Administration of $p$-hydroxybenzaldehyde carbonyl $\mathrm{C}^{14}$ (6.28 μmoles, 1.42 × $10^4$ c.p.m. per μmole) to $R$. rubrum resulted in a markedly different distribution of radioactivity in the cells, although the percentage uptake was approximately the same (8.5%). Most of the $\mathrm{C}^{14}$ found in the cells was not extracted into ether–ethanol (3:1), and the petroleum ether fraction contained only 2.37% of the counts taken up. The coenzyme $Q_0$ did not contain significant radioactivity. Apparently, in the conversion of $p$-hydroxybenzaldehyde to coenzyme $Q_0$, the side-chain carbon is lost.

The evidence that the $p$-hydroxybenzaldehyde ring can give rise to coenzyme $Q_0$, in direct manner was strengthened by degradation of the radioactive coenzyme $Q_0$ by the method of Bentley et al. (1). In a degradation carried out on coenzyme $Q_0$ isolated from $R$. rubrum grown with $p$-hydroxybenzaldehyde–U–C$^{14}$ as described above, the $3', 5'$-diacetoxy-4', 5'-dimethoxy-2'-methylphenylacetic acid, derived from the coenzyme $Q_0$ benzoquinone ring, was found to account for 86.5% of the total $\mathrm{C}^{14}$ in the coenzyme $Q_0$. Thus coenzyme $Q_0$ having a specific activity of 24.2 c.p.m. per μmole after the addition of carrier afforded the ring derivative with an activity of 20.9 c.p.m. per μmole. The coenzyme $Q_0$ side chain contained no detectable $\mathrm{C}^{14}$.

Although a relatively small amount of $p$-hydroxybenzaldehyde is taken up by the cells, these data suggest that once inside the cell $p$-hydroxybenzaldehyde is converted fairly directly to coenzyme $Q_0$. The data appear to exclude tyrosine as an intermediate in the conversion of $p$-hydroxybenzaldehyde to coenzyme $Q_0$. In further confirmation of this concept, the addition of unlabeled tyrosine to cells growing with $p$-hydroxybenzaldehyde–U–C$^{14}$ does not decrease the specific activity of the coenzyme $Q_0$ isolated. Phenol–U–C$^{14}$ did not prove to be an effective precursor of coenzyme $Q_0$, although the observed loss of the aldehyde carbon from $p$-hydroxybenzaldehyde suggests that the formation of coenzyme $Q_0$ involves a 6 carbon aromatic intermediate, which is subsequently hydroxylated, O-methylated, and C-methylated.  

Iwaski, Iwaski, and Gest (12) have found that $p$-hydroxybenzaldehyde and $p$-hydroxybenzoic acid are among several aromatic compounds exerted into the medium when $R$. rubrum is grown with limiting amounts of (NH$_4$)$_2$SO$_4$ as the sole nitrogen source. Pseudomonas species are known to metabolize $p$-hydroxybenzaldehyde by oxidation to $p$-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid (13). In Escherichia coli, $p$-hydroxybenzoic acid arises from the shikimic acid pathway at a branch point preceding the formation of phenylalanine and tyrosine (14). These considerations and the finding that labeled $p$-hydroxybenzaldehyde is converted to coenzyme $Q_0$ without apparent dilution by tyrosine suggest that in $R$. rubrum the coenzyme $Q_0$ benzoquinone nucleus is not formed directly from aromatic amino acids, but is derived from aromatic compounds which arise earlier in the shikimic acid pathway. The possibility must also be considered, however, that aromatic compounds similar to $p$-hydroxybenzaldehyde may arise via the acetate pathway (15).

