Evidence for an Essential Histidine in Carboxypeptidase Y.

REACTION WITH THE CHLOROMETHYL KETONE DERIVATIVE OF BENZYLXOXYCARBONYL-L-PHENYLALANINE*

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The possible role of histidine residues in the catalytic function of carboxypeptidase Y from bakers' yeast has been investigated using site-specific reagents. Among the reagents tested, benzyloxy-L-phenylalanylchloromethane (Z-PheCH₂Cl) was the most powerful inhibitor of the enzyme. It irreversibly inactivated both the peptidase and esterase activities with an apparent second order rate constant of 3.8 M⁻¹ s⁻¹; the D isomer caused essentially no effect on either activity. Inhibition by L-Z-PheCH₂Cl was retarded by certain competitive inhibitors of the enzyme. Using radioactive L-Z-PheCH₂Cl, the reaction with the enzyme was shown to be essentially stoichiometric. Diisopropylphosphorofluoridate (iPr₂PF)-inactivated enzyme failed to react with Z-PheCH₂Cl, and conversely, the Z-PheCH₂Cl-inhibited enzyme failed to react with radioactive iPr₂PF. Amino acid analyses of the Z-PheCH₂Cl-inactivated enzyme revealed the loss of essentially 1 residue, with a concomitant yield of a 0.62 residue of N'-carboxymethylhistidine. Since carboxypeptidase Y has a reactive serine at its active center, we concluded from these results that the mechanism involves a charge-relay system in the hydrolysis of peptide and ester substrates, as in chymotrypsin. An —SH group of carboxypeptidase Y was not affected during the reaction with L-Z-PheCH₂Cl.

The generic name "serine carboxypeptidase" has been proposed for carboxypeptidase Y and for the iPr₂PF-sensitive carboxypeptidases from plants, molds, and animal tissues, in order to distinguish them from "metal carboxypeptidases" to which carboxypeptidase A (EC 3.4.12.2) and B (EC 3.4.12.3) belong.

Carboxypeptidase Y has been obtained from bakers’ yeast and characterized as an enzyme of broad specificity (2–5). Its ability to release proline is especially useful for structural studies of proteins and peptides (3, 6). The active site of this enzyme also appears to be unique. The enzyme has no essential metals (10), but has a serine hydroxyl at the active center and, thus, differs from the pancreatic carboxypeptidases A and B. The uniqueness of carboxypeptidase Y has also been shown by its strong esterase activity toward the substrates of chymotrypsin, i.e. Ac-Tyr-OEt (2, 3), in contrast to the pancreatic enzymes which hydrolyze only ester substrates with a free carboxyl group in the leaving group, i.e. Bz-Gly-β-phenyllactic acid for A (7), and Bz-Gly-arginic acid for B (8). Thus, carboxypeptidase Y seems to be quite similar to chymotrypsin at its active site and in its mechanism, although the former enzyme is an exopeptidase, whereas the latter is an endopeptidase.

When we began this study, however, the following features made it difficult to define unequivocally carboxypeptidase Y as a serine enzyme. First, the enzyme displays activity at acidic pH values (4), whereas serine enzymes usually are active in the alkaline region. Second, as judged from the stoichiometric inhibition by p-HMB and chemical analyses of the enzyme (9), the enzyme has an —SH group, the functional role of which remains to be clarified.

In spite of these differences, however, the high reactivity of a serine residue is common to and the most prominent feature of both carboxypeptidase Y (10) and the serine enzymes (11). Thus, it was advisable to explore the structure of the active center of carboxypeptidase Y using methods analogous to those applied to serine enzymes. In the serine enzymes, a serine residue of the active center is linked in a charge-relay system to the imidazole ring of a histidine and the carboxylate anion of an aspartic acid enhancing the nucleophilicity of the serine hydroxyl (12). Reaction with site-specific reagents toward the serine enzyme, i.e. iPr₂PF and PhCH₂SO₃F for the serine residue and chloromethyl ketone reagents such as Tos-PheCH₂Cl and Tos-LysCH₂Cl (13) for the histidine, has consistently shown the mechanism of the charge-relay system.

