{ay}\) Reference 83.

\(^{az}\) Reference 84.

\(^{aa}\) Reference 85.

\(^{ab}\) Reference 86.

\(^{ac}\) Reference 87.

\(^{ad}\) Reference 88.

\(^{ae}\) Reference 89.

\(^{af}\) Reference 90.

\(^{ag}\) Reference 91.

\(^{ah}\) Reference 92.

\(^{ai}\) Reference 93.

\(^{aj}\) Reference 94.

\(^{ak}\) Reference 95.

\(^{al}\) Reference 96.

\(^{am}\) Reference 97.

\(^{an}\) Reference 98.

\(^{ao}\) Reference 99.

\(^{ap}\) Reference 100.

\(^{aq}\) Reference 101.

\(^{ar}\) Reference 102.

\(^{as}\) Reference 103.

\(^{at}\) Reference 104.

\(^{au}\) Reference 105.

\(^{av}\) Reference 106.

\(^{aw}\) Reference 107.

\(^{ax}\) Reference 108.

\(^{ay}\) Reference 109.

\(^{az}\) Reference 110.
is not too large for its binding region. Steric hindrance to binding of alkyl moieties does not seem to be the case, since the benzyl ester of N-acetylseryosine is hydrolyzed even more rapidly than the ethyl ester (13).

One may raise the question of how much bond angle distortion is necessary for enzyme action. If bond angles in lactones may be used as guides for ester distortion, the data in Table II are pertinent. Distortion of the C—O—C bond angle from 116° to 108° produces an order of magnitude increase in the rate of hydrolysis by OH−, and at most a similar increase in rate of hydrolysis attack by water. Further distortion to 94° does not appreciably increase the hydroxide rate, but does increase the rate of hydrolysis by water by at least a factor of 104. The 10-fold increase in the rate of hydroxide catalysis is insufficient to explain enzymatic catalysis, while the factor of 104 is the magnitude of rate enhancement often seen in enzymic reactions. However, the S2 mechanism of attack by water results in alkyl oxygen cleavage, and the experiments reported above show that this does not occur in enzymatic hydrolysis of esters. Thus, one is left with examples of bond angle distortion which, for one mechanism (OH− attack) do not provide rate enhancement corresponding to that seen in enzymes, and in the other mechanism (H2O attack) provide sufficient enhancement of rate but do not have the pattern of bond cleavage observed in the enzymatic reaction. This leads us to question whether bond angle distortion in the enzyme-substrate complex contributes significantly to catalysis of ester hydrolysis by proteases.

An alternative form of strain might be what we have called “electronic distortion.” This merely implies that interactions in the enzyme-substrate complex produce a distortion of the molecular orbitals around the sensitive bonds, thus raising the ground-state energy of the complex and increasing the probability of passage to the transition state. The types of interactions which we envision as responsible for this sort of distortion are dipole-dipole interactions and electrostatic perturbations.

The possible presence of a large electrostatic influence in the active site of chymotrypsin was shown by Zeffren and Reavill (17). They employed nuclear magnetic resonance spectroscopy to study the 19F chemical shift attendant upon binding N-trifluoroacetyl-DL-phenylalanine to chymotrypsin. In that paper they equated the active site environment in chymotrypsin to a 10 m NaCl solution. Subsequent measurements (18) have shown that equivalent perturbations of the 19F chemical shift N-trifluoroacetyl-DL-phenylalanine may be produced by small amounts of ionic materials in a nonpolar medium (e.g. 0.0175 m propylammonium acetate in methyl ether). Consistent with this interpretation of electronic distortion is the general finding of protein crystallographers that “the low dielectric constant of the nonpolar interior of protein molecules facilitates electrical interactions which are many times stronger than in water” (19).

The effect of dipoles in the active site upon enzymatic activity was also pointed out by Stauffer and Etson (20). In this case, introduction of a S — O dipole in place of the nonpolar methionine sulfide very close to the active site serine of subtilisin produced a drastic decrease in the rate of ester hydrolysis. The increased polar nature of the active site of oxidized subtilisin probably lessens the effect of the normal electrical interactions in this usually apolar region.

We have considered two kinds of substrate distortion by the enzyme: perturbation of the energy levels of the ester's ground-state molecular orbitals; and spatial or bond angle distortion. We conclude that the former probably contributes more to the enhanced rate of enzymatic hydrolysis than does the latter.

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REFERENCES
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