Structural Requirements for Nucleophilic Substrates of Carboxypeptidase Y*

William S. Lewis§ and Sheldon M. Schuster¶

From the §Department of Chemistry, University of Nebraska-Lincoln, Lincoln, Nebraska 68588-0304 and the ¶Department of Biochemistry and Molecular Biology, Box J-245, J. Hills Miller Health Center, University of Florida, Gainesville, Florida 32611

* This work was supported by BioNebraska, Inc., Lincoln NE. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

§ Present address: Diagnostic Products Corp., 5700 W. 96th St., Los Angeles, CA 90045.

¶ To whom correspondence should be addressed: 1301 Fifield Hall, University of Florida, Gainesville, FL 32611.

The composition and structural aspects of the amino and carboxylic acid groups required for incorporation into peptides by transpeptidation and inhibition of hydrolysis in carboxypeptidase Y-catalyzed reactions were studied. Separation of these two groups by even one carbon prevents incorporation by transpeptidation and does not inhibit incorporation of other amino acids into peptides. Substitution of phosphonic or sulfonic acids for the carboxylic acid group also results in loss of incorporation by transpeptidation. Only the sulfonic acid analog of glycine causes inhibition of hydrolysis and this inhibition is lost when serine is included in the reaction. a-Serine is not incorporated by carboxypeptidase Y, and its presence in the reaction mixture does not inhibit the incorporation of the L-isomer.

All of the naturally occurring amino acids except those with acidic side chains, glutamate and aspartate, have been incorporated into peptides by carboxypeptidase Y-catalyzed reactions (1). Although proline has not been reported to be incorporated as the free amino acid, proline amide is readily incorporated. Incorporation yields with these nucleophiles have been shown to vary greatly with the nature of the side chain and some patterns in this relationship have been identified. Generally, hydrophobic, aliphatic side chains are preferred while aromatic and hydrophilic side chains reduce incorporation. Positive charges and alcohols are tolerated on the side chain but negatively charged or amide side chains result in poor incorporation. Amino acid amides are preferred over free amino acids and are less influenced by the nature on the side chain. Dipeptides and secondary amines are not incorporated by carboxypeptidase Y (1).

The potential for using these reactions for incorporation of amino acid analogs or other nucleophilic groups into proteins and peptides makes the extension of these reactions to nucleophiles other than the naturally occurring amino acids desirable. A thorough understanding of the structural requirements for incorporation of the nucleophile by carboxypeptidase Y is required. Although the structural preferences of the enzyme for different side chain groups has been extensively investigated, there has been little systematic study of the structural requirements of the 1-amino carboxylic acid region common among amino acids. Few nucleophiles other than naturally occurring amino acids, or their carboxyl-blocked derivatives, have been tested in these reactions. The chemical and structural aspects of this region which make these molecules good substrates for carboxypeptidase Y are presented.

The information gained from these experiments, along with the side chain preferences described by Bredam (1), can provide a guideline for the design of labels possessing the desired physical or chemical properties which can be used for incorporation into proteins or peptides by carboxypeptidase Y-catalyzed reactions.

D-Amino acids have been shown to inhibit the hydrolysis of peptides by carboxypeptidase Y (2). Widmer et al. (1) reported that D-amino acids were not incorporated into peptides and stated that racemic mixtures could, therefore, be used for peptide synthesis. Synthesis of other types of nucleophiles is likely to form such racemic mixtures. If these mixtures can be used without resolution of the enantiomers, fewer purification steps would be needed. However, no studies have yet reported the effect of the D-isomer of a nucleophile on the incorporation of its L-isomer. The effect of the presence of the D-isomer on the carboxypeptidase Y-catalyzed hydrolysis of peptides and the transpeptidation of these peptides with the L-isomer are investigated.

Since ethanolamine and benzylamine have been reported to be incorporated by carboxypeptidase Y (1), the α-carboxyl group of amino acids does not appear to be required. In several ways, the carboxyl group may be more of a hindrance to incorporation since incorporation of most amino acids can be improved by shielding the charge of this group by derivatization to the ester or amide (3). The possibility also exists that the nucleophile with a free carboxyl group or an ester or amide derivative can bind to the enzyme in the peptide-binding site, competing with the peptide for this site. The carboxyl group may also be undesirable on some labels, making them unstable or providing functional groups with which the label can react. When present on the side chain of the nucleophile, as seen with aspartate and glutamate, they can prevent incorporation. The effects of elimination or replacement of this functional group by other acidic groups is reported, as are the effects of the proximity of these groups to the amino group.