To explore the possible role of histidine residues in carboxypeptidase Y, its inhibition by chloromethyl ketone reagents has been studied, especially with respect to the effects on the
enzyme of the chloromethyl ketone derivative of benzyloxy-carbonyl-L-phenylalanine (Z-PheCH₂Cl). Evidence is presented showing that the inactivation by Z-PheCH₂Cl is the result of specific alkylation of a histidine residue of the enzyme.

EXPERIMENTAL PROCEDURE

Materials—Carboxypeptidase Y (Lot OV73 11) was prepared from bakers' yeast (Oriental Yeast Co.) as described previously, then lyophilized (3, 10). Z-Phe-Leu* was purchased from Fluka, and Ac-Tyr-OEt and Ac-Phe-OEt from the Protein Research Foundation. Z-A-Trp-Leu, Leu-Phe, and Ac-Phe-OEt had previously been synthesized (5). iPr₂P and PhCH₂SO₂F were obtained from Sigma, and hydrochloric acid (28%) was prepared by dissolving the acid in isopropyl alcohol. [1,3,3,3]²IP₂ was prepared in propylene glycol (specific activity 0.6 mCi/mg) purchased from New England Nuclear, and U-¹⁴Cphenylalanine (specific activity 477 mCi/mmol) from the Radiochemical Centre. Tos-PheCH₂Cl was obtained from Nakarai Chemicals. Z-PheCH₂Cl was purchased from Fox Chemicals or synthesized as described below. Z-Ala-PheCH₂Cl and Z-Ala-Gly-PheCH₂Cl were kindly supplied by Drs. K. Morihara and T. Oka of the Shionogi Research Laboratories (14). Stock solutions (0.5 mM) of the chloromethyl ketone reagents were prepared by dissolving them in methanol. A mixture of N⁺, N⁻, and N⁺N⁻·Cm-histidines was prepared according to Crestfield et al. (1b) and was used, without purification, to identify each component on amino acid analyses.

Synthesis of L-Z-PheCH₂Cl, D-Z-PheCH₂Cl, and L-[¹⁴C]Z-PheCH₂Cl—The L isomer of Z-PheCH₂Cl was synthesized similarly to the chloromethyl ketone of Z-Lys by the method of Coggins et al. (18). Z-L-Phe (1.7 g) in tetrahydrofuran (6 ml) was converted into the mixed anhydride at -10° by the addition of triethylamine (0.9 ml) and ethyl chloroformate (0.64 ml). Then, an excess of cold ethereal diazomethane (84 ml of 0.16 M diazomethane) was added. After being maintained for 30 min at 0°, the mixture was washed with 0.1 M acetic acid and saturated aqueous NaHCO₃. The ethereal layer was dried over MgSO₄, filtered, and concentrated. Z-PheCH₂Cl was crystallized from ethanol (yield 1.2 g, m.p. 103-105°, literature m.p. 99-102°). The D isomer of Z-PheCH₂Cl was synthesized as described above, and concentrated from ethanol (m.p. 101-103°). Measurement of the optical rotations of the L-Z-PheCH₂Cl and D-Z-PheCH₂Cl synthesized revealed that the reagents were true enantiomers, having equal rotations of opposite sign ([α]₁₃₀ = -346 and +346, respectively, in 95.9% ethanol).

