Primary Structure of Bovine Carboxypeptidase B

INFERENCES FROM THE LOCATIONS OF THE HALF-CYSTINES AND IDENTIFICATION OF THE ACTIVE SITE ARGinine*

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SUMMARY

Bovine pancreatic carboxypeptidase B is a protein which is believed to have evolved with carboxypeptidase A by gene duplication from a common ancestral prototype enzyme. Whereas bovine carboxypeptidase A has but one disulfide bond, carboxypeptidase B has three. Reduced, S-aminoethylated carboxypeptidase B was maleylated and the product subjected to tryptic hydrolysis. The peptides with COOH-terminal arginine so produced were fractionated by gel filtration and by chromatography on DEAE-Sephadex A-25. Eleven of the 14 principal peptides possible were isolated. The peptides that contained the six S-aminoethylcysteine residues originating from the half-cystines in the enzyme were subjected to sequence analysis and were aligned on the basis of substantial homologies with sequences in carboxypeptidase A. Referred to the sequence of carboxypeptidase A, the half-cystines in carboxypeptidase B are at positions 66, 79, 138, 152, 161, and 166. Those at positions 138 and 161 are homologous with the two half-cystine residues in carboxypeptidase A. The remaining four half-cystines, when related to the known chain conformation of carboxypeptidase A, are in locations which would be stereochemically compatible with the presence of two additional disulfide bonds in the structure: one, as previously found, between half-cystines 66 and 79 and the other between half-cystines 152 and 166. One of the half-cystines is present in a peptide which terminates in an arginine residue recognized to be homologous with Arg-145 in carboxypeptidase A, a residue critical for the establishment of the specificity of carboxypeptidase A. The present experiments have permitted placement in sequence of an additional 85 residues in carboxypeptidase B, bringing the total residues so placed to 221. Of these residues, 100 are homologous with residues in carboxypeptidase A. These homologies, the configurational correlations revealed by the positions of the half-cystines, the conservation of at least two of the three residues involved in zinc binding (His-69 and Glu-72) and of the three functionally essential residues in the active site (Arg-145, Tyr-248 and Glu-270) all implicate the existence of a close structural homology with carboxypeptidase A.

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The pancreatic exopeptidases carboxypeptidase A and carboxypeptidase B are structurally and functionally closely related. Both enzymes possess essentially identical molecular weights, exhibit similar amino acid compositions, are zinc metalloproteins, and operate on their respective substrates by essentially the same mechanisms (for reviews, see References 1 and 2). The enzymes are clearly distinguished in their specificities and in this regard share the same relationship as trypsin and the chymotrypsins. These properties have led to the view that carboxypeptidase A and B, on the one hand, and the chymotrypsins and trypsin, on the other, represent examples of enzymes that have evolved by gene duplication from a single ancestral prototype (3). Implicit in this view is the concept that these pairs of enzymes must have the same over-all conformation, with the differences in specificity attributable to a relatively minor change in the distribution as to type of the side chains that comprise the substrate binding sites, most notably the presence of a carboxylic acid side chain in place of a neutral side chain in carboxypeptidase B and trypsin. Considerable support for this position developed with respect to the trypsin-chymotrypsin pair in advance of the x-ray analysis of bovine trypsin which later demonstrated unequivocally the close similarity in the tertiary structures of these two enzymes (4). Most notably, when the amino acid sequence of trypsin was fitted to the chain conformation found by x-ray crystallography for chymotrypsin, the correct pairing of the additional half-cystines in trypsin was observed and the catalytically essential amino acids were found in homologous locations (5). Moreover, the subsequent x-ray studies (4), as well as chemical modification (6), showed that the critical difference in specificity for trypsin is determined by the presence of Asp-189 (numbering as described in Reference 4) in the binding site.

Comparably detailed knowledge has yet to be developed for the carboxypeptidases. Thanks to the x-ray crystallographic studies of Lipscomb and his colleagues (7, 8) and to the primary structure elucidated by Bradshaw et al. (9), the structure of bovine carboxypeptidase A is known in some detail. Significant features of the structure are (7) the three ligands for the zinc atom provided by His-69, Glu-72 and His-196 (8); the specificity-determining arginine residue, Arg-145, which establishes an electrostatic bond to the COOH-terminal carboxyl group of the substrate (7); the catalytically important phenolic group of Tyr-248, which functions as a proton donor at the amino group of the COOH-terminal residue in the substrate, as that residue is cleaved (7); and the carboxyl group of Glu-270, a nucleophile in
the initial attack on the substrate carbonyl group at which cleavage will take place (7).

