CARBOXYPEPTIDASE B

II. MODE OF ACTION ON PROTEIN SUBSTRATES AND ITS APPLICATION TO CARBOXYL TERMINAL GROUP ANALYSIS*

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In a previous publication (1) the isolation, partial purification, and elucidation of the specificity of carboxypeptidase B were described. The enzyme was shown to hydrolyze lysine, arginine, ornithine, and homoarginine from the C-terminal position1 of synthetic peptide substrates.

In recent years carboxypeptidase A2 has been widely used for the identification of C-terminal amino acid residues and partial sequence analysis of proteins and peptides (2). Carboxypeptidase A was shown to act slowly, if at all, upon basic amino acid residues (3, 4), and, therefore, the identification of these amino acids was in question. Since carboxypeptidase B rapidly hydrolyzes lysine and arginine bonds (1), an investigation of the action of this enzyme upon a number of protein and peptide substrates was undertaken. This report describes the release of basic amino acids by carboxypeptidase B upon its reaction with protein substrates, singly and in conjunction with carboxypeptidase A.

Materials

Carboxypeptidase B was prepared as previously described (1) with one of the two following modifications.

Method I—An aliquot of 2.5 ml. of final stock solution of procarboxypeptidase B containing 4 to 5 mg. of protein per ml. was adjusted to pH 8.0

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1 The following abbreviations will be employed throughout this paper: C-terminal and N-terminal for the carboxyl terminal and amino terminal amino acid residues of proteins and polypeptides, DFP for diisopropyl phosphorofluoridate, DIP for diisopropylphosphoryl, S:E for the molar ratio of substrate to enzyme, DNP for dinitrophenyl.

2 For clarity of nomenclature Anson’s classical carboxypeptidase will be referred to as carboxypeptidase A.

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to 8.3 by the addition of 10 mg of K$_2$HPO$_4$. Trypsin (6.25 mg in 2.5 ml. of 10$^{-3}$ M HCl) was added, and the mixture was placed in a dialysis sack and dialyzed with stirring against large quantities of 0.2 M NaCl, pH 8.0, at 25$^\circ$. The dialysis was stopped after 1 hour. Tryptic activation was terminated by the addition of a 200-fold molar excess of DFP, and the activated mixture was then made up to a total of 10 ml. with 0.2 M NaCl. Method II was similar to Method I with the exception that dialysis was omitted. After 1 hour of incubation with trypsin, the activated solution was stirred with 600 mg. of a mixture of ion exchange resins (1:2 wet weight mixture of Dowex 50W-X8 (H form) and Dowex 2-X10 (OH form), both 50 to 100 mesh) for 20 to 30 seconds. Following removal of the ion exchange resins, the pH was quickly readjusted to 8.0, DFP was added (pH maintained at 8.0), and the volume was adjusted as in Method I. Method II yielded an extremely low enzyme blank. 0.5 ml. of these enzyme solutions was used per μmole of protein substrate after routine esterase assays for trypsin (5) showed no traces of residual trypsic activity (20 to 30 minutes after addition of DFP). In all experiments, preparations of carboxypeptidase B, starting from acetone powders, were made up and used on the same day. In the preliminary investigations reported in this paper no attempt was made to discern maximal and minimal concentrations of enzyme applicable to the method. Such data will be reported in a future publication.

Salmine—Two preparations were employed. One of them was kindly supplied by Dr. L. Weil of the Eastern Utilization Branch of the United States Department of Agriculture (Preparation I). The other was prepared in this laboratory (6), extreme care being taken during the preparation to avoid hydrolysis (Preparation II). Both protamines were in the sulfate form. Preparation II was also used as a control to ascertain the potency of the individual carboxypeptidase B preparations.

DIP-$\pi$-chymotrypsin was prepared by employing 0.2 M β-phenylpropionic acid by a method essentially the same as that reported by Dreyer and Neurath (7).$^3$

Chymotrypsinogen was obtained from the Worthington Biochemical Corporation, Freehold, New Jersey, as the damp filter cake of first crystals. It was recrystallized six times from ammonium sulfate, dialyzed salt-free against 10$^{-3}$ M HCl at 4$^\circ$, and lyophilized.

Trypsin and DIP-Trypsin—Four lots of Worthington crystalline trypsin were used (Lots TL-581, 433-C, 436, and TR-20-SF). They were all dialyzed salt-free and lyophilized when required. The salt-free enzyme powder (10 mg. per ml.) was dissolved in cold (0$^\circ$) 0.05 M tris(hydroxymethyl)-
aminomethane buffer, pH 8.0. Immediately thereafter a 200-fold molar excess of DFP in isopropanol was added, the pH being maintained between 7.8 and 8.0. Esterase determinations for trypsinic activity showed essentially complete inactivation in about 20 to 30 minutes. The solutions were quickly adjusted to pH 3.0 and dialyzed against large quantities of 10⁻³ M HCl at 4°.