EXPERIMENTAL PROCEDURES

Materials—Carboxypeptidase Y was a gift from Dr. Klaus Bredam (Carlsberg Laboratory, Copenhagen, Denmark). Cbz-Phenylalanine and Cbz-phenylalanyl-alanine were obtained from Sigma. Acetonitrile was obtained from Fisher. All other reagents were from Sigma.

Labeling Reaction—The standard substrate mixture for the reaction consisted of 2 mM N-carbobenzoxy-L-phenylalanyl-L-alanine

1 The abbreviations used are: Cbz, benzoxycarbonyl; HPLC, high performance liquid chromatography.
(Cbz-Phe-Ala), 0.5 mM nucleophile, and 50 mM sodium phosphate, adjusted to pH 7.5 with sodium hydroxide. The reaction was started by addition of 4 μl of a 2.6 mM solution of carboxypeptidase Y in water to 1.0 ml of substrate mixture. The mixture was incubated at 37 °C and 100-μl samples were removed at different times and immediately analyzed by HPLC.

HPLC Assay—Analyses were performed using a dual pump Beckman or ChemResearch HPLC and a Microsorb C3 column (15 × 0.46 cm). The method used for the separation of the peptides is a modification of a system used by Widmer et al. (1). The buffers were 50 mM triethylammonium formate, pH 5.0 (TEAF, buffer A) and 60% acetonitrile, 40% buffer A (buffer B). The peptides were separated using a linear gradient of 50–70% buffer B in 5 min at a flow rate of 1 ml/min. The Cbz-peptides were detected, using an ISCO UV/VIS monitor, by their absorbance at 230 nm. Absorbance data was collected and integration of the absorbance peaks was done on an Apple IIe computer using a program written by Dr. Bruce Partridge (BioNebraska, Lincoln, NE).

Data Analysis—The molar absorptivity of the peptides were determined to be 230 A/mol/liter, 372 A/mol/liter, and 342 A/mol/liter for Cbz-Phe (hydrolysis product), Cbz-Phe-Ala (substrate peptide), and Cbz-Phe-Ser (transpeptidation product), respectively. The peak areas in all of the samples were then corrected for the difference in absorbance by multiplying the peak area of each peptide peak by the molar absorptivity of the corresponding peptide. The percent yield was then calculated from these corrected peak areas as

\[
\% \text{ Yield} = \frac{\text{corrected peak area}}{\text{Total corrected areas of all peaks}} \times 100
\]

In experiments where peptides other than those listed above were produced, no correction for difference in molar absorptivity was made for any of the peptides in the chromatogram. These data are presented as the uncorrected value for the peak area.

\(\textit{d-Serine—}\) The substrate mixture was made using either L-serine or D-serine at concentrations of 0.5 M. The DL-serine mixture was made by mixing equal volumes of the substrate mixtures made with 0.5 M or 1 M D-serine with a substrate mixture containing the same concentration of L-serine.

Carboxylate Requirement—The substrate mixture was modified to contain the desired nucleophile at a concentration of 1 M. The nucleophiles used for this study were L-serine, ethanolamine, and 2-aminoacidopropyl (t-2-amino-1-propanol). The HPLC separation gradient was modified from that described above. A linear gradient from 50% buffer B to 55% buffer B in 20 min was used.

Carboxyl-amine Group Separation—The nucleophiles used were glycine, 3-aminoacaproic acid (3-ala), 4-aminobutyric acid, 3-aminovaleric acid, and 6-aminocaproic acid. Two different gradients were used for the HPLC separation of the products in these experiments. The HPLC gradient used for glycine, 5-aminovaleric acid, and 6-aminocaproic acid varied linearly from 50% buffer B to 70% buffer B over a period of 10 min while the gradient used for 3-aminopropanoic acid and 4-aminobutyric acid varied linearly from 45% buffer B to 65% buffer B in 20 min.

Inhibition of the transpeptidation reaction with serine by 3-aminopropanoic acid was measured using the substrate mixture containing varying concentrations of serine and 3-aminopropanoic acid as nucleophile as indicated under “Results.”

Sulfonic and Phosphonic Acids—Inhibition of hydrolysis of Cbz-Phe-Ala and incorporation of sulfonic and phosphonic amino acid analogs by transpeptidation was assayed utilizing the indicated amino acid analog as the nucleophile.

