Common Acylcarboxypeptidase A Intermediates for Ester Substrates Containing Different Leaving Alcohols*

(Received for publication, December 30, 1985)

Junghun Suh, Suk-Bong Hong, and Shin Chung

From the Department of Chemistry, Seoul National University, Seoul 151, Korea

In the carboxypeptidase A-catalyzed ester hydrolysis of the trans-α-(benzoylaminocinnamoyl) derivatives of both L-mandelate and L-β-phenyllactate, kcat stands for the breakdown of an additional enzyme-substrate complex (ES'). The pH dependence of kcat indicates that ES' is the anhydride acylcarboxypeptidase A formed by the nucleophilic attack of Glu-270 for both of the ester substrates. Furthermore, the very similar kcat values for the two ester substrates which share a common acyl moiety strongly suggest that common acyl enzyme intermediates are involved, whose breakdown process is little affected by the cleaved alcohol portions. This provides the most direct evidence that has been reported for the nucleophilic mechanism of the carboxypeptidase A action.

In the mechanistic study of carboxypeptidase A (peptidyl-l-amino-acid hydrolase, EC 3.4.17.1), whether the carboxylate of Glu-270 acts as a nucleophile or a general base has been investigated. It is most direct that has been reported.

**EXPERIMENTAL PROCEDURES**

Materials—BACM was prepared according to the method using sodium tert-butoxide which has been employed in the preparation of BACPL (6). The product was recrystallized from 1,2-dichloroethane hexane, m.p. 165-166 °C. Carboxypeptidase A, was purchased as a suspension in toluene/water from Worthington. Preparation of the stock solutions and the assay of the enzyme activity were performed as reported previously (6).

**Kinetic Measurements**—Reaction rates were measured with a Beckman 5260 UV/VIS spectrophotometer. Temperature was controlled within ±0.1 °C with a Lauda Brinkman circulator. pH measurements were carried out with a Fisher Accumet Model 525 pH meter. Buffers used were Mops at pH 5.0-7.0, Hepes at pH 7.5-8.0, and boric acid at pH 8.0-9.5. Buffer solutions contained 0.5 M sodium chloride and 0.05 M buffer. The substrate was converted to the sodium salt prior to the preparation of the stock solutions. The UV spectrum of the product solution of the carboxypeptidase A-catalyzed hydrolysis of BACM was identical with that of the solution containing trans-α-(benzoylaminocinnamate and L-mandelate.

**RESULTS AND DISCUSSION**

The values of kcat and KMapp for the carboxypeptidase A-catalyzed hydrolysis of BACM were estimated from the initial rates (v0) measured under the condition of S0 >> E0, as reported previously (6). The plot of E0/v0 against 1/S0 (Equation 1) leads to the values of kcat and KMapp.

\[
E_0/v_0 = \frac{1}{k_{cat}} + \frac{1}{k_{Mapp}} \left( \frac{1}{S_0} \right)
\]

The pH dependence of kcat at 25 °C is illustrated in Fig. 1. The kinetic parameters for BACM measured at pH 7.5 are compared with those for other substrates in Table I. The existence of an acyl enzyme intermediate can be demonstrated by the isolation and the characterization of the intermediate. Although accumulation of intermediates has been achieved in the carboxypeptidase A-catalyzed hydrolysis of dansyl-containing depsipeptides or oligopeptides at ~0 °C, examination with spectrophotometry, EPR spectroscopy, or spectrofluorometry did not provide any decisive information regarding the structure of the intermediates (9, 10). Therefore, whether the accumulating intermediates were covalent acyl enzyme intermediates or mere enzyme-substrate complexes was not resolved. Trapping of a labile acyl enzyme intermediate with an external trapping reagent or an intramolecular trapping group can also prove the nucleophilic mechanism. Several attempts to trap acylcarboxypeptidase A intermediates have been unsuccessful (6, 11, 12), presumably because the entrance of the trapping reagents into the active site is blocked in the acylcarboxypeptidase A intermediate or because the enzymatic pathway of the breakdown of the inter-
mediates is much faster than the attack of the trapping reagents.

Alternatively, "decomposition at the same rate of a common intermediate formed from different substrates" (13) can provide evidence for the covalent nature of the intermediates. In order to obtain unambiguous results using two different substrates that contain the same acyl moiety, the breakdown of the intermediate must be rate-determining for both of the substrates. Furthermore, the breakdown process should not be effected appreciably by the cleaved leaving groups.

Hydrophobic side chains of the C-terminal portions of the substrates are required for the activity of carboxypeptidase A, and, consequently, the L-β-phenylalactate esters are superior to the L-mandelate esters as substrates for carboxypeptidase A (14). Ester substrates BACM and BACP, therefore, should differ markedly in the reactivity toward carboxypeptidase A, and this is confirmed by the much greater value of $k_{cat}/K_{m app}$ for BACP (Table I). The α-acylamino groups of the ester substrates affect the kinetic behavior significantly. Kinetic results obtained for the p-substituted trans-cinnamoyl esters of L-β-phenylalactate agree with the rate-determining formation of the acylcarboxypeptidase A intermediate (6). Introduction of α-acylamino group to the trans-cinnamoyl derivatives of L-β-phenylalactate moves the rate-determining step to the deacylation step (6). As will be discussed later, the kinetic results of the present study indicate that the deacylation step is also rate-determining in the hydrolysis of L-mandelate ester BACM. Furthermore, the present results reveal that the rates for the deacylation process are comparable for BACM and BACP although the binding to carboxypeptidase A and the acylation rate is much less efficient for BACM.

