Identification of a Glutamic Acid at the Active Center of Bovine Carboxypeptidase B*

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SUMMARY

The glutamic acid residue at the active center of bovine carboxypeptidase B was labeled with $\alpha$-N-bromoacetyl-$\text{L}$-arginine and the alkylated protein was hydrolyzed with pepsin. A $^{14}$C-labeled peptide fraction was isolated in a 52% yield by gel filtration on Sephadex G-25 followed by ion exchange chromatography on CM-cellulose. Compositional and end group analysis, in addition to enzymatic hydrolysis with carboxypeptidases A and B, aminopeptidase M, and trypsin, suggest the following amino acid sequence: Thr-Phe-Glu-Leu-Arg-Asp-Lys-Gly-Arg-Tyr-Gly-Phe. A comparison with the glutamic acid-containing sequence at the active sites of carboxypeptidases A and B revealed nearly complete homology.

A bovine carboxypeptidase B was shown previously to contain a carboxyl group at the active center (1). This group and a tyrosine residue that is essential for full enzymatic activity (2) exhibit similarities in function to amino acids in the active center of bovine carboxypeptidase A (3-5). In addition, the amino acid sequence of a peptide containing the functional tyrosyl residue of carboxypeptidase B was shown by us (2) to be nearly identical with a corresponding region in carboxypeptidase A (3, 4). The present report shows that the peptide of carboxypeptidase B containing the glutamic acid labeled by an affinity reagent has an amino acid sequence nearly identical with the corresponding sequence of bovine carboxypeptidase A. This glutamic acid in carboxypeptidase A has been implicated as a functional group by x-ray crystallography (3) and affinity labeling (5).

EXPERIMENTAL PROCEDURES

Methods

Protein Preparation and Determination—Carboxypeptidase B was prepared from pancreatic juice as described previously (2). Activity was measured at 254 nm by the hydrolysis of hippuryl-L-arginine (Schwarz-Mann) or hippuryl-L-phenyllactic acid (Cyclo) by the procedure of Kycia et al. (6). Protein concentrations of carboxypeptidase B were obtained assuming an absorbance index of $E_{1%}^{\text{cm}}$ (280 nm) = 21 (7). Absorbance measurements were made with a Gilford model 2000 spectrophotometer.

Preparation of $\text{BrAc-N-}[^{14}$C$]\text{Arg}$ Carboxypeptidase B—Carboxypeptidase B (200 mg in 40 ml of 0.1 M Tris-HCl, pH 7.0) was alkylated with a 40-fold molar excess of $\text{BrAc-N-}[^{14}$C$]\text{Arg}$ (1). The reaction mixture was desalted on Sephadex G-25 and fractionated on $L$-leucyl-$L$-arginine-Sepharose 4B columns (1). The inactive nonretarded fraction was concentrated to 20 ml in an ultrafilter (Amicon Corporation) with a PM-10 membrane at 40 p.s.i. The concentrate was dialyzed against four 2-liter changes of distilled water. Protein concentration was estimated from absorption at 280 nm, and appropriate aliquots were removed for determination of radioactivity and enzymatic activity. The salt-free protein was then lyophilized.

Peptic Digestion—The lyophilized protein was suspended in 5% formic acid (7 ml/100 mg of protein) and digested with pepsin (Worthington) at 37° (molar substrate to enzyme ratio, 66:1). In some digestions, a second equivalent aliquot of pepsin was added at 8 hours. The clear digestion mixture obtained after 24 hours was frozen or placed directly on a Sephadex G-25 column.

Amino Acid Analyses—Acid hydrolysates of 0.5 mg of protein or 0.02 to 0.1 μmole of peptide were prepared with twice distilled, constant boiling HCl of known ammonia content in evacuated, nitrogen-flushed tubes at 110° for 24 hours. Analyses were performed with an automatic amino acid analyzer (8). The pH 3.24 and 4.24 buffers contained 3% and 3.75% l-propanol, respectively (9). For peptide analyses, all amino acids were determined on one column. The pH 3.24 and 4.24 buffers were prefilled through an ammonia filtration column (Durram Chemical Corp.) and a third buffer, 0.3 M sodium citrate, pH 6.5, 0.9 M in NaCl, was used to elute the basic amino acids. Analyses by this method were performed with a column (0.9 × 50 cm) of Aminex A-6 resin (Bio-Rad).