REFERENCES


On the Question of an Acyl-enzyme Intermediate in the Chymotrypsin-catalyzed Hydrolysis of Hydroxamic Acids*

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(Received for publication, June 18, 1963)

That the reactions of esters of hippuric acid catalyzed by chymotrypsin pass through an acyl-enzyme intermediate has been demonstrated by the fact that in 0.1 M NH$_4$OH solution the same fractions of 10 esters of hippuric acid were converted to hippurylhydroxamic acid (1). These reactions are depicted

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† Predoctoral Fellow of the United States Public Health Service.
in Scheme 1. As indicated in Scheme 1, O-acylhydroxylamines

\[
\begin{array}{c|c|c}
\text{O-Acylhydroxylamine} & \text{NH}_2\text{OH} \\
\text{Ester} & \text{acyl enzyme} & \text{NH}_2\text{OH} \\
\text{H}_2\text{O} & k_4 & k_5 \text{NH}_2\text{OH} \\
\text{Acid Hydroxamic acid} & \\
\end{array}
\]

are anticipated transient side products which can revert to the acyl-enzyme and thence to acid and hydroxamic acid, or can be converted to the hydroxamic acid by a nonenzymic pathway catalyzed by hydroxylamine (2). This conversion is especially rapid at pH 6.2 and can be used to identify the O-acylhydroxylamines. The O-acylhydroxylamines also hydrolyze spontaneously, but this reaction appears to be relatively slow at pH 6.6.

If this scheme is correct, the enzymic pathway for degrading the O-acylhydroxylamine should predominate with high enzyme concentrations, and the nonenzymic pathway, in which mostly hydroxamic acid is obtained, should predominate at low enzyme concentrations (Table I). In this way the relative speeds of the three paths leading from the acyl-enzyme can be quantitatively evaluated in accordance with Scheme 1.\(^1\) For example, in 1 M NH\(_2\)OH, 25\(^\circ\), pH 6.6, \(\Gamma/2 = 1\) M, about 0.39 of acetyltyrosyl-enzyme (derived from the ethyl ester) goes to the O-acylhydroxylamine, 0.16 to hydroxamic acid, and 0.45 to acid. On the other hand, tyrosyl-enzyme yields relatively little O-acylhydroxylamine, since there is little change in the fraction of ester converted to hydroxamic acid over a wide range of enzyme concentration.

To test the validity of the scheme further, O-hippurylhydroxylamine was prepared in solution by reaction of p-nitrophenyl hippurate with hydroxylamine in a manner similar to the preparation of O-acylhydroxylamine (3). This compound was a very good substrate for chymotrypsin (\(\alpha\)- and \(\delta\)-); in fact, better than the alkyl esters. The fraction of this compound converted to hydroxamic acid by the enzymic pathway was the same as found for hippurate esters.

Using the O-acylhydroxylamine test, we have demonstrated the formation of O-hippurylhydroxylamine and its subsequent disappearance during the chymotrypsin-catalyzed reaction of hippurylhomocholine with water and hydroxylamine.

A particularly interesting test of the acyl-enzyme theory has been described by Caplow and Jencks (4) in which acetyltyrosine hydroxamic acid is used as a substrate in the presence of hydroxylamine. The theoretical scheme expected for this situation is shown in Scheme 2, where \(S\) is acetyltyrosine hydroxamic acid, \(E\) is acetyltyrosyl-enzyme, \(P_1\) is hydroxylamine, \(P_2\) is acetyltyrosine, and \(P_3\) is O-acetyltyrosylhydroxylamine. The nonenzymic pathway (dotted arrow) is not important when sufficient enzyme is used. The quantity \(kP_1/k_5\) is known from the ratio of hydroxamic acid produced to that of acid determined with the ethyl ester as substrate \((k_5P_2/k_5\) is also known). With the hydroxamic acid as substrate, \(k_5 \ll k_5\) (also \(k_5 < k_5\)), and therefore the partial reversion of the acyl-enzyme to hydroxamic acid shows up as an inhibition of the rate of hydrolysis. The inhibition should be precisely the same as the fraction of hydroxamic acid formed from an ester. We thought that the failure to observe this identity might have arisen from insufficient enzyme during the ester hydrolysis,\(^2\) and we therefore made measurements at high enzyme concentrations.

