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Amino Acid Sequence of Crayfish (*Astacus fluviatilis*) Carboxypeptidase B†

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ABSTRACT: The complete amino acid sequence of carboxypeptidase B from the crayfish *Astacus fluviatilis* has been determined. The S-carboxymethylated protein was cleaved with cyanogen bromide and with trypsin, before and after citraconylation of lysine residues. Peptides were purified by gel filtration followed by reverse-phase high-performance liquid chromatography and analyzed by a liquid-phase sequencer. The enzyme contains 303 amino acid residues and has a molecular weight of 33 899 (without zinc). The crayfish enzyme shows 44.7% sequence identity with bovine carboxypeptidase B as compared to 44.0% identity with bovine carboxypeptidase A. The constituents of the active site of the bovine enzymes are present in corresponding positions in the crayfish enzyme. Isoleucine-255, which, in carboxypeptidase A, is located at the pit of the substrate binding pocket, is occupied by aspartic acid in the crayfish enzyme, as in bovine carboxypeptidase B. When compared to bovine and rat carboxypeptidases, the amino acid sequence of the crayfish enzyme displays one deletion, two insertions, and 35 amino acid replacements but is fully compatible with the X-ray structure of bovine carboxypeptidase B. The present analysis affords the first detailed view into the evolution of carboxypeptidases at the invertebrate level.

Bovine pancreatic carboxypeptidase A (EC 3.4.12.2) is the most thoroughly characterized metallopeptidase and has served as a prototype in the study of the mechanism of action of metalloenzymes in general [for the most recent review, see Vallee et al. (1983)]. The amino acid sequences (Bradshaw et al., 1969; Titani et al., 1975) and the X-ray structures (Lipscomb et al., 1968; Schmid & Herriott, 1976) of both bovine carboxypeptidases A and B (EC 3.4.12.3) are known. By comparison, little is known of carboxypeptidases of other mammalian species (Folk, 1970; Reeck & Neurath, 1972; Everitt & Neurath, 1980; Quinto et al., 1982) and even less of those of invertebrate origin (Gates & Travis, 1973; Zwilling et al., 1979, 1980). To fill this gap of knowledge, and to establish a data base for a study of the evolution of carboxypeptidases, we have undertaken an investigation of the amino acid sequence of crayfish (*Astacus fluviatilis*) carboxypeptidase. This species was chosen for several reasons: (1) crayfish lies on the evolutionary pathway from which decapode crustacea and mammals diverged some 700 million years ago; (2) preliminary analysis of the amino-terminal sequence of crayfish carboxypeptidase indicated significant structural homology to bovine carboxypeptidase A (Zwilling et al., 1979); (3) in view of our recent findings that bovine and crayfish trypsins are homologous and evidently have diverged from a common ancestor (Titani et al., 1983), it appeared of interest to compare the rates of evolution of two enzymes that originated from analogous structural milieus, the pancreas in the case of the mammalian enzymes and the hepatopancreas in the case of the crayfish enzymes.

In this paper, we report the complete amino acid sequence of crayfish (*Astacus fluviatilis*) carboxypeptidase B and evaluate its homology to bovine and rat carboxypeptidases. On the basis of a comparison of bovine and crayfish carboxypeptidases and trypsins, respectively, we present arguments about the rates of evolution of these pancreatic proteases.

Materials and Methods

Crayfish carboxypeptidase originates in the hepatopancreas of the animal and is secreted into the stomachlike cardia where it is present in the active form at approximately 1 mg/mL digestive fluid. No evidence for the presence of a zymogen form has been obtained thus far.

Digestive fluid was collected from the cardia of a large number of animals by the method previously described for crayfish trypsin (Zwilling & Neurath, 1981) and stored in the frozen state until used. Carboxypeptidase was isolated by affinity chromatography on immobilized potato inhibitor as described by Ager & Hass (1977). Details of the isolation procedure have been previously published (Zwilling et al., 1979). Material prepared in this fashion was homogeneous as judged by sodium dodecyl sulfate gel electrophoresis, two-dimensional immunoelectrophoresis, and amino-terminal sequence analysis (Zwilling et al., 1979).