Another preparation of DFP-inhibited trypsin from Lot TL-581 was made under the exact conditions described above with the exception that the solution was made 0.10 M in CaCl₂ and 0.10 M in β-phenylpropionic acid. It was designated as Lot TL-581-Ca.

Trypsinogen Activation Mixtures—Trypsinogen, Worthington Lot Tg-521, was completely activated under conditions reported by Neurath et al. (8); activity and change in optical rotation were in accordance with the reported data. In another study, 0.1 M β-phenylpropionic acid, a competitive inhibitor of chymotrypsin (3), was used to suppress possible hydrolysis by chymotrypsin. In all activation experiments, at time intervals of 1, 2, 3, and 24 hours, aliquots were withdrawn, and DFP in 200-fold molar excess was added to terminate the reaction, the mixtures being kept at 0°. Loss of activity was followed by esterase assays. After complete DFP inactivation the pH was lowered to 3, and the contents were centrifuged when required, followed by dialysis against 10⁻³ M HCl at 4°.

Carboxypeptidase A—Starting materials were Worthington, three times crystallized, Lots CO-561, CO-568, and CO-569. All preparations contained varying degrees of carboxypeptidase B activity when assayed against hippuryl-L-arginine. Subsequent recrystallizations by the gradient dialysis techniques and isoelectric crystallizations as described by Neurath, Elkins, and Kaufman (9), and modified by Neurath and Schwert (cf. (3)), removed all but traces of this activity.

Protein concentrations in these experiments were determined spectrophotometrically; extinction coefficients were $E_{280}^{1%} = 20.6$ for chymotrypsinogen (10), $E_{280}^{1%} = 14.4$ for trypsin, $E_{280}^{1%} = 13.9$ for trypsinogen (11). Carboxypeptidase A concentrations were determined at 278 nm, assuming a molar extinction coefficient of $8.6 \times 10^4$ (12).

Light Meromyosin—These crystalline preparations, prepared by the short term action of trypsin on myosin (13), were kindly supplied by Dr. K. Laki of this laboratory.

Performic acid oxidized chick globin was kindly supplied by Dr. Jean Rotherham of the National Heart Institute, National Institutes of Health. The dialyzed and lyophilized salt-free preparation was completely soluble in distilled water at pH 8.2.

Poly-L-lysine·HBr (46 mer) was synthesized in this laboratory (14).
EXPERIMENTAL

Carboxypeptidase B preparations and substrates, in amounts previously specified, were incubated for various time periods under direct pH control at 25° and pH 8.0 to 8.2. In certain experiments carboxypeptidase A, S:E = 25:1 prepared as previously reported for end group analysis (4) was added at specific times. When protein substrates were DIP enzymes, a 50-fold molar excess of DFP was added to the substrate solution prior to adjusting the pH to 8.0. Esterase assays against their specific synthetic substrates (3) showed no traces of active enzymes after such treatment.

The reactions were terminated by adding aliquots directly to an excess of Dowex 50-X12 (H form). The mixtures were then treated, and the amino acids were eluted from the resin as previously described (4). In certain experiments, where indicated, the mixtures were dinitrophenylated, and the amino acids were identified and estimated according to the method of Fraenkel-Conrat et al. (2).

Initial identifications of a portion of the ion exchange resin eluents were carried out on two-dimensional paper chromatograms by employing the technique of Irreverre and Martin (15). The chromatograms, run on Schleicher and Schuell No. 598 filter paper, were lightly sprayed on one side with a sensitive Sakaguchi reagent for arginine. Following this procedure the entire paper was dipped in 0.25 per cent ninhydrin in acetone containing 5 per cent (v/v) glacial acetic acid for identification of other amino acids.

For quantitative purposes, arginine was determined by a colorimetric Sakaguchi procedure developed in this laboratory by Hayden and Irreverre. When necessary lysine and other amino acids were determined quantitatively by the ion exchange method of Moore and Stein (16) or the DNP technique of Fraenkel-Conrat et al. (2).

Results

Salmine—Table I shows the results obtained upon the incubation of carboxypeptidase B preparations, of equal potency against synthetic substrates, with two preparations of salmine sulfate. The results of the addition of carboxypeptidase A after 2 hours prior incubation with carboxypeptidase B are also illustrated.