Structural information published previously concerning bovine carboxypeptidase B is in keeping with the view that the two carboxypeptidases are configurationally related proteins. Several segments of the peptide chain in carboxypeptidase B have been characterized by sequence analysis and, as pointed out by Reeck et al. (10), display a significant structural homology to sequences in carboxypeptidase A. From a functional standpoint, the greatest interest attaches to three segments of the chain so characterized: (a) a 32-residue segment containing a disulfide bond (positions 65 through 96, when numbered as in carboxypeptidase A) described in previous work from this laboratory (11) and recognized later, when the sequence of carboxypeptidase A was found by Bradshaw et al. (12), to contain two of the zinc ligands in the molecule, His-69 and Glu-72; (b) a segment of 12 residues (positions 246 through 257), including the catalytically important Tyr-248, identified by Plummer (13); and (c) a segment of 12 residues (positions 268 through 279), including the functionally significant Glu-270, also identified in Plummer's laboratory (14, 15). Work by Neurath and his colleagues (16, 17) has shown that the active site-directed reagent N-bromosuccinimide methylates both carboxypeptidase A and carboxypeptidase B by introduction of one molecule of reagent at a dipeptide sequence, Phe-Glu, which in all likelihood relates to residues 269 and 270 in both enzymes.

The experiments reported in the present communication were undertaken in connection with studies aimed at the determination of the complete amino acid sequence of bovine carboxypeptidase B and are presented separately because they document conservation of the functionally important arginine residue in the active site, Arg-145, and locate the remaining half-cystine residues in the carboxypeptidase B molecule at positions 138, 152, 161, and 166, as will be seen, a structural feature entirely consistent with the view that the carboxypeptidases have essentially identical tertiary structures.

RESULTS AND DISCUSSION

In previous work from this laboratory a series of tryptic peptides was obtained from reduced, S-aminoethylated carboxypeptidase B (18). These peptides contained a preponderance of the polar residues and included sequences for approximately two-thirds of the residues in the peptide chain. Moreover, they appeared to provide an accounting for all of the cysteine and cystine in carboxypeptidase B. Attempts to achieve a more complete representation of the residues in the molecule by CNBr cleavage have met with partial success. The two smallest fragments thus derived have already been described (11, 19). One (CN-1) is a peptide of 14 residues that comprises the COOH terminus of the molecule (19); the second (CN-2) is the 32-residue, disulfide-containing fragment to which reference has already been made (11). This fragment provides a placement for one of the three disulfide bonds in carboxypeptidase B.

In the present experiments, particulars concerning which will be found in the "Supplement," i.e., the reduced, S-aminoethylated protein was maleylated in 6 m guanidine hydrochloride and the product was subjected to trypptic hydrolysis. The resulting fragments, all of which were soluble at pH 8, were fractionated first by gel filtration on Sephadex G-25 and G-50, and subsequently by chromatography on DEAE-Sephadex A-25 at pH 7 with gradients of increasing NaCl concentration. The peptide fractions so obtained were desalted with the aid of Bio-Gel P-2 columns and, when necessary, were deblocked by treatment with pyridine-acetic acid buffer at pH 3.3 for 30 hours at 30°.

The results are most conveniently presented with reference to Fig. 1 and Table I. Since there are 13 arginine residues in carboxypeptidase B (21), 14 principal fragments may be expected when the maleylated protein is cleaved with trypsin. As Table I shows, 11 of these fragments have been obtained in a sufficiently homogeneous state to allow sequence analysis. The remaining three have been located (see "Supplement"), but will require further purification before they can be subjected to sequence analysis. However, within the limits of error of amino acid analyses with relatively large peptide fragments generated in this way, it is now evident that the three peptides that remain to be purified to homogeneity, together with those in Table I, will provide a complete accounting of the residues in carboxypeptidase B.

Of more immediate consequence is the fact that the fragments in Table I provide a representation of the seven S-aminoethylcysteines in the reduced, S-aminoethylated protein. Fragments I-3b and V (see Fig. 1) include the half-cystines present in the previously characterized disulfide fragment (11). The sequences in fragments I-3b and V align well with positions 37 through 84 in carboxypeptidase A, with a total of 22 homologous residues. Further evidence for the correctness of this precise alignment derives from the fact that fragment I-3j is clearly, by virtue of the sequence established by Reeck et al. (10) for residues 3 through 33, the NH₂-terminal fragment. The composition of fragment I-3j shows that it contains residues 3 through 33 plus 1 residue each of serine and arginine. Moreover, when fragment I-3j was treated with carboxypeptidase A and B, the presence of the terminal sequence Ser-Arg was revealed, establishing residue 36 as arginine and showing that fragment I-3b starts at position 37.

