The yield of hydroxamic acid was determined according to the procedure of Caplow and Jencks (4) by stopping the reaction after completion of the enzymatic hydrolysis of the ethyl ester. Under these conditions less than 2% of the hydroxamic acid formed was hydrolyzed by the enzyme. Experimental conditions: 25°, 0.2 M Tris-HCl buffer.

<table>
<thead>
<tr>
<th>Free NHOH</th>
<th>pH</th>
<th>$E_0 \times 10^4$</th>
<th>$S_0 \times 10^4$</th>
<th>$\Delta V/2$</th>
<th>Yield of acetyl-L-tyrosine (H-dioxyacetic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.423</td>
<td>0.10</td>
<td>90.5</td>
<td>5.00</td>
<td>0.8</td>
<td>18.5</td>
</tr>
<tr>
<td>0.423</td>
<td>6.16</td>
<td>1.49</td>
<td>6.01</td>
<td>0.8</td>
<td>39.1</td>
</tr>
<tr>
<td>0.8</td>
<td>8.00</td>
<td>94.4</td>
<td>4.93</td>
<td>2.6</td>
<td>30.0</td>
</tr>
<tr>
<td>0.8</td>
<td>8.00</td>
<td>9.05</td>
<td>5.03</td>
<td>2.6</td>
<td>36.7</td>
</tr>
<tr>
<td>0.8</td>
<td>8.00</td>
<td>0.97</td>
<td>5.04</td>
<td>2.7</td>
<td>44.6</td>
</tr>
<tr>
<td>0.6</td>
<td>7.22</td>
<td>1.95 - 2.48</td>
<td>5.05</td>
<td>2.7</td>
<td>50†</td>
</tr>
<tr>
<td>0.8</td>
<td>8.27</td>
<td></td>
<td>5.05</td>
<td>2.7</td>
<td>49†</td>
</tr>
</tbody>
</table>

*Enzyme solutions were spectrophotometrically titrated with cinnamoylimidazole.
† From (4).

(from $V_N/V_W$) and the hydroxamic acid (from the decrease of $V_{max}$) found previously (4) bear out prediction a. The free enzyme in these experiments may be calculated from the equation $K_m$ (app)/$S = \text{free enzyme}/\text{bound enzyme}$. With use of stated values of $K_m$ (app) and $S$ (4), this ratio is 0.14 for acetyl-L-tyrosine ethyl ester and 0.31 for acetyl-L-tyrosine hydroxamic acid. Thus, for a given $E_0$, more free enzyme will exist in the reaction of the hydroxamic acid than in the reaction of the ester. Furthermore, the absolute concentration of the $E_0$ was 500-fold greater in the former experiment (9). Therefore, the difference in free enzyme in the two experiments was over 1000, leading to the observed differences in partitioning. Prediction a is further borne out by the experiments listed in Table I, describing the partitioning in the simultaneous hydrolysis and hydroxylaminolysis of acetyl-L-tyrosine ethyl ester at 100-fold different enzyme concentrations. Prediction b is borne out by the pH dependence of the partitioning in this reaction found previously (4) (see Table I). Prediction c is borne out by the small dependence of the partitioning of this reaction on substrate concentration.

Although chymotrypsin-catalyzed hydrolysis and hydroxylaminolysis reactions are complicated (Equation 3 may not be a full description), their main features are amenable to interpretation in terms of a mechanism involving an acyl-enzyme intermediate. In contrast to reactions involving hydroxylamine as an added nucleophile, reactions involving methanol as an added nucleophile are straightforward kinetically and chemically, and also give results which are compatible with the acyl-enzyme hypothesis (10).

Both Caplow and Jencks (9) and Epand and Wilson (11) have independently found that a higher enzyme concentration leads to a lower ratio of hydroxamic acid. The latter workers also propose the theory embodied in Equation 3 to explain this result.

*W. P. Jencks, personal communication.

The Kinetics of α-Chymotrypsin-catalyzed Hydrolysis and Alcoholysis*

Myron I. Bender, Gerald E. Clement, Claude R. Gunter, and Ferenc J. Kézdy

From the Department of Chemistry, Northwestern University, Evanston, Illinois

(Received for publication, June 20, 1963)

Since the suggestion of Koshland and Herr (1) that water analogues be used to determine the role of water in enzymatic reactions, many such investigations have been carried out with α-chymotrypsin. Alcohols have been the favorite water analogues. In contrast to dipolar aprotic organic solvents (2), alcoholic nucleophiles can participate directly in chymotrypsin reactions. For example, Balls and Wood found an increase in the rate constant of deacylation of acetyl-α-chymotrypsin in the presence of ethanol and isolated ethyl acetate from the reaction mixture (3). Bender and Glasson found that the exchange reaction between N-acetyl-L-phenylalanine methyl-14C ester and unlabeled methanol of the solution was catalyzed by α-chymotrypsin (4). Thus it is seen that alcohols participate as nucleophiles in chymotrypsin-catalyzed reactions of both non-specific and specific substrates, and indeed are water analogues.