The steady state solution for the velocity derived from the scheme for high enzyme concentrations follows the Michaelis-Menten equation with

\[
V_{\text{max}} = \frac{k_4k_5E^3}{k_1 + k_5 + k_5P_1 + k_5P_2\text{NH}_2\text{OH}/k_1}
\]

\[
K_m = \frac{(k_5 + k_5)(k_5P_1 + k_5P_2\text{NH}_2\text{OH})}{k_5 + k_5 + k_5P_1 + k_5P_2\text{NH}_2\text{OH}/k_1}
\]

The easiest quantity to measure precisely is \(V_{\text{max}}/K_m\). It is the slope of a semilogarithmic plot of \(S\) versus \(t\) when \(S \ll K_m\). The expected result is

\(\text{Table I}\)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Fraction of ester converted to hydroxamic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-L-tyrosine ethyl ester, pH 6.6,</td>
<td></td>
</tr>
<tr>
<td>25(^\circ), (\Gamma/2 = 1) M, 1 M NH(_2)OH</td>
<td>6.0 \times 10^{-1} 0.268</td>
</tr>
<tr>
<td></td>
<td>1.6 \times 10^{-2} 0.313</td>
</tr>
<tr>
<td></td>
<td>8 \times 10^{-4} 0.500</td>
</tr>
<tr>
<td></td>
<td>1.0 \times 10^{-4} 0.544</td>
</tr>
<tr>
<td>Acetyl-L-tyrosine ethyl ester, pH 8.50,</td>
<td></td>
</tr>
<tr>
<td>25(^\circ), 5 M NaCl, 2 M NH(_2)OH</td>
<td>2.4 \times 10^{-1} 0.510</td>
</tr>
<tr>
<td></td>
<td>1.2 \times 10^{-1} 0.500</td>
</tr>
<tr>
<td></td>
<td>1.2 \times 10^{-1} 0.628</td>
</tr>
</tbody>
</table>

\(1\) The fraction of ester converted to hydroxamic acid equals

\[
\frac{k_4(\text{NH}_2\text{OH})}{k_5 + k_4(\text{NH}_2\text{OH})}
\]

at high enzyme concentrations and

\[
\frac{(k_4 + k_5)(\text{NH}_2\text{OH})}{k_5 + (k_4 + k_5)(\text{NH}_2\text{OH})}
\]

at low enzyme concentrations.

\(2\) The importance of enzyme concentration has also been recognized by Kezdy, Clement, and Bender and by Caplow and Jencks in this Journal.
The studies with acetyl-L-tyrosine hydroxamic acid were carried out at pH 6.6, 25°, p/2 = 1 M, with an enzyme concentration of 0.24 mg per ml. The studies with L-tyrosine hydroxamic acid were carried out at pH 8.5, 25°, 3 M NaCl, with an enzyme concentration of 2.3 mg per ml. The enzyme used with L-tyrosine hydroxamic acid was Worthington chromatographically pure; all other experiments were with Worthington three times crystallized α-chymotrypsin.

<table>
<thead>
<tr>
<th>Molarity of hydroxamic acid</th>
<th>Fraction of ester converted to hydroxamic acid</th>
<th>1/</th>
<th>Vmax/Km</th>
<th>(Vmax/Km)/ (Vmax/Km)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl-L-tyrosine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydroxamic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.340 ± 0.009</td>
<td>1.51</td>
<td>0.0484 ± 0.0013</td>
<td>1.47</td>
</tr>
<tr>
<td>1</td>
<td>0.502 ± 0.006</td>
<td>2.01</td>
<td>0.0283 ± 0.0012</td>
<td>1.05</td>
</tr>
<tr>
<td>2</td>
<td>0.613 ± 0.013</td>
<td>2.58</td>
<td>0.0208 ± 0.0006</td>
<td>2.63</td>
</tr>
<tr>
<td>L-Tyrosine hydroxamic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.437 ± 0.007</td>
<td>1.78</td>
<td>0.0139 ± 0.0005</td>
<td>1.74</td>
</tr>
<tr>
<td>0.5</td>
<td>0.610 ± 0.006</td>
<td>2.57</td>
<td>0.0100 ± 0.0001</td>
<td>2.42</td>
</tr>
<tr>
<td>1</td>
<td>0.708 ± 0.014</td>
<td>4.31</td>
<td>0.00370 ± 0.0000</td>
<td>4.24</td>
</tr>
</tbody>
</table>