The protein was reduced with dithiothreitol and was S-carboxymethylated with either cold or 1-^{14}C-labeled iodoacetic acid (New England Nuclear). CM-protein[1] was cleaved with cyanogen bromide or with trypsin after labeling of methionyl residues with [^{14}C]methyl iodide (New England Nuclear) (Sasagawa et al., 1983). [^{14}C]CM-protein was digested with

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[1] Abbreviations: CM, carboxymethyl; HPLC, high-performance liquid chromatography; Pth, phenylthiohydantoin.

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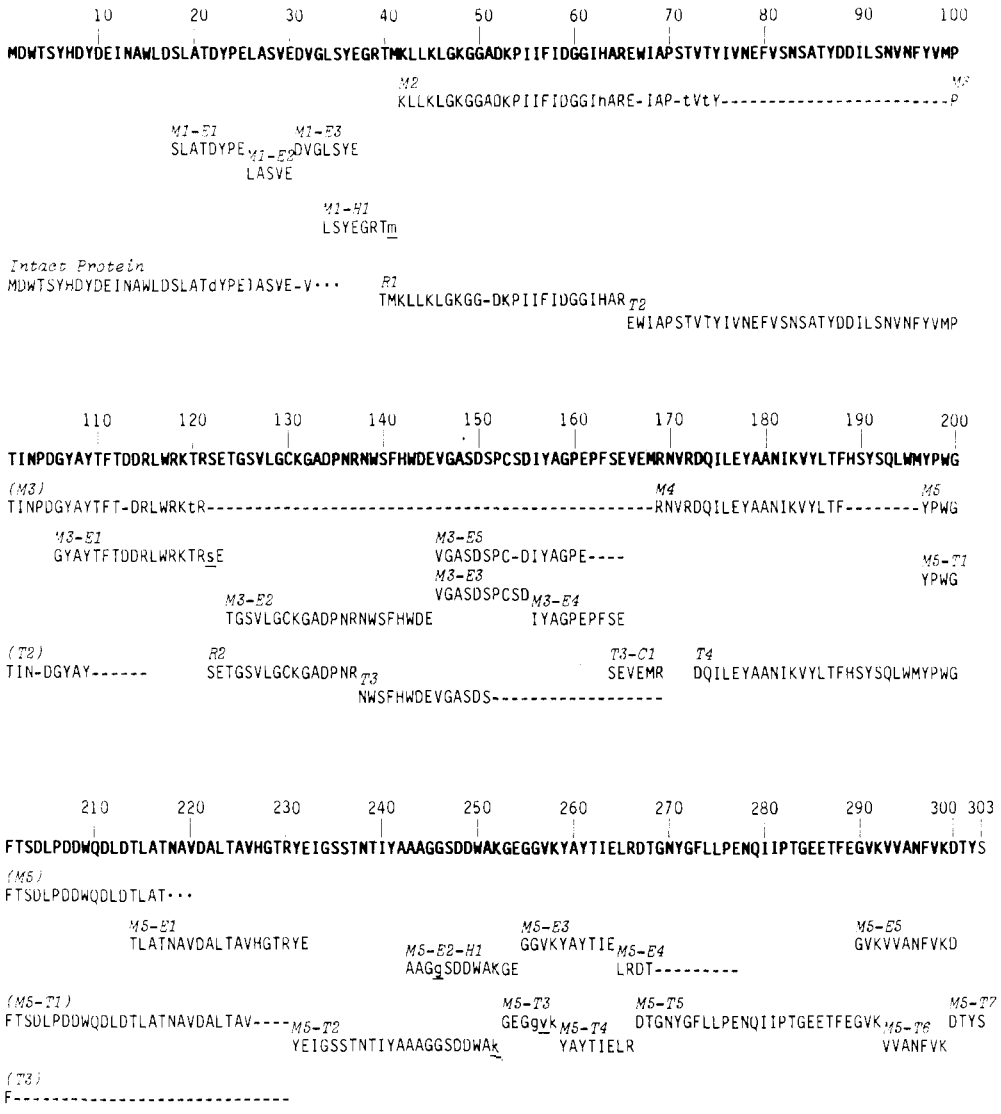