Radioactivity Analyses—Incorporation of $\text{BrAc-N-}[^{14}$C$]\text{Arg}$ into carboxypeptidase B was determined on suitable aliquots with a liquid scintillation counter (Nuclear-Chicago, model 720). The extent of alklylation was estimated from the counts per min per amole of protein divided by the counts per min per amole of...
alkylating agent. Radioactivity was monitored during column chromatography by a liquid radiochromatography system (Nuclear-Chicago, model 4526) with a 2-ml flow cell (Nuclear-Chicago, model 6782) and a recorder.

**Purification of Peptides**

**Gel Filtration**—An aliquot of the peptide digest containing peptides equivalent to 80 mg of protein was applied to a column of Sephadex G-25 (1.9 × 238 cm), particle size 44 to 63 μm, prepared in 0.1 N acetic acid. The effluent was collected in 6-ml fractions at a flow rate of 10.5 ml per cm² per hour. All manipulations were at room temperature. Peptides were located by absorbance measurements at 230 nm. Radioactive peptides were detected with the coupled liquid radiochromatography system mentioned above and their effluent tube position was verified by removal of suitable aliquots for liquid scintillation counting. Pertinent fractions were combined as indicated in Fig. 1 and lyophilized.

Subsequent steps requiring a desalting of individual peptides or peptide fragments were accomplished on Sephadex G-25 (0.9 × 194 cm, 44 to 63 μm) or G-10 (0.9 × 270 cm, 40 to 120 μm) in 0.1 N acetic acid. The effluent was collected in 3-ml fractions at a flow rate of 18.9 ml per cm² per hour. All manipulations were at room temperature. Peptides were located by absorbance measurements at 230 nm. Radioactive peptides were detected with the coupled liquid radiochromatography system mentioned above and their effluent tube position was verified by removal of suitable aliquots for liquid scintillation counting. Pertinent fractions were combined as indicated in Fig. 1 and lyophilized.

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**Sequence Determination**

**Edman Degradation**—The procedure of Gray (10) was performed on the peptides with the modification of Elzinga et al. (11), whereby samples of peptides were pipetted into individual tubes equal in number to the steps of sequential degradation. Volatile reagents and by-products were removed in an aluminum block maintained at 54° in a vacuum chamber (12). After its final cycle, the residue in each tube was suspended in 300 μl of 0.2 N acetic acid and incubated at 45° for 1 hour (13). Water (300 μl) was added and the residues were extracted twice with 3 ml of n-butyl acetate after thorough agitation with a Vortex mixer. The aqueous layer was lyophilized and the residue was transferred into hydrolysis tubes with two 500-μl aliquots of 6 N constant boiling HCl for amino acid analysis.

**Hydrolysis by Aminopeptidase M**—Samples containing 0.03 to 0.05 μmole of peptide were lyophilized and redissolved in 100 μl of 0.1 M sodium acetate, pH 5.6, or 0.1 M sodium phosphate, pH 6.0. A 50-μl portion of 0.1% aminopeptidase M (P-L Biochemical or Henley and Co.) was added, and the digest was incubated at 25°. After an appropriate time interval, the reaction was terminated with 0.7 ml of 0.2 M sodium citrate, pH 2.2. The resultant solution and appropriate controls were subjected directly to amino acid analysis.

**Hydrolysis by Carboxypeptidase A and B**—Crystalline car-
bovine pancreatic juice by the method of Cox et al. (14) was dissolved in 1 M NaCl to make a 0.9% solution. Reaction mixtures were incubated at 25°C in 0.1 M sodium acetate, pH 5.6 (2). A digestion study consisted of a series of tubes containing 0.03 pmole of peptide in 150 μl of water. To each tube were added 25 μl of 0.5 M sodium acetate, pH 5.6, 25 μl of the 0.9% carboxypeptidase A solution, and 1 drop of toluene. Appropriate enzyme blanks were included.

For reaction mixtures in which the digestion by carboxypeptidase A was followed by carboxypeptidase B, an aliquot of 25 μl of a 0.7% solution of carboxypeptidase B was added after 24 hours of incubation with carboxypeptidase A.

The hydrolysis in individual tubes was stopped by the addition of 0.6 ml of 0.2 M sodium citrate, pH 2.2. The total solution was then subjected directly to amino acid analysis.