Fragment I-5a, a dodecapeptide, was found to align with positions 134 through 145 in carboxypeptidase A, with 6 residues in locations of homology, including Arg-145, the residue which provides a critical electrostatic interaction with the terminal carboxyl group of a substrate. Fragment I-2c, with 39 residues, including 3 of S-aminoethylcysteine, likewise was found to align well with positions 146 through 184, with 22 residues in locations of homology.

The 7th S-aminoethylcysteine residue is present in fragment I-3f, which, being devoid of arginine, is the COOH-terminal fragment. Fragment I-3f contains 39 residues. It covers a span of residues already placed in sequence by the work of Kimmel and Plummer (19), Wintersberger (20), and Elainga et al. (19), and has the composition to be expected from the sequence. The S-aminoethylcysteine at position 290 is derived from the single cysteine residue in carboxypeptidase B (20). Of the small fragments in Table I, fragments II-1 and IV describe this paper and the JBC Document number, the form desired (microfiche or full size photocopy of 22 pages), and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014, and must be accompanied by a remittance to the order of the journal in the amount of $2.50 for microfiche or $3.30 for photocopy.
Fig. 1. A partial amino acid sequence of bovine carboxypeptidase B (B) aligned to maximize homology with the complete sequence of carboxypeptidase A (A) determined by Bradshaw et al. (9). Homologous residues are underlined. Residues shown by Lipscomb et al. (7) to be located in α helices are indicated by ______, those located in the internal β structure by ______. The helices carry the letter designations of Lipscomb et al. (7). Principal peptides obtained in the present experiments are shown immediately below the sequence for carboxypeptidase B and are identified, as in Table I, with a Roman numeral prefix. Peptides derived from these principal fragments by further cleavage with chymotrypsin and trypsin carry additional designations of C and T, respectively. The results of experiments on carboxypeptidase-catalyzed hydrolyses with these peptides are shown by the symbol —— inserted immediately above the peptide studied. Results obtained on Edman degradation of these various peptides are shown similarly by the symbol ——. Peptides isolated previously (18) from a tryptic hydrolysate of reduced, S-aminoethylated carboxypeptidase B are designated with the prefix AE-T—. A number of these peptides were subjected to sequence analysis in unpublished ex-
FIG. 1.

As already pointed out by Bradshaw et al. (12), examination of the tertiary structure of bovine carboxypeptidase A as revealed by x-ray crystallography (7) shows that half-cystines located at positions 66 and 79 would be at the correct distance apart to permit establishment of a disulfide bond between them (see Fig. 2). This and the homology of the residues included between these two positions in the disulfide loop from carboxypeptidase B (11) were regarded by these authors as good evidence that the carboxypeptidases are homologous proteins. The present results provide a substantial amplification of this view. In carboxypeptidase A there is a single disulfide bond connecting half-cystines 66 and 79.

The sequence from 280 through 293 to Wintersberger (20). CN-1 and CN-2 are peptides sequenced previously in this laboratory (11, 21) and were obtained by CNBr cleavage of reduced, S-aminoethylated carboxypeptidase B. (CN-2)T-6 is a tryptic peptide derived from CN-2.

These same peptides were also obtained from a tryptic hydrolysate of reduced, S-aminoethylated carboxypeptidase B (18) and subjected independently to sequence analysis (see Fig. 1). Fragment II-2 has been assigned to positions 273 through 276 on the basis of its composition and the sequences of dipeptides AE-T-35 and AE-T-36 isolated previously (Fig. 1 and Reference 18). Assignment of fragments III and VI must await further experiments.

Unpublished experiments by Dr. M. Elzinga in this laboratory.
TABLE I

Amino acid composition of tryptic peptides from maleylated, reduced, S-aminoethylated bovine carboxypeptidase B

The compositions are expressed in terms of molar ratios of the constituent amino acids in 22-hour acid hydrolysates. Tryptophan was determined separately by the method of Liu (see Reference 38 in "Supplement") with a hydrolysis time of 48 hours. Except for fragment I-3f, the calculations were based on the assumption that each peptide contained 1.0 residue of arginine. Values less than 0.1 residue have been omitted.