* The expected value is 1 + (kP1/k0); see the text.

\[
\frac{V_{\text{max}}/K_{\text{m}}}{V_{\text{max}}/K_{\text{m}}} = 1 + \frac{kP_1}{k_0}
\]

assuming \( k_1 \gg k_0 \), where the subscript \( e \) indicates measurements in the absence of hydroxylamine. Using the values of \( kP_1/k_0 \), the ratio of hydroxamic acid formed to acid formed, determined with the ethyl ester at high enzyme concentrations, we find very good agreement between theory and experiment (Table II). The experiments with acetyltirosine hydroxamic acid were done at pH 8.5 because we found interference from the reversibility of the hydrolysis of the hydroxamic acid at pH 6.6. At this pH, the apparent equilibrium constant is

\[
\text{Total hydroxamic acid} / \text{Total acid} \times \text{Total hydroxylamine} = 0.09
\]

In essence, our results prove that the ratio of hydroxamic acid produced to acid is the same for an ester and the corresponding hydroxamic acid, at least at low substrate concentrations, and they thereby extend the previous work with hippurates over a much wider range in reaction velocity; the ester is hydrolyzed 100 times more rapidly than the hydroxamic acid.

With the use of high enzyme concentrations to reduce the relative importance of nonenzymic side reactions, we have shown that the incisive experiment devised by Caplow and Jencks substantiates the acyl-enzyme theory when work is done with low substrate concentrations. However, these authors present in this Journal data obtained by them at high substrate concentrations which do not fit the requirements of the theory well enough to be considered to substantiate the theory, even though high enzyme concentrations were used. It is clear that if we are unable to explain their data in a manner consistent with the acyl-enzyme theory, the status of the theory with regard to hydroxamates will once again be uncertain.

A similar treatment of this problem is also given in this Journal by Kényt, Clement, and Bender.

REFERENCES


The Apparent Absence of an Acyl-enzyme Intermediate in Certain Chymotrypsin-catalyzed Reactions—A Correction*

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From the Graduate Department of Biochemistry, Brandeis University, Waltham 54, Massachusetts

(Received for publication, June 10, 1963)

It was recently reported that experiments designed to trap the presumed common acyl-enzyme intermediate formed in the chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester and N-acetyl-L-tyrosine hydroxamic acid with hydroxylamine gave different results with the two substrates, and it was concluded that these differences were not consistent with the acyl-enzyme mechanism, as it is ordinarily formulated, for the reactions of both of these substrates (1). Since such trapping experiments require that the experimental conditions be identical for both substrates, careful attention was paid to the effects of variations in buffer, ionic strength, pH, and the concentrations of substrate and hydroxylamine on the trapping, and it was shown either that these conditions were the same in both experiments or that small variations did not cause a change in the trapping. However, one variable that was not controlled in the two experiments was the enzyme concentration, because the experiments with ATE involved the measurement of reaction rates which were 100 to 1000 times larger than those observed with ATH.

It has now been found that the yield of ATH formed in the chymotrypsin-catalyzed reaction of ATE in the presence of a constant concentration of hydroxylamine is a function of the enzyme concentration (Table I). Although the rates of hydrolysis and hydroxylaminolysis of ATE are too fast to measure directly at the higher enzyme concentrations, the fraction of hydroxylaminolysis, determined from the fraction of ATH formed at the end of the reaction, decreases markedly with increasing

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† National Institutes of Dental Research Postdoctoral Fellow.

The abbreviations used are: ATE, acetyl-L-tyrosine ethyl ester; ATH, acetyl-L-tyrosine hydroxamic acid.
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