Z-L-¹⁴C Phe was synthesized from [U-¹⁴C]phenylalanine and benzyloxy-carbonyl chloride in the conventional manner, and was used for synthesis without crystallization. The radioactive phenylalanine (25 μCi) had been previously mixed with normal phenylalanine (33 mg). Z-L-Phe (1.7 g) in tetrahydrofuran (6 ml) was converted into the mixed anhydride at -10° by the addition of triethylamine (0.9 ml) and ethyl chloroformate (0.64 ml). Then, an excess of cold ethereal diazomethane (84 ml of 0.16 M diazomethane) was added. After being maintained for 30 min at 0°, the mixture was washed with 0.1 M acetic acid and saturated aqueous NaHCO₃. The ethereal layer was dried over MgSO₄, filtered, and concentrated. Z-PheCH₂Cl was crystallized from ethanol (yield 1.2 g, m.p. 103-105°, literature m.p. 99-102°). The D isomer of Z-PheCH₂Cl was synthesized as described above, and concentrated from ethanol (m.p. 101-103°). Measurement of the optical rotations of the L-Z-PheCH₂Cl and D-Z-PheCH₂Cl synthesized revealed that the reagents were true enantiomers, having equal rotations of opposite sign ([α]₁₃₀ = -346 and +346, respectively, in 95.9% ethanol).

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RESULTS

Effects of Various Chloromethyl Ketone Reagents on Carboxypeptidase Y Activity—The effects of chloromethyl ketone derivatives of Tos-Phe, Z-Phe, Z-Ala-Phe, and Z-Ala-Gly-Phe on the peptidase and esterase activities of carboxypeptidase Y were tested as a 20-fold molar excess of reagents to protein for 8 hours of incubation. After 8 hours, Tos-PheCH₂Cl, Z-PheCH₂Cl, and Z-Ala-Gly-PheCH₂Cl showed about 15%, 50%, and 25% inactivation by both assays, respectively, while Z-Ala-PheCH₂Cl had essentially no effect on either activity. The inactivation apparently followed first order kinetics.

The second order rate constants for inactivation were calculated by dividing the apparent first order rate constants by the inhibitor concentrations. The constants are shown in Table I and compared with those for chymotrypsin (11). Z-PheCH₂Cl was the most effective inhibitor for carboxypeptidase Y. Although the inactivation rate was somewhat slower than that with chymotrypsin, it was much faster than that with a model compound, acetylhistidine (4.5 x 10⁻⁴ M⁻¹ s⁻¹) (25). Replacement of the Z group by Tos resulted in a weaker inhibition of the enzyme.

Inhibition by Z-PheCH₂Cl: Effects of Its Enantiomer, Reversible Inhibitors, and pH—The first order rate plot for inactivation by the L isomer of Z-PheCH₂Cl is shown in Fig. 1. Here, 85% inactivation was observed after 22 hours. Parallel losses in both peptidase and esterase activities occurred. The inactivation was irreversible, as evidenced by the fact that the inactivated enzyme regained no activity when diluted or dialyzed against water. The D isomer of Z-PheCH₂Cl caused essentially no inactivation (Fig. 1).

The presence of competitive inhibitors of carboxypeptidase Y, Z-Phe-d-Leu, Ac-Phe-OEt, and trans-cinnamic acid, at concentrations equal to or greater than their respective K values (5), reduced the inactivation rate by L-Z-PheCH₂Cl in both peptidase and esterase assays (Fig. 2).

The pH dependency of the second order rate constant for the inactivation by L-Z-PheCH₂Cl revealed a broad bell-shaped curve with maximum at pH 5.5 to 6.5 (Fig. 3).

Stoichiometry and the Site of the Reaction of Z-PheCH₂Cl—When L-¹⁴C Z-PheCH₂Cl was mixed with carboxypeptidase Y in 10-fold molar excess, the radioactivity incorporated increased with time of incubation and in parallel with the disappearance of enzyme activity (Fig. 4). By extrapolation to total inactivation, the radioactivity bound to the enzyme was estimated as 0.9 mol of radioactive Z-PheCH₂Cl/mol of enzyme. This value essentially accounts for a stoichiometric reaction between Z-PheCH₂Cl and the enzyme, when one corrects for the loss of activity due to denaturation during the prolonged incubation (see under "Experimental Procedure"). (The loss has tentatively been corrected as a dashed line in Fig. 4.)