When the simple Michaelis-Menten scheme is modified to include an additional intermediate ("ES") as in Equation 2, the expressions of steady-state kinetic parameters $k_{cat}$ and $K_{m app}$ become Equations 3 and 4, respectively, as shown previously (6).

$$E + S \rightarrow ES \rightarrow ES' \rightarrow E + P_1$$

$$k_{cat} = k_1k_2/k_3 + k_0 + k_{-3}$$

$$K_{m app} = (k_0k_3 + k_{-1}k_3 + k_1k_{-3})/k_3k_0 + k_5 + k_{-3}$$

When ES' accumulates, $[ES'] > [ES]$ and $k_0 > (k_3 + k_{-3})$. Under these conditions, $k_{cat}$ and $K_{m app}$ are expressed by Equations 5 and 6, respectively.

$$k_{cat} = k_0$$

$$K_{m app} = K_m(k_1 + k_3)/k_3$$

If $K_m$ is approximated to be similar to $k_0 (= k_{-1}/k_1)$, an equilibrium constant relating ES to E and S, as demonstrated in the hydrolysis of dansyl-containing substrates (9), Equation 7 is obtained for the reaction in which ES' accumulates.

$$K_{m app} = K_m(k_0 + k_{-3}/k_3)$$

This can be ascribed to the partial protonation of a histidine residue coordinating to the active-site zinc ion and the consequent partial dissociation of the zinc ion (15).

Fig. 1. The plot of $k_{cat}$ against pH for the carboxypeptidase A-catalyzed hydrolysis of BACM at 25 °C.

Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_{cat}$</th>
<th>$K_{m app}$</th>
<th>$k_{cat}/K_{m app}$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BACM</td>
<td>7.4 s⁻¹</td>
<td>0.74 M</td>
<td>3.8 × 10⁶ s⁻¹</td>
<td>This study</td>
</tr>
<tr>
<td>O-(trans-p-Nitrocin-namoyl)-L-mandelate</td>
<td>5.1 10⁻⁴ M</td>
<td>8.8 6.8 10⁶ s⁻¹</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>O-(p-Nitrobenzoyl)-L-mandelate</td>
<td>1.7</td>
<td>13</td>
<td>1.3 × 10⁵</td>
<td>8</td>
</tr>
<tr>
<td>BACP</td>
<td>0.46</td>
<td>0.0022</td>
<td>2.1 × 10⁶</td>
<td>6</td>
</tr>
</tbody>
</table>

An expression of $K_{m app}$ which is equivalent to Equation 6 is misprinted in Ref. 6 (Table V, case C).

The slightly lower $k_{cat}$ at pH 5.5 was also observed with BACP. This can be ascribed to the partial protonation of a histidine residue coordinating to the active-site zinc ion and the consequent partial dissociation of the zinc ion (15).
Mechanism of Carboxypeptidase A

The process would be subjected to general base catalysis by the modifier. Mediation occurs mainly through a water path. The possibility that the breakdown of the common acyl-enzyme intermediate, 

\[ \text{acids, which were originally taken to support the general base role of Glu-270 (pK}_a = 6-7)^4 \text{ (15-21). If } k_{\text{cat}} \text{ represents the breakdown (} k_b \text{) of the anhydride intermediate (Equation 5), ionization of Glu-270 should not be reflected in the pH profile of } k_{\text{cat}} \text{ as is observed in the pH profiles of BACM as well as BACPL. The pH independence of } k_{\text{cat}} \text{ in the alkaline range further indicates that the breakdown of the anhydride intermediate occurs mainly through a water path. }

The } k_{\text{cat}} \text{ values for BACM and BACPL are not much different (Table I), despite the large difference in the substrate specificity manifested by } k_{\text{cat}}/K_{\text{m,app}}. \text{ This indicates that the breakdown of the common acyl-enzyme intermediate, trans-\(\alpha\)-(benzoylamino)cinnamoylcarboxypeptidase A, is little affected by the nature of the cleaved alcohol portions. As mentioned above, the anhydride intermediate is hydrolyzed mainly by the attack of a water molecule. The possibility that this process would be subjected to general base catalysis (Scheme 1) by the cleaved leaving group has been noted (5, 23). In addition, the results of } ^{18}\text{O}-\text{exchange experiments with a dipeptide in the presence of added amino acids or hydroxy acids, which were originally taken to support the general base role of Glu-270 (4, 24), were explained in terms of the very efficient general base catalysis by amino acids (} XH = NH}_2 \text{) in Scheme 1 (5). Since the hydrophobic side chain (} R \text{) would interact with the hydrophobic pocket in Scheme 1, the distance between the basic group (} XH \text{) and the reaction center in Scheme 1 would be different for the benzyl and phenyl side chains. Similar } k_{\text{cat}} \text{ values for BACM and BACPL, therefore, suggests that the general base catalysis in Scheme 1 is not very effective for ester substrates (} XH = OH \text{). }

REFERENCES


---

4 The pK_a of the Glu-270 carboxyl group has been measured in the study of its inactivation by affinity labeling with N-bromosuccinyl-N-methyl-L-Phe (22). Thus, the pK_a of about 7.9 was observed for the ionization of Glu-270 in the complex (EI) of carboxypeptidase A with the modifier.