Sulfonic acid and phosphonic acids of the form

\[
\text{H}_2\text{N-CH}_2-(\text{CH}_2)_n-\text{SO}_2\text{H}
\]

\[
\text{H}_2\text{N-CH}_2-(\text{CH}_2)_n-\text{PO}_2\text{H}
\]

were tested in which \(n\) varied from 0 to 2. The nucleophiles used were 1-aminomethanesulfonic acid (\(n = 0\)), 2-aminomethanesulfonic acid (taurine, \(n = 1\)), and 3-aminopropanesulfonic acid (\(n = 2\)) for the sulfonic acids and those used for the phosphonic acid nucleophiles were 1-aminomethanephosphonic acid (\(n = 0\)), 2-aminoethanephosphonic acid (\(n = 1\)), and 3-aminothrepanephosphonic acid (\(n = 2\)).

The HPLC separations were accomplished as described above except that the gradient varied linearly from 50 to 70% buffer B in 10 min. Inhibition by 1-aminomethanesulfonic acid of hydrolysis of Cbz-Phe-Ala and transpeptidation of the peptide by serine was studied as described above for 3-aminopropanoic acid.

**RESULTS**

\(\textit{d-Serine—}\) The hydrolysis of Cbz-Phe-Ala was slower in the presence of 0.5 M L-serine or when no serine was present than in the presence of 0.5 M D-serine (Fig. 1A). The rate of hydrolysis in the presence of 1 M DL-serine is between these limits. It appears from these data that D-serine may increase the rate of hydrolysis of the peptide. The transpeptidation reaction (Fig. 1B) proceeds at the same rate in the presence of 1 M DL-serine as when 0.5 M L-serine is present and at a slower rate when 0.5 M DL-serine is present. No transpeptidation product was observed when 1 M D-serine was present. The incorporation of serine is proportional to the concentration of L-serine and independent of the presence of D-serine. No activation of the transpeptidation reaction by D-serine was observed, as was seen for the hydrolysis reaction.

Carboxylate Requirement—The rate of hydrolysis of Cbz-Phe-Ala in the presence of ethanolamine is the same as the rate in the presence of L-serine but is slower when 2-aminopropanoic acid is present as the nucleophile (Fig. 2A). The transpeptidation in the presence of these nucleophiles follows a different pattern (Fig. 2B). The transpeptidation product is formed much faster with L-serine than with either ethanolamine or 2-aminopropanol. Ethanolamine is incorporated at about half the rate of 2-aminopropanol.

Carboxyl-amine Group Separation—The rate of hydrolysis was studied in the presence of a series of nucleophiles with the general formula

\[
\text{H}_2\text{NCH}_2-(\text{CH}_2)_x-\text{COOH}
\]

where \(x\) varied from zero to four. The concentration of the hydrolysis product, Cbz-Phe, at different incubation times in the presence of each of these nucleophiles is shown in Fig. 3. Similar hydrolysis rates are observed for all the nucleophiles tested except 3-aminopropanoic acid. This nucleophile caused a large decrease in the rate of hydrolysis of the peptide substrate. The effect observed with 3-aminopropanoic acid was later determined to be due to improper adjustment of the pH of the substrate mixture (see below). The transpeptidation reaction with each of these nucleophiles was also studied. No
transpeptidation with serine were studied. No inhibition of the hydrolysis reaction or the transpeptidation reaction were observed at any concentration of serine, as shown in Fig. 7B. For the aminosulfonic acids, hydrolysis was almost completely inhibited by 1-aminomethanesulfonic acid and showed some inhibition from 3-aminopropanoic acid while the compound intermediate between these two, 2-aminoethanesulfonic acid, showed no inhibition at all (Fig. 7A). For the aminophosphonic acids, no inhibition of the hydrolysis reaction was observed (Fig. 7B). No incorporation of either the aminosulfonic acids nor the aminophosphonic acids via a transpeptidation reaction was observed for any of the compounds tested.

The effects on the formation of the hydrolysis and transpeptidation products as a function of time at different concentrations of serine and 1-aminomethanesulfonic acid are shown in Figs. 8 and 9, respectively. In the absence of serine, the hydrolysis reaction is inhibited but increasing the concentration of serine in the reaction mixture relieves some of this inhibition. A similar effect is observed with the transpeptidation reaction where a higher concentration of serine shows less inhibition by 1-aminomethanesulfonic acid. A large inhibition of the hydrolysis reaction without serine present in the mixture is completely eliminated when the concentration of serine is 1.0 M. Less of an effect is seen in the transpeptidation reaction, in part because the reaction rate is zero in
FIG. 7. Effect of functional group separation distance on inhibition of hydrolysis. Concentration of hydrolysis product in the reaction mixture at various incubation times with different concentrations of serine and aminomethanesulfonic acid. Concentrations of serine used were 0.5 M (A), 0.5 M (B), and 1.0 M (C). Concentrations of aminomethanesulfonic acid were 0 M (+), 0.5 M (x), and 1.0 M (o).