* When this manuscript was written, Kuhn et al. (24) mentioned the inhibition of carboxypeptidase Y by Tos-PheCH₂Cl.
TABLE I
Rate of carboxypeptidase Y and chymotrypsin inactivation by some active site-directed reagents at 25° and pH 7

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Carboxypeptidase Y</th>
<th>Chymotrypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k (M⁻¹ s⁻¹)</td>
<td></td>
</tr>
<tr>
<td>Tos-PheCH₂Cl</td>
<td>1.6⁵</td>
<td>7.7⁵</td>
</tr>
<tr>
<td>Z-PheCH₂Cl</td>
<td>3.8⁴</td>
<td>60⁶ (11)⁶</td>
</tr>
<tr>
<td>Z-Ala-PheCH₂Cl</td>
<td>0⁶</td>
<td>(1.4)⁶</td>
</tr>
<tr>
<td>Z-Ala-Gly-PheCH₂Cl</td>
<td>0.6⁵</td>
<td>(100)⁶</td>
</tr>
<tr>
<td>iPr,PF</td>
<td>93.3⁶</td>
<td>45⁵</td>
</tr>
<tr>
<td>PhCH₂SO,F</td>
<td>16.1⁵</td>
<td>248⁸</td>
</tr>
</tbody>
</table>

⁴Carboxypeptidase Y (2.3 × 10⁻⁶ M) and chloromethyl ketone reagents (4.8 × 10⁻⁵ M) were incubated at 25° in 0.09 M sodium phosphate, pH 7.0, containing 9.1% methanol. Aliquots of 20 and 50 µl were removed for the activity assay with Z-Phe-Leu and Ac-Tyr-OEt, respectively. Inactivation was followed for 8 hours with aliquots removed at 2-hour intervals. Peptidase and esterase activities were lost in parallel during incubation with the respective inhibitor. About a 5% loss of activity was observed during incubation for 22 hours in the absence of inhibitors. This loss of activity was excluded from calculations.

Ref. 11.


Ref. 10.

Amino acid analyses were performed with native and l-Z-PheCH₂Cl-treated carboxypeptidase Y after performic acid oxidation. The latter enzyme lost about 90% of its activity during incubation with Z-PheCH₂Cl. Results are shown in Table II. Essentially no difference was observed with respect to the amino acid content of the two enzymes, except for the content of histidine. Carboxypeptidase Y appeared to lose 1 of the 8 histidine residues through the reaction with Z-PheCH₂Cl. This was further confirmed as follows.

Tos-PheCH₂Cl-inhibited chymotrypsin gives rise to N'-histidine upon performic acid oxidation. The yield is much less than 1 mol/mol of the enzyme, since oxidation of the ketone group may occur at either side of the α carbon atom (26). Performic acid oxidation of Z-PheCH₂Cl-inhibited carboxypeptidase Y gave 0.62 residue of N'-Cm-histidine (Table II). Neither N'-nor N''-Cm-histidine was detected on amino acid analyses. Along with the loss of approximately 1 histidine residue, this constitutes evidence that inactivation of the enzyme by Z-PheCH₂Cl proceeds through the alkylation of a single histidine residue at the N' position.

A methionine residue of α-chymotrypsin is alkylated by chloromethyl ketone reagents (27). However, amino acid analyses of Z-PheCH₂Cl-treated carboxypeptidase Y after performic acid oxidation revealed that the methionine content was practically unchanged, as determined from methionine sulfone (Table II). The reaction site of chloromethyl ketone reagents has also been reported to be a cysteine residue in thiol enzymes (28-31). However, the -SH group of carboxypeptidase Y was not affected during the reaction with Z-PheCH₂Cl, as judged from the Ellman reaction and from determination of S-Cm-cysteine (Table II). This was further confirmed by the constant content of cysteic acid in the native and Z-PheCH₂Cl enzymes. The tyrosine content was also not affected during the reaction with Z-PheCH₂Cl, as judged from amino acid analyses performed on samples without performic acid oxidation.