Preparation II, in later stages of these investigations, was employed to estimate enzyme potency against protein substrates. For compari-

4 Irreverre, F., Kominz, D., and Hayden, A., to be published.
5 Hayden, A., and Irreverre, F., to be published.
6 Units of carboxypeptidase B are defined as $20 \times K_0$, where $K_0$ = the zero order rate constant with 0.025 M hippuryl-L-arginine as substrate.
sons on an equivalent activity basis, stock enzyme preparations in a sufficient amount to liberate 2 equivalents of arginine per mole of salmine substrate in 3 hours were employed, except when otherwise specified.

Chymotrypsinogen and Trypsinogen—Since neither chymotrypsinogen nor trypsinogen yields C-terminal groups to carboxypeptidase A (4, 17) or to the technique of hydrazinolysis (Akabori (18)), incubation of these substrates with carboxypeptidase B followed by, or simultaneously with, carboxypeptidase A was performed for as long as 6 hours. In no case could liberation of any amino acids by these enzymes be detected.

DIP-\(\pi\)-Chymotrypsin—The results of the incubation of carboxypeptidase B and carboxypeptidase A, singly and in combination with one another, with DIP-\(\pi\)-chymotrypsin, are shown in Table II. The amount of the carboxypeptidase B preparation employed in these experiments was of sufficient potency to release 2 equivalents of arginine from salmine Preparation II in 2 hours.

DIP-Trypsin—In Table III the hydrolysis of various preparations of DIP-trypsin by carboxypeptidase B is illustrated. Conditions employed were identical to those in the preceding experiments with DIP-\(\pi\)-chymotrypsin.

The action of carboxypeptidase A on two lots of the DIP-trypsins, Lots TL-581-Ca and TR-20-SF, was essentially in complete accord with the previous results reported by Davie and Neurath (17); only faint traces of lysine and other minor amino acid spots appeared on two-dimensional chromatograms.

Trypsinogen Activation Mixtures—Since commercial trypsin yielded such large quantities of lysine following 2 hours of incubation with carboxypeptidase B, it was of interest to examine the C-terminal groups of trypsin formed by the tryptic activation of trypsinogen over an extended period of time.

During activation (120 minutes) and for 22 hours following activation,
no significant amounts of lysine were released by carboxypeptidase B from the dialyzed trypsinogen activation mixtures. These results were unaltered by the presence of 0.1 M β-phenylpropionic acid in the activation mixtures.

Performic acid-oxidized chick globin, when incubated with Worthington carboxypeptidase A, Lot 3561 (three times crystallized), S:E = 25, pH 8.2, yielded within 15 minutes 1 equivalent each (based on the molecular weight of 65,000), of lysine and histidine as well as lesser quantities of alanine, leucine, and other minor amino acid spots. Following recrystallizations of the enzyme as described under “Materials,” the recrystallized preparation when incubated with globin substrate yielded 1 equivalent of histidine, smaller quantities of leucine and alanine, but only slight traces of lysine. Contrariwise, the supernatant solutions from the recrystallizations, upon incubation with the substrate, released approximately 1 equivalent of lysine, but only small amounts of histidine, leucine, and alanine.

### Table II

**Equivalents of Amino Acids Per Mole DIP-π-Chymotrypsin Liberated by Carboxypeptidase A and Carboxypeptidase B**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Time</th>
<th>Arginine</th>
<th>Serine*</th>
<th>Leucine*</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>2</td>
<td>0.7</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>0</td>
<td>&quot;</td>
<td>0.10-0.15</td>
</tr>
<tr>
<td>B + A†</td>
<td>(2 + 1)</td>
<td>0.7</td>
<td>0.3</td>
<td>0.25-0.30</td>
</tr>
</tbody>
</table>

* Estimated by DNP method of Fraenkel-Conrat et al. (2).  
† S:E = 25.

### Table III

**Lysine Liberation from DIP-Trypsin by Carboxypeptidase B**

<table>
<thead>
<tr>
<th>Lot No. DIP-trypsin preparation</th>
<th>Equivalents of lysine liberated with time</th>
<th>Other amino acids identified after 120 min.†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min.*</td>
<td>120 min.†</td>
</tr>
<tr>
<td>581</td>
<td>0.93</td>
<td>3.4</td>
</tr>
<tr>
<td>433-C</td>
<td>0.97</td>
<td>3.4</td>
</tr>
<tr>
<td>436</td>
<td>0.94</td>
<td>3.3</td>
</tr>
<tr>
<td>TR-20-SF</td>
<td>0.97</td>
<td>3.4</td>
</tr>
<tr>
<td>TL-581-Ca</td>
<td>0.94</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Determined by ion exchange chromatography according to Moore and Stein.  
† Estimated by the DNP technique of Fraenkel-Conrat et al. (2).  
‡ Qualitatively identified by paper chromatography. The amino acids listed were observed in all enzymatic hydrolysates of all trypsin preparations.
A purified preparation of carboxypeptidase B yielded essentially the same results as were obtained with the supernatant materials. Amino acid analyses were performed by the DNP technique of Fraenkel-Conrat et al. (2).