FIGURE 1: Summary proof of the sequence of crayfish carboxypeptidase B. The one-letter code (see Table I) designates amino acid residues unambiguously identified by Edman degradation (capital letters), those tentatively identified (lower case letters), or those placed by composition (underlined lower case letters). Those not identified in each peptide are shown by dashes, except for the intact protein and M5, where the continuation of the peptide is indicated by dots. Cyanogen bromide fragments, tryptic peptides, and tryptic peptides after citraconylation are designated by the prefix M, T, and R, respectively. Subpeptides generated by α-chymotrypsin, staphylococcal protease, and thermolysin are designated by the prefix C, E, and H, respectively. The final sequence derived is shown at the top in boldfaced letters.

trypsin after citraconylation of lysyl residues (Atassi & Habeeb, 1972).

A Hamilton polystyrene column PRP-1 (4.1 × 150 mm), a gift from Hamilton Co., was used for separation of peptides by HPLC in a reverse-phase mode as described by Sasagawa et al. (1982). The column was equilibrated with 3 mM NH_4HCO_3 adjusted to pH 9.5 with 1 N NH_4OH, and peptides were eluted by increasing concentrations of acetonitrile.

Other materials and methods were the same as those previously described in the determination of the amino acid sequence of crayfish trypsin (Titani et al., 1983).

Results

Strategy. Edman degradation of the intact CM-protein (30 nmol) yielded the amino-terminal sequence of 32 residues, including tentative identifications of Asp-22 and Leu-26 and ambiguous identification of residue 31 (see Figure 1). In order to extend and complete the remaining sequence analysis, a complete set of cyanogen bromide (M) fragments was isolated, save for the amino-terminal homoserine. Most of the sequence was obtained by analysis of these five M fragments and their subpeptides (245 out of 303 residues; 81%). These M fragments were then ordered by isolation and analysis of methionine-containing tryptic peptides selectively isolated from the CM-protein radiolabeled at methionyl residues. Selected portions of the sequence were also obtained by analysis of CM-Cys-containing tryptic peptides isolated from citraconylated [^{14}C]CM-protein. A complete summary proof of the sequence of 303 residues of the protein is shown in Figure 1.

Cyanogen Bromide Fragments. Twenty-five milligrams of CM-protein (600 nmol) was cleaved with cyanogen bromide in 70% formic acid at room temperature for 15 h. The digest was resolved by gel filtration in the presence of 6 M guanidine hydrochloride at pH 8 into three major fractions as shown in Figure 2a. Fraction I contained one fragment, M5 derived from the carboxyl-terminus, and was recovered by dialysis against water. Fraction II contained two fragments, M2 and M3, which were further separated by reverse-phase HPLC as shown in Figure 2B as follows: the pool (approximately 50 mL) containing guanidine hydrochloride and NH_4HCO_3 was acidified to pH 3–4 with 88% formic acid and injected into a single column by multiple injection, and the peptides were then eluted by appling a gradient of acetonitrile. M1 and M4 in fraction III were also separated by HPLC, in a similar

Table I: Amino Acid Compositions[a] of Crayfish Carboxypeptidase and Its Cyanogen Bromide Fragments (Residues per Molecule)