Amino acid analysis of carboxypeptidase Y from Oriental Yeast showed a slight difference from that of the enzyme from Fleischmann's yeast (3). One less histidine and 2 less methionine residues were found in the former enzyme.

Interrelationships of the Reactions of p-HMB, iPr,PF, and Z-PheCH₂Cl with Carboxypeptidase Y. Carboxypeptidase Y has a serine (9, 10), a histidine, and a cysteine (9) residue which stoichiometrically, respectively, reacted with iPr,PF.
The presence of competitive inhibitors. (In spite of the small enzyme (4, 5). (b) Inactivation by Z-PheCH,Cl is reduced in for unknown reason(s).) (c) The pH dependence of the inactivation is similar to the pH dependence of the enzymatic value, Leu-Phe had no effect on reducing the inactivation rate, was also largely prevented. The appreciable amounts of radioactive Z-PheCH,Cl bound/mol of enzyme should be corrected for the 9% remaining activity and the 10% loss by denaturation during incubation with the inhibitor, giving 0.93 mol/mol.

If Z-PheCH,Cl treatment was followed by reaction with [14C]iPr2PF, the incorporation of radioactive iPr2PF was prevented. (The observed value of 0.13 mol of [14C]iPr2PF/mol of enzyme is in accord with the 9% remaining activity after pre-treatment with Z-PheCH,Cl.) Conversely, if the enzyme was first inactivated with iPr2PF, the reaction of L-[14C]Z-PheCH,Cl was prevented. If p-HMB was added, or, if the enzyme was heated-denatured, the reaction with radioactive Z-PheCH,Cl was also largely prevented. The appreciable amounts of radioactive incorporated into the heat-denatured enzyme may be accounted for by nonspecific reactions of [14C]iPr2PF with various side chains of the denatured protein (15).

**DISCUSSION**

The reaction of Z-PheCH,Cl and p-HMB. The interrelationships of these reactions are interesting (Table III). The stoichiometry of the reaction with radioactive iPr2PF and l-Z-PheCH,Cl is shown in the table. The observed value of 0.74 mol of [14C]Z-PheCH,Cl bound/mol of enzyme should be corrected for the 9% remaining activity and the 10% loss by denaturation during incubation with the inhibitor, giving 0.93 mol/mol.

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**Table II**

| Residues            | Native enzyme | Z-PheCH,Cl enzyme | Fleischmann's yeast enzyme
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>residues/molecule</td>
<td>residues/molecule</td>
<td>residues/molecule</td>
</tr>
<tr>
<td>Lysine</td>
<td>19.0</td>
<td>18.9</td>
<td>19</td>
</tr>
<tr>
<td>Histidine</td>
<td>5.2</td>
<td>3.3</td>
<td>9</td>
</tr>
<tr>
<td>Arginine</td>
<td>9.5</td>
<td>9.6</td>
<td>10</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td>10.9</td>
<td>11.1</td>
<td>11</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>65.5</td>
<td>66.1</td>
<td>65</td>
</tr>
<tr>
<td>Methionine sulfone</td>
<td>4.5</td>
<td>4.7</td>
<td>7</td>
</tr>
<tr>
<td>Threonine</td>
<td>18.2</td>
<td>19.4</td>
<td>18</td>
</tr>
<tr>
<td>Serine</td>
<td>30.2</td>
<td>30.4</td>
<td>30</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>41.0</td>
<td>40.7</td>
<td>41</td>
</tr>
<tr>
<td>Proline</td>
<td>25.8</td>
<td>26.5</td>
<td>25</td>
</tr>
<tr>
<td>Glycine</td>
<td>35.2</td>
<td>36.4</td>
<td>35</td>
</tr>
<tr>
<td>Value</td>
<td>26.8</td>
<td>30.4</td>
<td>30</td>
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<tr>
<td>Isoleucine</td>
<td>20.5</td>
<td>20.4</td>
<td>20</td>
</tr>
<tr>
<td>Leucine</td>
<td>35.0</td>
<td>35.1</td>
<td>37</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>22.5</td>
<td>22.0</td>
<td>27</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>21.6</td>
<td>21.6</td>
<td>24</td>
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<tr>
<td>N'-Cm-histidine</td>
<td>0</td>
<td>0.62</td>
<td>2</td>
</tr>
<tr>
<td>N' and N''-Cm-histidine</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S-Cm-cysteine</td>
<td>0.80</td>
<td>0.70</td>
<td>1</td>
</tr>
<tr>
<td>SH group (DTNB)</td>
<td>1.04</td>
<td>0.85</td>
<td>1</td>
</tr>
<tr>
<td>*Ref. 3.</td>
<td></td>
<td></td>
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<tr>
<td>*Values for cysteic acid (99), threonine (91), and serine (91) were corrected for 5%, 5%, and 10% destruction, respectively, during acid hydrolysis.</td>
<td></td>
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<td></td>
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<tr>
<td>*Calculated using the glycine color value (23).</td>
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<tr>
<td>*S-Cm-cysteine and the SH group were determined without performic acid oxidation.</td>
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<td></td>
</tr>
</tbody>
</table>