Preliminary experiments with carboxypeptidase B were carried out on light meromyosin. In all cases the only major amino acid released by carboxypeptidase B preparations was lysine. Poly-L-lysine, when incubated with carboxypeptidase B, yields only free lysine. Quantitative estimations were not performed.

Table IV summarizes some of the data pertaining to the effect of carboxypeptidase B on various protein substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Carboxypeptidase B</th>
<th>Carboxypeptidase A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chymotrypsinogen</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Trypsinogen</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Commercial trypsin</td>
<td>Lysine</td>
<td>0</td>
</tr>
<tr>
<td>Activated trypsinogen*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DIP-α-chymotrypsin</td>
<td>Arginine</td>
<td>0</td>
</tr>
<tr>
<td>Salmine</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td>Performic acid-oxidized chick globin</td>
<td>Lysine</td>
<td>Histidine</td>
</tr>
<tr>
<td>Light meromyosin</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td>Poly-L-lysine (46 mer)</td>
<td>&quot;</td>
<td>0</td>
</tr>
</tbody>
</table>

* Trypsin-activated, see the text for the details.

DISCUSSION

The present experimental data extend to protein substrates our knowledge of the specificity of carboxypeptidase B. It is evident that carboxypeptidase B may be employed for the determination of basic C-terminal amino acid residues in proteins and peptides. With the consideration in mind of the limitations of other enzymatic methods of end group analysis, one may employ carboxypeptidase B, either alone or coupled with carboxypeptidase A, to elucidate C-terminal amino acid patterns of certain proteins and peptides.

The inability of carboxypeptidase B to liberate free basic amino acids from trypsinogen or chymotrypsinogen, coupled with negative results in previous attempts to obtain C-terminal residues via carboxypeptidase A (4, 17) or chemical techniques (18), demonstrates either that these zymo-

7 Estimated from the intensity of the ninhydrin color to be about 1 equivalent.
gens contain C-terminal amino acids which are not identifiable by the present methods of C-terminal group analysis or that they contain no free α-carboxyl grouping.

Since the initial reports of a C-terminal lysine in DIP-trypsin (19) were in conflict with the negative results reported by chemical methods (18), a more complete study in which carboxypeptidase B was employed was undertaken. The experimental results indicate that many preparations of commercial trypsin, although crystalline, are chemically heterogeneous. These data support the findings that most of the tryptic activity remains intact during the initial stages of autolysis (20) and suggest that perhaps the trypsin molecule is held intact by chemical cross-linkages during the initial stages of autolysis. On the other hand, trypsin resulting from a more rapid activation of trypsinogen contains virtually no lysine available to carboxypeptidase B. This is compatible with the hypothesis of Davie and Neurath that the hydrolysis of the ...lys.isoleu bond in trypsinogen coupled with release of hexapeptide is sufficient to impart enzymatic activity (11).

The C-terminal sequence of amino acids of DIP-π-chymotrypsin heretofore inferred to be ...leu.ser.arg (7) has now been verified through the use of carboxypeptidase B coupled with carboxypeptidase A. Since the best preparations of carboxypeptidase B to date contain a minimum of 4 per cent carboxypeptidase A contamination (1), the appearance of trace quantities of other amino acids is probably attributable to the latter enzyme. The appearance of small quantities of leucine following incubation of DIP-π-chymotrypsin with carboxypeptidase A is indicative of small quantities of δ-chymotrypsin impurity (7).

The results achieved with the performic acid-oxidized chick globin confirm and extend to protein substrates the previous observation that C-terminal histidine is not removed by carboxypeptidase B (1).

The few examples of C-terminal group analysis presented here as realized by carboxypeptidase B are only representative examples of the potential value of this enzyme in protein and peptide structure studies. The use of carboxypeptidase B in conjunction with other methods opens the way to C-terminal sequence analysis of peptides obtained during tryptic digestion of protein material as exemplified by elucidation of the C-terminal sequence of DIP-π-chymotrypsin.

8 One significant side light of the specificity of carboxypeptidase B is the release of S-(β-aminoethyl)cysteine from a tryptic digest of beef insulin which had been reduced and allowed to react with β-bromoethylamine as outlined by Lindley (21). Tietze et al. (22).
SUMMARY

The specificity requirements of a new proteolytic enzyme, carboxypeptidase B, have been examined by employing protein substrates. The enzyme hydrolyzes lysine and arginine, but not histidine, from the carboxyl terminal position of polypeptide chains. It can therefore be used to determine carboxyl terminal lysyl and arginyl residues in proteins and polypeptides.

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