Hydrolysis by Trypsin—The peptide (1.5 pmol) in 0.03 M sodium acetate, pH 1.5, was adjusted to pH 5.6 with 0.1 N NaOH and to 0.06 M with 1.0 M sodium acetate, pH 5.6. An aliquot of 1-1-tosylamide-2-phenylethyl chloromethyl ketone trypsin (15) equivalent to 1.84 mg was added (substrate to enzyme ratio, 20:1). After an 8-hour incubation at 25°C, the hydrolysis was stopped with 0.1 ml of 4 N HCl and the mixture was placed on a Sephadex G-25 column (0.9 × 194 cm).

RESULTS

Preparation of BrAc-d-[5,4C]Arg-alkylated Carboxypeptidase B—Carboxypeptidase B was alkylated with BrAc-d-[5,4C]Arg as described earlier (1). The protein contained 1.09 moles of alkylating agent per mole of enzyme and was enzymatically inactive (1). The larger scale preparations required for peptide isolation were accomplished by increasing the volumes of reagents without changing the concentration of enzyme (5 mg per ml) or of alkylating agent (20-fold excess) from the previous study.

Peptic Digestion of 14C-Alkylated Protein—Digestion with pepsin resulted in extensive cleavage of the alkylated carboxypeptidase B. When a suspension of the lyophilized protein in 5% formic acid was incubated with pepsin, the mixture was clarified in 4 hours. The digestion was allowed to continue for 24 hours.

Gel Filtration—A preliminary fractionation of the peptic peptides was obtained by gel filtration on a Sephadex G-25 column equilibrated in 0.1 N acetic acid (Fig. 1). Fractions containing radioactive peptides were located during chromatography with the liquid scintillation flow cell system and their position was verified by aliquots removed for scintillation analysis. Radioactivity was located in three peaks, labeled I, II, and III, containing 20, 71, and 9%, respectively, of the total radioactivity. The major fraction, Peak II, was subjected to further chromatography. Preliminary studies were also done on Peak I and will be discussed under "Sequence Studies."

CM-Cellulose Chromatography—The absorbance curve (Fig. 2) demonstrates the separation obtained by chromatography of radioactive Peak II on CM-cellulose. The radiochromatography system detected four radioactive peaks which were pooled. Peaks A, B, and D were minor components and contained only 6% of the total radioactivity. Peak C contained 94% of the recovered 14C-label (73% of the radioactivity applied to the column) and was used to determine the amino acid sequence containing the alkylated carboxyl group. This peak will be referred to hereafter as peptide C.

The amino acid composition of peptide C is reported in Table I. This composition was found repeatedly for different preparations with the exception that the values for threonine and phenylalanine varied somewhat. This variation was explained by sequence analysis and will be discussed with those studies.

TABLE I

| Amino acid | Peptide C
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>1.00</td>
</tr>
<tr>
<td>Threonine*</td>
<td>0.15</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.06</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.85</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.01</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.90</td>
</tr>
<tr>
<td>Phenylalanine*</td>
<td>1.44</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.06</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.94</td>
</tr>
</tbody>
</table>

* Separate aminopeptidase M and carboxypeptidase A digestion of this preparation of peptide C indicated that 32% of the phenylalanine was on the NH2-terminal end and 68% on the COOH-terminal end. Aminopeptidase M digestion rapidly released the threonine. While the total amount of phenylalanine and threonine varied between preparations of peptide C, threonine was always present as 0.3 residue or less and phenylalanine as 1.4 ± 0.5 residues. The amounts of enzymatically released threonine and phenylalanine were always in excellent agreement with those observed after hydrolysis in 6 N HCl.

Sequence Studies

The sequence of amino acids for peptide C and the steps employed to establish this sequence are summarized in Fig. 3. The methods are given in detail below.