The reaction of Z-PheCH,Cl and Tos-PheCH,Cl toward carboxypeptidase Y is somewhat slower than that toward chymotrypsin; unlike the serine proteases (14, 16, 33-35), the chain length of the chloromethyl ketone reagents (peptide chloromethyl ketones) is not effective in enhancing the inactivation (see Table I). Nevertheless, the following evidence shows that Z-PheCH,Cl reacts with the active center of carboxypeptidase Y in an enzymatically promoted reaction. (a) The optical isomer of L-Z-PheCH,Cl has essentially no effect on enzyme activity, in accord with the stereospecificity of the enzyme (4, 5). (b) Inactivation by Z-PheCH,Cl is reduced in the presence of competitive inhibitors. (In spite of the small Ki value, Leu-Phe had no effect on reducing the inactivation rate, for unknown reason(s).) (c) The pH dependence of the inactivation is similar to the pH dependence of the enzymatic hydrolysis of some peptides. (d) A single mole of [14C]Z-PheCH,Cl is incorporated into the enzyme molecule with complete loss of activity, while no incorporation occurred into the heated-denatured enzyme (13). (e) The iPr2PF-inactivated enzyme fails to react with Z-PheCH,Cl, and, conversely, the Z-PheCH,Cl-inhibited enzyme fails to react with iPr2PF. In addition, the reaction site of Z-PheCH,Cl was unequivocally shown to be the N' position of a histidine residue of the enzyme. Carboxypeptidase Y has 1 serine residue which is reactive toward iPr2PF at twice the rate of an active serine of chymotrypsin (9, 10) (see also Table I). In addition, the behavior presented above closely parallels the behavior of chymotrypsin. Thus, Z-PheCH,Cl reacts with the enzyme via a noncovalent enzyme-substrate complex to produce an alkylated histidine, as has been explained in detail for chymotrypsin by Baker (36). Furthermore, it is reasonable to assume the presence of a
proteases, which are easily alkylated by these reagents. The -SH group exhibited no reactivity toward the -SH group. These proper-
ties are in marked contrast to those of -SH groups of thiol enzymes. Thus, carboxypeptidase Y and probably the family of enzymes described above should more appropriately be referred to by the generic name "serine carboxypeptidases" to distinguish them from "metal carboxypeptidases" to which this term is closely associated with acid proteases. Acid proteases, i.e. pepsin, renin, and a number of mold enzymes, have recently been shown to have a common catalytic center, aspartic acid (65). According to the classification of proteases by Hartley (56) (serine, thiol, metal, and acid proteases), carboxypeptidase Y should be a serine protease, but neither a metal nor an acid protease. The enzyme also differs from thiol proteases. Thus, carboxypeptidase Y and probably the family of enzymes described above should more appropriately be referred to by the generic name "serine carboxypeptidases" to distinguish them from "metal carboxypeptidases" to which carboxypeptidases A and B belong.