FIG. 8. Effect of serine and aminomethanesulfonic acid on the hydrolysis reaction. Concentration of hydrolysis product in the reaction mixture at various incubation times with different concentrations of serine and aminomethanesulfonic acid. Concentrations of serine used were 0 M (A), 0.5 M (B), and 1.0 M (C). Concentrations of aminomethanesulfonic acid were 0 M (+), 0.5 M (x), and 1.0 M (o).

FIG. 9. Effect of serine and aminomethanesulfonic acid on the transpeptidation reaction. Concentration of transpeptidation product in the reaction mixture at various incubation times with different concentrations of serine and aminomethanesulfonic acid. Concentrations of serine used were 0 M (+), 0.5 M (x), and 1.0 M (o).
aration of the functional groups of the nucleophile by more than one carbon prevents the incorporation in the transpeptidation reaction but hydrolysis proceeds normally.

Phosphonic acid analogs of amino acids are not incorporated by transpeptidation and do not inhibit the hydrolysis reaction. No evidence was seen which would indicate that these nucleophiles bind to the enzyme at all. Although the possibility that one or more of the aminophosphonic acids are incorporated and the products are not separated from the other peptides cannot be excluded, it appears unlikely that this is the case. If the transpeptidation product coeluted with the substrate peptide, the formation of the hydrolysis product would be expected to be nonlinear with respect to time since it is unlikely that significant amounts of the transpeptidation product would accumulate if the rate of hydrolysis of the transpeptidation product were the same as that of the substrate. Coelution of the transpeptidation product with the hydrolysis product would not be expected since the substitution of a phosphonic acid for a carboxylic acid would be expected to make the resulting peptide more hydrophilic than the substrate.

The aminosulfonic acids behave differently than either the aminocarboxylic acids or the aminophosphonic acids. No incorporation of the aminosulfonic acids was observed. Although the possibility that the transpeptidation product is not separated from the other peptides cannot be ruled out completely, it is unlikely for the same reasons as in the case of the aminophosphonic acids. The effects of the aminosulfonic acids on the hydrolysis of the substrate peptide are more complex than those caused by the other nucleophiles tested.

The smallest molecule of the series, in which the amino group is attached to the same carbon as the acid group, inhibits the hydrolysis almost completely while separation of these groups by one carbon unit eliminates the inhibition and separation by two carbons results in the reappearance of inhibition. The inhibition seen with 1-aminomethanesulfonic acid may be caused by nonproductive binding in the amino acid-binding site since inhibition is relieved by the addition of serine which would compete for this site. The loss of inhibition resulting from separation of the amino and sulfonic acid groups may indicate that this separation places these groups too far apart to fit in the binding site. Inhibition caused by further increasing this separation distance is likely a result of a different binding configuration, probably either a configuration in which only one of the two functional groups occupies the binding site or one resulting from binding to a different site. Neither the aminophosphonic acids nor the aminosulfonic acids appear to be suitable as labeling groups for proteins or peptides but the latter may be useful in designing inhibitors for the enzyme.

Widmer et al. (1) reported that two of the nucleophiles tested above, 2-aminoethanesulfonic acid and 2-aminoethane-phosphonic acid, are incorporated into peptides by transpeptidation. The peptide they used was a peptide ester in which the ester group was removed during the carboxypeptidase Y-catalyzed reaction and replaced with the nucleophile. It is therefore possible that these two nucleophiles, and possibly others, are incorporated into peptides by carboxypeptidase Y but may require a better leaving group than the alanine used here to produce detectable yields. If this is the case, these groups may be usable for protein labeling in some situations, but since the leaving group of the protein to be labeled is often determined by the sequence of that protein, application of these groups would be limited to only certain proteins or synthetic peptides.

As a result of the studies presented here, designs for protein labels should conform as closely as possible to the following specifications. The nucleophile should possess a terminal carboxyl group as its only carboxyl group and an amino group on the carbon adjacent to this carboxyl group. Sulfonic acids and phosphonic acids are not tolerated in place of the carboxylic acid group, although the possibility that ester or amide derivatives of these groups are accepted was not explored. The carbon containing the amino group should be in the \textit{L} configuration.

Acknowledgments—We thank Dr. Fred Wagner for many helpful discussions and suggestions during the course of this work. We also thank Dr. Klaus Breddam for the gift of carboxypeptidase Y.

REFERENCES