	M1	M2	M3	M4	M5	summary of M1 to M5	whole protein
residue no.[b]	1-41, 2-41	42-99	100-168	169-196	197-303		
Asp (D) + Asn (N)	6.7 (7)	7.3 (8)	9.2 (10)	3.0 (3)	14.2 (15)	40.4 (43)	40.6 (43)
Thr (%)	2.7 (3)	2.9 (3)	4.4 (5)	1.0 (1)	10.2 (12)	21.2 (24)	21.9 (24)
Ser (S)	3.5 (4)	3.5 (4)	5.9 (7)	1.8 (2)	5.0 (5)	19.7 (22)	19.6 (22)
Glu (E) + Gln (Q)	4.0 (4)	2.3 (2)	4.8 (5)	2.8 (3)	9.0 (9)	22.9 (23)	23.9 (23)
Pro (P)	1.0 (1)	1.7 (2)	5.6 (6)	0 (0)	4.0 (4)	12.3 (13)	13.1 (13)
Gly (G)	2.1 (2)	5.0 (5)	5.9 (6)	0.3 (0)	11.3 (12)	24.6 (25)	24.7 (25)
Ala (A)	*3.0* (3)	*4.0* (4)	*4.0* (4)	*2.0* (2)	*10.0* (10)	*23.0* (23)	*23.0* (23)
$^1/_2$-Cys (C)[c]	0 (0)	(0)	1.7 (2)	0 (0)	0.2 (0)	1.9 (2)	1.97 (2)
Val (N)	2.5 (2)	4.2 (5)	3.1 (3)	1.9 (2)	5.4 (7)	17.1 (19)	18.7 (19)
Met (M)[d]	0.3 (1-2)	0.2 (1)	0.2 (1)	0.3 (1)	0 (0)	1.0 (4-5)	4.58[e] (5)
Ile (I)	1.0 (1)	5.2 (7)	2.1 (2)	1.8 (2)	4.5 (5)	14.6 (17)	15.2 (17)
Leu (L)	4.0 (4)	4.0 (4)	2.1 (2)	3.0 (3)	7.0 (7)	20.1 (20)	20.6 (20)
Tyr (Y)	3.6 (4)	2.5 (3)	2.8 (3)	2.8 (3)	6.1 (7)	17.8 (20)	19.7 (20)
Phe (F)	0 (0)	2.6 (3)	3.1 (3)	1.0 (1)	4.1 (4)	10.8 (11)	11.1 (11)
His (H)	1.3 (1)	1.0 (1)	0.6 (1)	1.0 (1)	1.2 (1)	5.1 (5)	5.08 (5)
Lys (K)	0.3 (0)	3.5 (4)	1.8 (2)	0.9 (1)	3.6 (4)	10.1 (11)	10.7 (11)
Arg (R)	1.1 (1)	1.1 (1)	4.1 (4)	2.1 (2)	2.6 (2)	11.0 (10)	10.3 (10)
Trp (W)[d]	ND[f] (2)	ND (1)	ND (3)	ND (1)	ND (3)	ND (10)	ND (10)
no. of residues	40-41	58	69	28	107	302-303	303
yield (%)	53	14	43	23	68		

[a] By amino acid analysis (24-h acid hydrolysis) or (in parentheses) calculated from the sequence (Figure 1). [b] For residue numbering, see Figure 1. [c] Analyzed as CM-cysteine. [d] Analyzed as homoserine. [e] Analyzed as methionine. [f] Not determined (ND).