Acknowledgments—Thanks are due for consultation with Dr. Takeshi Igarashi and Dr. Shô Takahashi on the synthesis of Z-PheCHCl and for the generous cooperation of Dr. Kazuyuki Morihara and Dr. Tatsushi Oka in providing samples of Z-Ala-PheCHCl and Z-Ala-Glu-PheCHCl. We also appreciate the counsel of Dr. Seiyo Sano throughout this study.

REFERENCES

*V Bai, and R Hayashi, in preparation.

**TABLE III**

Reactions of carboxypeptidase Y with iPr,PF, p-HMB, and Z-PheCHCl

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incorporation into the enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[14C]Z-PheCHCl</td>
</tr>
<tr>
<td>5 × 10^{-4} M [14C]Z-PheCHCl</td>
<td>0.74</td>
</tr>
<tr>
<td>10^{-4} M [14C]iPr,PF</td>
<td>0.09</td>
</tr>
<tr>
<td>5 × 10^{-4} M Z-PheCHCl, followed by 10^{-4} M [14C]iPr,PF</td>
<td>0.13</td>
</tr>
<tr>
<td>10^{-4} M iPr,PF, followed by 5 × 10^{-4} M [14C]Z-PheCHCl</td>
<td>0.13</td>
</tr>
<tr>
<td>10^{-4} M p-HMB, followed by 5 × 10^{-4} M [14C]Z-PheCHCl</td>
<td>0.35</td>
</tr>
<tr>
<td>Heat, followed by 5 × 10^{-4} M [14C]Z-PheCHCl</td>
<td>0.38</td>
</tr>
</tbody>
</table>

charge-relay system in carboxypeptidase Y. This charge-relay system in the enzyme would operate in the enzymatic hydrolysis of both peptide and ester substrates, since inactivations by iPr,PF (10) and Z-PheCHCl closely paralleled loss of the two activities in many respects.

Interestingly, chymotrypsin is an endopeptidase while carboxypeptidase Y is an exopeptidase. This difference may arise from features of the active site other than the catalytic center. As an inherent characteristic of carboxypeptidases, carboxypeptidase Y may have a specific binding site for the terminal carboxylate anion of the substrates (4, 5), corresponding to arginine 145 of carboxypeptidase A (57). The pH dependence for the enzymatic hydrolysis of the peptide differs between carboxypeptidase Y and chymotrypsin. Carboxypeptidase Y has two ionizable groups with pK values of 4.4 and 6.5 for the hydrolysis of the peptide substrate, and an ionizable group with pK 5.9 for the hydrolysis of the ester substrate, while chymotrypsin has two ionizable groups with pK values of around 6.8 and 8.5 for the hydrolysis of Ac-Trp-OEt and Ac-Trp-NH₂ (38). These features are also reflected in the optimum pH for inactivation by Z-PheCHCl. pH 5.5 to 6.5 for carboxypeptidase Y, and 7.2 for chymotrypsin (39). These differing features of the pH dependence probably arise from a different environment around the charge-relay system (40).

Certain functions have been considered for the -SH group of carboxypeptidase Y; the enzyme is completely inactivated by a stoichiometric reaction of p-HMB (9). The -SH group is not available to react with either iodoacetate or iodoacetamide unless the protein is denatured (9). Furthermore, Z-PheCHCl exhibited no reactivity toward the -SH group. These properties are in marked contrast to those of -SH groups of thiol proteases, which are easily alkylated by these reagents.

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*Y Bai, and R Hayashi, in preparation.*
Evidence for an essential histidine in carboxypeptidase Y. Reaction with the chloromethyl ketone derivative of benzylloxycarbonyl-L-phenylalanine.

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