Henceforward, the place of alcohols in the chymotrypsin mechanism has not been specified. In the preceding communication (5), the added nucleophile, hydroxylamine, was shown to participate as a competitor of water for the acyl-enzyme intermediate. In the present communication, it will be shown that the added nucleophile, alcohol, also participates as a competitor of water for the acyl-enzyme intermediate, without the complications pursuant to hydroxylamine.

The effect of methanol on the kinetics of a discrete deacylation

* This research was supported by grants from the National Institutes of Health. This is the 22nd paper in the series dealing with the mechanism of action of proteolytic enzymes.
† Alfred P. Sloan Foundation Research Fellow.
‡ National Institutes of Health Postdoctoral Fellow.
step, the deacylation of cinnamoyl α-chymotrypsin, was first investigated. In methanol-water solutions at pH 8.5, the deacylation of cinnamoyl α-chymotrypsin was shown spectrophotometrically to produce cinnamate ion and methyl cinnamate. The partitioning of the acyl-enzyme to carboxylic acid ion and methyl ester can be envisioned kinetically in two ways: (a) competitive reactions of water and methanol from solution (Equation 1) or (b) competitive formation of complexes of acyl-enzyme and water or methanol followed by reaction (Equation 2).

\[\begin{align*}
E + S &\rightarrow ES^- \\
\text{ES'} &\rightarrow E + P_2 \\
\text{ES'W} &\rightarrow E + P_2 \\
\text{ES'M} &\rightarrow E + P_2 \\
\text{ES} &\rightarrow E + P_2 \\
\end{align*}\]

Equation 3 predicts that \(k_{obs} = k_1[H_2O] + k_2[MeOH]\) will be a linear function of the methanol concentration; Equation 4 predicts that \(k_{obs} = k_3[H_2O]/K_W + k_4[MeOH]/K_M\) will in general not be a linear function of the methanol concentration but rather that a saturation by methanol will be observed at some point. Experimentally the former is found (see Fig. 1). Spectrophotometric analysis of the products allows the calculation of \(k_1 = k_1[H_2O]\) and \(k_2 = k_2[MeOH]\). Both \(k_1\) and \(k_2\) are linear functions of methanol concentration, the latter because the water concentration is a linear function of the methanol concentration; furthermore, \(k_3\) and \(k_4\) are independent of methanol concentration. Thus the predictions of Equations 1 and 3 are found experimentally. The ratio of reactivities of water and methanol (\(k_1/k_2\) in 5% methanol) in the solvolysis of cinnamoyl α-chymotrypsin, involving intrinsic general base catalysis (8), and of \(p\)-nitrophenyl 5-nitrosalicylate, a model system involving intramolecular general base catalysis (7), are 0.47 and 0.36, respectively. Both processes presumably proceed by similar mechanisms, and the water to methanol reactivity bears out this hypothesis. Thus no kinetic evidence at present requires the presence of a complex between the nucleophile and the acyl-enzyme, and these data are in accord with a simple competition for the acyl-enzyme between methanol and water from solution.1

Since methanol reacts with the acyl-enzyme in a completely straightforward way, we investigated the effect of alcohol on the α-chymotrypsin-catalyzed hydrolysis of specific substrates under normal enzymatic conditions (\(E_0 \ll S_0\), measuring initial rates). By expanding Equation 1, the complete enzymatic reaction may be written

\[\frac{k_2[H_2O]}{k_2[H_2O] + k_3[H_2O] + k_4[MeOH]} E + S \rightarrow ES^- \rightarrow P_2\]

where \(P_2\) represents an alcoholic portion of the ester substrate, \(S\); \(P_2\) represents the carboxylic acid; and if the nucleophil \(N\) is the same as \(P_2\), then \(P_2\) must be equivalent to \(S\) (enzyme-product complexes are omitted). From Equation 5, Equations 6 and 7 can be derived under the usual steady state assumption.

\[\begin{align*}
dP_2/dt &\frac{k_2(k_3' + k_2' + k_4')}{k_3' + k_2' + k_4'} E + S \\
\end{align*}\]

The catalytic rate constant (\(k_{cat}\)) is equal to the complex constant in the numerator of each equation, and the apparent \(K_m(\text{app})\) is equal to \(K_m \times \text{the complex constant in the denominator. Although Equations 6 and 7 are not tractable, there are five simplifying conditions which lead to predictable and testable equations of all known data.