Carboxypeptidases A and B—Hydrolysis of peptide C by carboxypeptidase A at pH 5.6 resulted in rapid release of less than 1 residue of phenylalanine per mole of peptide and a slower concomitant release of 1.0 residue each of tyrosine and glycine. No other amino acids were detected after incubation for 24 hours. If an aliquot of carboxypeptidase B was added at this time, 1.0 residue of arginine and trace amounts of other amino acids were released in 1 hour. Since carboxypeptidase A should release glycine concomitant with tyrosine if the glycine were COOH-terminal to tyrosine in the sequence, these studies establish the sequence shown in Fig. 3. A tryptic cleavage, that will be discussed later, allowed the isolation of two COOH-terminal peptides, a dipeptide of Tyr-Gly and a tripeptide of Tyr-Gly-Phe, indicating that partial cleavage of the COOH-terminal

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phenylalanine had occurred during the peptic digestion. Edman degradation of the tripeptide confirmed the order of amino acids assigned by carboxypeptidase A cleavage.

Aminopeptidase M—Aminopeptidase M (P-L Biochemicals) was incubated with peptide C at pH 6.0 for 24 hours and the mixture was subjected to analysis on the amino acid analyzer for free amino acids. All amino acids were recovered in amounts similar to the ratios in Fig. 1, except for glutamic acid and arginine which were present as >0.10 and 2.0 moles, respectively. The values for threonine and phenylalanine were similar to those obtained from acid hydrolysis. Aspartic acid was released as free aspartic acid and not as asparagine. A new peak was observed 15 min after phenylalanine on amino acid analysis. This was the only peak that proved to be radioactive on coupling the amino acid analyzer with the liquid scintillation flow cell system.

The digestion of peptide C by aminopeptidase M was studied for shorter periods. When peptide C was incubated with aminopeptidase M (Henley and Co.) for 1 hour at pH 5.6, the following amino acids were released (based on leucine = 1.00 residue): threonine, 0.11; phenylalanine, 0.74; arginine, 0.73; and the new radioactive peak appearing after phenylalanine. The new peak was isolated from a preparative scale aminopeptidase M digestion mixture containing 0.1 M sodium acetate, pH 5.6, 0.94 mole of peptide C, and 1.23 mg of aminopeptidase M. After 1.25 hours at 25°C, the digestion mixture was chromatographed on a CM-cellulose column under conditions identical with those in Fig. 2. The radioactive peak (Peak 1) was slight retarded and eluted at 90 ml. The only peak containing absorbance at 230 nm eluted at approximately 300 ml that was also asymmetric with respect to ninhydrin analysis. Aspartic acid was released as free aspartic acid and not as asparagine. A new peak was observed 15 min after phenylalanine on amino acid analysis.

Analysis of radioactive Peak 1 prior to acid hydrolysis revealed it to be the new peak that eluted on the amino acid analyzer after phenylalanine. Following acid hydrolysis of Peak 1, a 1:1 ratio of arginine to glutamic acid was obtained. The radioactivity was associated with arginine. The molar absorbance of Peak 1 was 76% that of glutamic acid. The small amount of threonine observed (0.06 residue) was also noted prior to acid hydrolysis and represents a slight contamination by the threonine released by aminopeptidase M.

Amino acid analysis of Peak 2 indicated a complete loss of COOH-terminal phenylalanine and a partial loss of glycine and tyrosine. Since the latter two residues were not detected as free amino acids, a minor contaminating enzyme that releases peptide fragments must be present (2).

Incubation of the 14C-labeled Peak 1 (Fig. 1) with aminopeptidase M for 24 hours at 25°C, followed by chromatography on the amino acid analyzer, indicated that all the radioactivity was also associated with an alkylated glutamic acid. This region was not studied further.

Tryptic Cleavage of Peptide C—Peptide C was incubated with trypsin for 8 hours and the mixture was subjected to gel filtration on Sephadex G-25. The results are seen in Fig. 4A. Amino acid analyses of each absorbance peak were determined and results indicated that all material eluting before 50 ml were trypsin or trypsin degradation products. Two radioactive peaks were detected, A and B, and their fractions were pooled. Peak B also contained the salt present in the sample. The two absorbance peaks that were retarded relative to Peak B were pooled as peptides T-1 and T-2.

Peak A was lyophilized and subjected to chromatography on CM-cellulose under the conditions described under "Methods." The results are presented in Fig. 4B. Chromatographic analysis indicated the presence of one asymmetric radioactive peak (T-6) at approximately 300 ml that was also asymmetric with respect to ninhydrin analysis. Three other peptide fragments, designated T-3 to T-5, were detected on ninhydrin analysis. The fractions containing peptide T-6 were combined into two pools, termed T 6a and T 6b.