| Peptide | I-2c | I-3c | I-3f* | I-3f| I-3a | IIc | IId | IIIe | IVf | Vg | VI |
|---------|------|------|-------|-----|-----|-----|-----|-----|-----|-----|-----|
| Lysine  | 2.2  | 3.1  | 1.3   | 1.4 | 0.8 |     | 1.0 |     |     |     |     |
| Aminomethyl cysteine | 3.0  | 1.0  | 1.1   | 1.1 |     |     |     |     |     |     |     |
| Histidine | 1.0  | 1.0  | 1.1   | 1.0 |     |     |     |     |     |     |     |
| Arginine | 1.0  | 1.0  | 1.0   | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Aspartic acid | 4.9  | 3.0  | 0.4   | 3.9 | 2.2 | 1.0 |     |     |     |     |     |
| Threonine | 1.1  | 2.8  | 3.2   | 3.9 | 2.0 | 1.0 | 1.0 |     |     |     |     |
| Serine   | 4.8  | 2.0  | 2.3   | 2.8 | 1.8 | 1.0 |     | 0.9 |     |     |     |
| Glutamic acid | 4.0  | 0.1  | 6.0   | 5.9 | 0.1 |     | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Proline  | 1.0  | 2.1  | 2.1   | 2.1 |     |     |     |     |     |     |     |
| Glycine  | 3.1  | 5.0  | 1.3   | 1.4 | 2.3 |     | 1.0 |     |     |     |     |
| Alanine  | 6.1  | 3.2  | 1.4   | 2.2 |     | 1.0 |     |     |     |     |     |
| Valine   | 1.1  | 2.1  | 3.2   | 1.4 |     | 1.0 |     |     |     |     |     |
| Methionine | 1.1  | 1.1  | 1.0   | 0.1 |     |     |     |     |     |     |     |
| Isoleucine | 1.9  | 2.0  | 2.1   | 2.2 |     |     |     |     |     |     |     |
| Leucine  | 0.2  | 3.0  | 4.3   | 1.8 | 1.1 |     |     |     |     |     |     |
| Tyrosine | 1.1  | 0.8  | 2.5   | 2.1 |     | 1.0 |     |     |     |     |     |
| Phenylalanine | 1.9  | 2.9  | 1.1   | 0.3 |     |     |     |     |     |     |     |
| Tryptophan | 1.0  |     |       |     |     | 1.8 |     |     |     |     |     |
| Total residues | 38* | 36   | 32    | 34  | 12  | 4   | 4   | 3   | 4   | 13  | 2   |

* Composition and treatment with carboxypeptidase showed this fragment to derive from the COOH-terminal end of the protein.

The molar ratios shown are based on assumption the hydrolysate contained 6.0 residues of glutamic acid.

† Composition and treatment with carboxypeptidase showed this fragment to derive from the amino-terminal end of the protein.

‡ Identified previously (11) as Glu-Ala-Val-Arg.

§ Identified previously from two tryptic dipeptides (18) as Asp-Lys-Gly-Arg.

# Identified previously as Ser-Thr-Glu.

I Identified previously as Glu-Trp-Ile-Ser-Pro-Ala-Phe-Cys (aminoethyl)-Glu-Trp-Phe-Val-Arg.

This peptide was found by sequence analysis to contain 39 residues by virtue of the presence of 6 serines, not 5, as suggested by amino acid analysis. The extensive destruction of serine is unexplained.

cystines 138 and 161 (7). Since half-cystines 138 and 161 are here shown to be conserved in carboxypeptidase B (Fig. 1), this disulfide bond is undoubtedly also conserved. The remaining two half-cystines in carboxypeptidase B, at positions 152 and 166 (Fig. 1), must therefore, by difference, be involved in the formation of the third disulfide bond in the molecule. Examination of the structure of carboxypeptidase A (Fig. 2) shows that half-cystines at positions 152 and 166 are, in fact, at just the right distance to be linked by a disulfide bond. The interesting structural correlations revealed by the positions of the half-cystines in carboxypeptidase B, the conservation of at least 2 of the 3 residues involved in zinc binding (His-69 and Glu-72) and of the 3 functionally essential residues in the active site, Arg 145, Tyr-248, and Glu-270 (14-17), coupled with the correspondence of 100 of the 221 residues so far placed in sequence all speak to a close structural homology with carboxypeptidase A.

Two notable features of the folded polypeptide chain in carboxypeptidase A are an internal, largely buried P-pleated sheet (7), composed of eight adjacent chain segments, and a so-called random chain region, from residues 122 to 174, which forms most of one side of the active site pocket (7). Five of the β structure segments and most of the random chain region are represented in the portion of the carboxypeptidase B molecule so far analyzed. Four of the β structure segments are the four closest to the amino terminus and comprise residues 32 to 37, 46 to 54, 61 to 67, and 103 to 111. The fifth, closest to the COOH terminus, comprises residues 265 to 271 (see Fig. 2). Of these five segments, the fifth shows the highest level of correspondence, residues 265 to 271, which include the active site Glu-270, are the same in both enzymes. The first four display significantly less homology, but all substitutions of internal residues are for residues of like type. Least homology is displayed in the first segment, 32 to 37, but this contains several external residues. The second, 46 to 54, shows significant variation only at the ends; there is conservation of apolar side chains between positions 47 and 52. Similar features characterize the third and fourth segments. It should be noted that Lys 51, Asp 65, and Asp 104 in these first four segments have externally disposed side chains, that of Asp-65 forming part of the active site cavity.