1. If one measures \(dP_1/dt\) and if \(k' > k_0\), then \(k_{cat} = k_1\) and \(K_{m(\text{app})} = K_m\). The chymotrypsin-catalyzed hydrolyses of \(N\)-acetyl-d-tyrosine amide (9) and of \(\text{glycyl-L-tyrosine amide}\) (10) are predicted to fall in this category.2 In agreement with prediction, in the former reaction, \(k_{cat}\) is independent of methanol concentration and \(K_m\) increases slightly with methanol concentration, whereas in the latter reaction, \(k_{cat}/K_m\) decreases slightly with methanol concentration.3 If the amide were to bypass the acyl-enzyme and react directly with water or methanol, \(k_{cat}\) should be dependent on either the water or the methanol concentration as the deacylation of cinnamoyl chymotrypsin is (Fig. 1). Since 20.8% methanol does not decrease \(k_{cat}\) of \(N\)-acetyl-d-tyrosine amide, the amide reaction must follow Equation 5.

2. If one measures \(dP_2/dt\) and if \(k_3' \gg k_0\), then at low \(N\), \(k_{cat} = k_1\) and as \(N\) increases, \(k_{2} = k_{cat}\). This conclusion predicts a change in the rate-determining step under these conditions which obtained in a study of the α-chymotrypsin-catalyzed hydrolysis of \(p\)-nitrophenyl acetate (11). In agreement with prediction, \(k_{cat}\) is approximately 24 times smaller than \(k_2\) at 0% methanol whereas \(k_{cat} = k_2\) at 0.25 mole fraction of methanol.

3. If one measures \(dP_2/dt\) and if \(k' \gg k_0\), \(k_{cat}\) can be expressed

1 Jencks and Caplow found linearity in the disappearance of furfuryl chymotrypsin with increasing methanol concentration and have independently reached the conclusion that this evidence provides no support for a binding site for the acyl acceptor in this reaction (personal communication from Dr. Jencks).

2 This conclusion is reached by analogy with the kinetic constants and the pH-rate profile in the hydrolysis of acetyl-l-tryptophan amide (8).

3 The slight increase in \(K_m\) and slight decrease in \(k_{cat}/K_m\) may be, and have been, explained on the basis of known competitive inhibition and dielectric effects caused by methanol (2).
yielding results which indicate that $k_2 = 0.49 \text{ sec}^{-1}$ and $k_0 = 0.32 \text{ sec}^{-1}$. In opposition to these results, Bernhard, Coles, and Nowell (14) claim that the kinetics of the hydrolysis and hydroxylaminolysis of methyl hippurate is not compatible with a scheme such as Equation 5. However, on the basis of the preceding communication (5), the use of hydroxylamine as a water analogue must be carefully scrutinized.

In summary, the data employing alcohols as added nucleophils show wide variation in the observed effects, but all known observations may be correlated simply by use of the hypothesis, embodied in Equation 5, of the partitioning of an acyl-enzyme intermediate by water and alcohol. It is not apparent how a one-step mechanism without an acyl-enzyme could account for the wide variations in the effect of alcohols on the kinetics. On the other hand, universal applicability of Equation 5 strongly

**Table I**

<table>
<thead>
<tr>
<th>Derivative</th>
<th>$k_2/k'$</th>
<th>$k_3/k''$</th>
<th>$K_m$ (M)</th>
<th>$k_d$ (M$^{-1}$ sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amide</td>
<td>12.6</td>
<td>16.6</td>
<td>$5.00 \times 10^6$</td>
<td>474</td>
</tr>
<tr>
<td>Ethyl ester</td>
<td>19.4</td>
<td>17.3</td>
<td>$1.38 \times 10^6$</td>
<td>28</td>
</tr>
<tr>
<td>Methyl ester</td>
<td>19.4</td>
<td>17.3</td>
<td>$1.18 \times 10^6$</td>
<td>28</td>
</tr>
</tbody>
</table>

* The physical constants for the derivatives are those reported in (8).
* From (8).
* In order to calculate the $K_m$, the $K_m$ (app) observed at each alcohol concentration was corrected for the known effect due to competitive inhibition and dielectric effects of the alcohol (6).
* Ethanolysis.
* Methanolysis.
suggests that a two-step mechanism involving an acyl enzyme intermediate is the general reaction path for the hydrolysis of both specific and nonspecific substrates by α-chymotrypsin.