Amino acid analysis of Peak B revealed small amounts of glutamic acid, leucine, and phenylalanine, and larger amounts of glycine and arginine. Peak B was made 0.1 N in HCl and applied to a column of Dowex 50-X8, H+ form (0.9 × 4.5 cm). The column was washed with 10 ml of 0.5 N HCl and the peptides were eluted with freshly prepared 0.2 N pyridine. The peptide fraction was lyophilized and applied to a column of CM-cellulose identical with that in Fig. 4B. The only prominent peak eluted at 220 ml and was designated as T-7.

Amino acid analyses of the tryptic peptides are listed in Table III. Enzymatic and chemical analyses of the peptides provided the amino acid sequence of peptide C and these will be discussed for each peptide.

Peptides T-1 and T-2 represent the variable COOH-terminal sequence of peptide C. Peptide cleavage of carboxypeptidase B resulted in partial loss of the COOH-terminal phenylalanine and the isolation of two tryptic fragments containing tyrosine. The Edman degradation of peptide T-2 is presented in Table IV and verified the sequence proposed from the carboxypeptidase A digestion of peptide C.

Peptide T-3 gave an amino acid analysis consistent with that of a des-Thr-Phe derivative of the NH2-terminal end of peptide.
TABLE IV
Results of subtractive Edman degradations on tryptic fragments of peptide C

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequences determined</th>
<th>Amino acid Peptide</th>
<th>Cycles of Edman degradation(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tyr-Gly-Phe</td>
<td>1.04 0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tyr</td>
<td>&gt;0.02 0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phe</td>
<td>1.00 1.00</td>
</tr>
<tr>
<td>T-5</td>
<td>Asp-Lys-Gly-Arg</td>
<td>Asp</td>
<td>0.02 &gt;0.02 0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lys</td>
<td>0.98 0.98 &gt;0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arg</td>
<td>1.00 1.00 1.00</td>
</tr>
</tbody>
</table>

\(a\) The residue removed in each step is indicated by bold face.

\(b\) All values are related to a phenylalanine and an arginine content of 1 pmole per pmole of peptide for peptides T-2 and T-5, respectively.

\(c\) The value for lysine could be increased to 0.69 pmole per pmole of peptide by increasing the acid hydrolysis to 48 hours.

TABLE V
Release of free amino acids from peptide T-6a by aminopeptidase M

The results are expressed in terms of micromoles of free amino acids \((\times 10^2)\). Aliquots of the digestion mixture were diluted to 0.825 ml with 0.2 M sodium citrate, pH 2.2, and were applied directly to the amino acid analyzer.

<table>
<thead>
<tr>
<th>Hydrolysis time (min)(a)</th>
<th>Threonine</th>
<th>Glutamic acid</th>
<th>Leucine</th>
<th>Phenylenalanine</th>
<th>Arginine (\gamma)-((\alpha-N\text{-glycolyl}))-\text{-arginine ester}</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.62</td>
<td>0.15</td>
<td>0.07</td>
<td>0.80</td>
<td>0.05</td>
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<tr>
<td>20</td>
<td>0.90</td>
<td>0.65</td>
<td>0.17</td>
<td>1.32</td>
<td>0.11</td>
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<tr>
<td>40</td>
<td>1.21</td>
<td>1.13</td>
<td>0.46</td>
<td>1.66</td>
<td>0.36</td>
</tr>
<tr>
<td>60</td>
<td>1.23</td>
<td>1.89</td>
<td>0.83</td>
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<td>0.84</td>
</tr>
<tr>
<td>120</td>
<td>1.25</td>
<td>2.41</td>
<td>1.91</td>
<td>1.87</td>
<td>1.93</td>
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<td>180</td>
<td>1.31</td>
<td>2.70</td>
<td>2.35</td>
<td>1.83</td>
<td>2.39</td>
</tr>
<tr>
<td>240</td>
<td>1.24</td>
<td>2.71</td>
<td>2.61</td>
<td>1.73</td>
<td>2.69</td>
</tr>
</tbody>
</table>

\(a\) The conditions for hydrolysis were similar to those in "Methods," but the rate was diminished by increasing the peptide volume to 600 \(\mu\)l and by decreasing the amount of aminopeptidase M to 25 \(\mu\)l of a 0.08% solution.