Over half of the residues placed in the random chain region of carboxypeptidase A are in homologous locations. Moreover, there is a remarkable conservation of residues as to type in this region. The sequence around the active site arginine (Arg-145) is striking. Residues 142 through 151 are either homologous or show conservative replacements. These same features are seen in the segment from residue 160 to residue 173.

The α helical regions so far covered display the greatest level of residue variation. Inasmuch as the helices are predominantly
FIG. 2. The conformation of the peptide chain in bovine carboxypeptidase A (data from Lipscomb et al. (7)). The 6 half-cystines in carboxypeptidase B are at positions 66, 79, 138, 152, 161, and 166, when the partial sequence is aligned as shown in Fig. 1. Of these 6 residues, those at positions 138 and 161 are homologous on the “outside” of the internal β-pleated sheet core of carboxypeptidase A, this observation is readily rationalized. Moreover, many of those residues conserved in the helices, as, for example, Ile-18, Trp-73, Leu-100, Val-179, or Phe-182, are residues with internal side chains.

Finally, we wish to comment on the regions where the peptide chain changes direction sharply. The first of these is the segment at positions 30 to 31 (Fig. 2), which terminates the first a helix (helix A). It is significant that the helix-breaking proline at position 30 is conserved. The turn at positions 55 to 58 which connects the second and third segments of the β structure involves a tripeptide sequence, Gly-Ser-Asn, present in both enzymes. The next turn, from residues 68 to 72, loops the chain around the zinc atom and contains the two zinc ligands, His-69 and Glu-72. The two substitutions in this turn are conservative. The turn between helices B and C, which includes residues 89 to 93, displays conservation of the Tyr-Gly sequence at residues 90 to 91. Helix C turns directly into the fourth segment of β structure. The critical residue here is 102, serine in carboxypeptidase A and lysine in carboxypeptidase B. The region between the fourth β structure segment and the next α helix, helix D, residues 111 to 114, does not involve a major change in direction of the chain, but is in the form of a small loop. There is little correspondence between the enzymes in this region. Moving to the COOH-terminal end of the molecule, it will be seen that there is a relatively long segment of the chain, residues 272 to 287, between the last element of the β structure and the final helix, helix H. This segment has a complex random chain folding, as does the longer random chain segment between 122 and 174, and, like the latter, it is a region of unusually high residue correspondence in the two structures. Indeed, this segment of 16 residues contains 12 that are identical in both enzymes.

These studies are being continued with a view to the development of the complete amino acid sequence of carboxypeptidase B and construction of a three-dimensional model which will enable a more thorough examination of the factors that govern conservation of the over-all structural configuration of the pancreatic carboxypeptidases.

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The principal nomenclature for these depressions, depres-
sion-thickened, aldehydesulfonation, succinate-sulfonation, and fatty-enzyme products from them, have been standardized by the Nomenclature Committee of the American Society for Biochemistry and Molecular Biology. These abbreviations are used in the following discussion of the structural properties of these products and are defined in the legend to the figure. 

Absorption and Amino Acid Composition: The procedure was essentially as described by Cohn and Edsall (1). The protein samples were dissolved in 10 mM sodium phosphate buffer, pH 7.0, and aliquots were taken for absorbance measurements at 280 nm. The amino acid compositions were determined by automatic amino acid analyzers. The results are expressed as micromoles of each amino acid per milligram of protein.

Chromatography: The protein samples were applied to a column of Sephadex G-25 to remove low molecular weight impurities. The column was equilibrated with 0.1 M acetic acid, and the protein sample was eluted with the same buffer. The fractions were collected and assayed for protein content.

The results of the amino acid analysis and chromatography are presented in Table 1. The amino acid compositions of the various protein samples are listed in the table. The data are expressed as micromoles of each amino acid per milligram of protein.

The absorbance measurements and amino acid compositions of the various protein samples are shown in Figure 1. The absorbance measurements are expressed as absorbance units at 280 nm. The amino acid compositions are expressed as micromoles of each amino acid per milligram of protein.
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