REFERENCES

Benzoate Derivatives as Intermediates in the Biosynthesis of Coenzyme Q in the Rat*

Robert E. Olson, Ronald Bentley, A. S. Aiyar,† G. Honcen Dilamari, Philip H. Gold,‡ Virginia R. Kemai, and C. M. Springer

From the Department of Biochemistry and Nutrition, Graduate School of Public Health, University of Pittsburgh, Pittsburgh 13, Pennsylvania

(Rceived for publication, June 26, 1963)

In the rat, the benzoquinone moiety of coenzyme Q is known to be derived from the phenylalanine acids (1, 2). The detailed intermediary metabolism of phenylalanine leading to the formation of the quinone moiety, however, is unknown. The work reported here indicates that the side chains of phenylalanine and tyrosine are totally lost in the biosynthesis of coenzyme Q, and that certain benzoate derivatives may function as intermediates in the biosynthesis. Further, formate has been established as the source of the ring-bound methyl group as well as the methoxyl methyl groups. These unexpected findings have led to some novel concepts of amino acid metabolism.

The source of the ring-bound methyl group as well as the methoxyl methyl groups. These unexpected findings have led to some novel concepts of amino acid metabolism.

The source of the ring-bound methyl group as well as the methoxyl methyl groups. These unexpected findings have led to some novel concepts of amino acid metabolism.

The details of experiments both in vivo and in vitro have been previously described (3). A number of possible coenzyme Q precursors were administered intraperitoneally to 150-g rats; the animals were killed after 3 hours, and the total body coenzyme Q was then isolated and crystallized with carrier to constant specific activity. As further evidence of radiochemical purity, diacetyl coenzyme Q, hydroquinone was prepared by reductive acetylation and was also crystallized to constant specific activity. These data are presented in Table I. The pure diacetoxy-4',5'-dimethoxy-2'-methylphenylacetic acid (m.p. 137°) was used as a ring fragment and the bis-dinitrophenylhydrazone of levulinaldehyde as a side chain fragment. The relative incorporation of precursor radioactivity into the ring fragment and the side chain were calculated on the assumption that the terminal isopropylidene group had the same relative specific activity as the levulinaldehyde.

The incorporation of label into coenzyme Q from all precursors studied was of the same order of magnitude, ranging from 0.0035% with formate-Cl4 to 0.0220% with L-tyrosine-U-Cl4. Acetate, D-tyrosine C14, and D-phenylalanine C14 label predominantly the polysoprene side chain, whereas L-tyrosine-U-Cl4, L-phenylalanine-U-Cl4, ring-labeled benzoate-C14, and formate-C14 label predominantly the diacetoxyphenylacetic acid fragment. The increased incorporation of label from tyrosine-U-Cl4 as compared with phenylalanine-U-Cl4 into coenzyme Q suggested that benzoate was probably not on the main pathway of coenzyme Q biosynthesis. A possible sequence for ring formation is therefore as follows:

\[
\text{Phenylalanine} \rightarrow \text{tyrosine} \rightarrow \text{p-hydroxybenzoate} \rightarrow \text{coenzyme Q} \rightarrow \text{benzoate}
\]

To gain additional information about the role of p-hydroxybenzoate, "swamping" experiments were conducted in vitro with liver slices. Slices of rat liver from normal albino rats (150 to 200 g) were incubated for 3 hours at 38° in pH 7.4 bicarbonate buffer with 95% O2-5% CO2 as the gas phase. After incubation, the coenzyme Q was isolated from the benzene-purifiable fraction as previously described, and was crystallized with carrier to constant specific activity. The results are shown in Table II. The diluting effect of cold mevalonate on the incorporation of label from acetate-1-C14 into coenzyme Q was dramatic. In contrast, p-hydroxybenzoate had no effect on the incorporation of mevalonate carbon into coenzyme Q. When tyrosine was the source of label, however, p-hydroxybenzoate almost completely inhibited the incorporation of activity, strongly supporting the hypothesis that p-hydroxybenzoate is on the main pathway from tyrosine to coenzyme Q.

Although C-methylations of benzenoid molecules have not heretofore been demonstrated in animal tissues, the above data suggested to us that such a methylation might occur in coenzyme Q biosynthesis. The diacetoxyphenylacetic acid fragment obtained from coenzyme Q labeled with formate-C14 was further degraded to determine the extent to which formate carbon en-
The Kinetics of α-Chymotrypsin-catalyzed Hydrolysis and Alcoholysis

Myron L. Bender, Gerald E. Clement, Claude R. Gunter and Ferenc J. Kézdy


Access the most updated version of this article at http://www.jbc.org/content/238/9/PC3143.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/238/9/PC3143.citation.full.html#ref-list-1