\(b\) Glutamic acid was determined on the amino acid analyzer as its \((\alpha-N\text{-glycolyl})\)-arginine ester.

C has spontaneously lost its \(^{14}\text{C}\) label. It was present as an impure minor component and was not studied further.

Peptide T-4 was a dipeptide resulting from the cleavage of both the lysine and the two arginines in peptide C by trypsin at pH 5.5. The other dipeptide resulting from this cleavage was isolated as T-7.

Peptide T-5 represented the center tetrapeptide obtained from the tryptic cleavage of peptide C at the two arginines only. Edman degradations of this peptide gave the sequence listed in Table IV and confirms the amino acid sequence of fragments T-4 and T-7.

The two fractions of peptide T-6 were studied separately. Fraction T-6a had an amino acid analysis considerably enriched in threonine and appeared to be a mixture of \(^{14}\text{C}\)-alkylated tri- to pentapeptides. Fraction T-6b analyzed as a homogeneous tetrapeptide. Preliminary aminopeptidase M studies indicated that a time study on the digestion of peptide T-6a would be quite informative and the data from such a study are shown in Table V. Threonine was released most rapidly, followed by...
phenylalanine. There was a much slower release of the substituted glutamic acid even though nearly 50% of it was present as the NH$_2$-terminal amino acid in the peptide mixture. The remaining dipeptide of leucyl-arginine cleaved even more slowly. Incubation of T-6a with carboxypeptidase A resulted in no release of amino acids. Digestion with carboxypeptidase B yielded an almost simultaneous release of leucine and arginine. The latter data, coupled with the fact that the peptide T-6a resulted from tryptic cleavage of peptide C, indicate that arginine must occur on the COOH-terminal end.

**DISCUSSION**

The amino acid sequence of the peptide containing the glutamic acid at the active center of bovine carboxypeptidase B was determined by established procedures. The only complication in this study resulted from the hydrolysis by pepsin of the peptide bond formed from the NH$_2$ or the COOH group of phenylalanine (for discussion, see Reference 17). This variable cleavage provided several peptides containing the active center glutamic acid.

Bovine carboxypeptidases A and B are now recognized as enzymes having many features in common (1, 2, 18, 19). Hass and Neurath (5) reported that carboxypeptidase A can be alkylated at the active center glutamic acid by an affinity alkylating reaction similar to that described for BrAc-d-Arg and carboxypeptidase B. The amino acid sequence determined for carboxypeptidase B can now be compared with a similar sequence containing the active center glutamic acid of bovine carboxypeptidase A located at position 270 (3, 4). This comparison is as follows:

**Carboxypeptidase A:**

\[
\text{Thr-Phe-Glu-Leu-Arg-Asp-Thr-Gly-Arg-Tyr-Gly-Phe}
\]

**Carboxypeptidase B:**

\[
\text{Thr-Phe-Glu-Leu-Arg-Lys-Gly-Arg-Tyr-Gly-Phe}
\]

These sequences show almost complete homology. The only difference is the substitution of a lysine for the threonine at position 274 of carboxypeptidase A.

It was shown earlier by us (2) that the active center tyrosine containing peptides of carboxypeptidases A and B differed by only two amino acids out of a sequence of 12. A proline and an aspartic acid in carboxypeptidase B replaced the respective glutamine and isoleucine residues in carboxypeptidase A. If the sequences of both active center peptides are compared, only three amino acids differ between the enzymes. Furthermore, Reeck et al. (10) have postulated that the aspartic acid substituted in the active center tyrosyl peptide of carboxypeptidase B may function as the anionic site of substrate binding.

**Porcine carboxypeptidase B** has been shown to have significant structural homology to both bovine enzymes. Roholt and Pressman (20) isolated two peptides containing the active center tyrosyl residue from iodinated porcine carboxypeptidase B and found that their amino acid sequences were identical with that of the bovine enzyme. More recently, Sokolovsky (21) isolated a similar peptide from nitrated porcine carboxypeptidase B. These studies all furnish further proof that the zinc proteases of pancreatic juice could have evolved from a common ancestral gene.

**Acknowledgments**—We would like to express our appreciation to Dr. J. Dodds and Dr. M. Abelseth, Griffin Laboratory, Division of Laboratories and Research, New York State Department of Health, for performing the cannulations required for the isolation of pancreatic juice for this study.

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