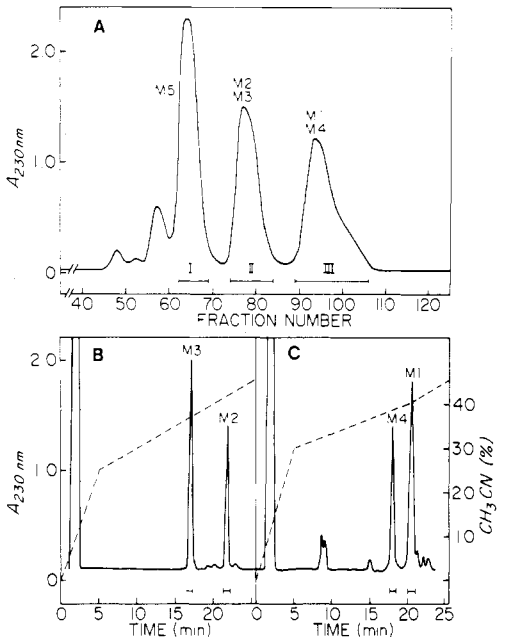

FIGURE 2: (A) Fractionation of a cyanogen bromide digest of CM-protein (600 nmol) on a column (2.5 × 110 cm) of Sephadex G-75 Superfine in 0.1 M NH_4HCO_3, pH 8. Fractions of 5 mL were collected at a flow rate of 10 mL/h and pooled as indicated by horizontal bars. (B) Fraction II in (A) was applied to a Synchropak RP-P column (0.41 × 24 cm) equilibrated with 0.1% trifluoroacetic acid (see the text) and eluted at a flow rate of 1 mL/min by a gradient of acetonitrile. Peptides were recovered by lyophilization. (C) Fraction III in (A) was separated in a similar manner as in (B).

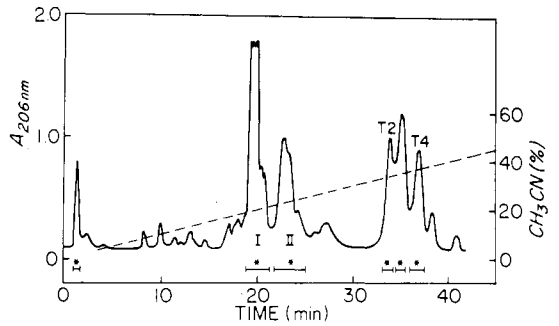

FIGURE 3: Separation of ^{14}C-radiolabeled methionine-containing peptides from a tryptic digest of CM-protein (85 nmol). The digest in 0.1 M NH_4HCO_3, pH 8, was applied to a Hamilton PRP-1 column (4.1 × 150 mm) equilibrated with 3 mM NH_4HCO_3–NH_4OH, pH 9.5, and eluted at a flow rate of 1 mL/min by a gradient of acetonitrile as shown by the broken line. Peptides were recovered by lyophilization. Asterisks indicate the pools containing radioactive peptide(s).

manner, as shown in Figure 2C.

The amino acid compositions and yields of the five isolated M fragments are listed in Table I. M2 and M4 are extremely hydrophobic and were difficult to isolate without reverse-phase HPLC. Their low yields, 14 and 23%, appear to be due to this hydrophobicity. M1, which appeared to be homogeneous as judged by its HPLC elution profile, was actually a mixture of two fragments, residues 1–41 and 2–41, generated by incomplete cleavage of the amino-terminal methionyl residue, as described below.

Radiolabeled Methionine-Containing Tryptic Peptides. Three milligrams of CM-protein (85 nmol) was labeled with [^{14}C]methyl iodide and then digested with trypsin in 0.1 M NH_4HCO_3, pH 8 at 37 °C for 3 h. The digest was separated at pH 9.5 on a Hamilton PRP-1 column by a reverse-phase mode (Figure 3). In addition to peptides T2 and T4, two more radiolabeled peptides, T1 and T3, were obtained by further purification of fractions I and II at low pH on a Synchropak RP-P column by reverse-phase HPLC.

Radiolabeled CM-cysteine-Containing Tryptic Peptides. Fifteen milligrams of [^{14}C]CM-protein (430 nmol) was citraconylated and then digested by trypsin in 0.1 M NH_4HCO_3–NH_4OH, pH 8.8 at 37 °C for 1 h. The digest was separated by gel filtration. Further purification of three radioactive fractions by reverse-phase HPLC yielded three [^{14}C]-CM-cysteinyl peptides, R2, R3, and R2-3, and one methionine-containing (nonradioactive) peptide, R1.

Sequence Analysis. Sequence analysis of M1 yielded two sequences, Asp-Trp-Thr--- and Met-Asp-Trp--- at approximately 2:1 ratio, indicating that the fragment is a mixture of two fragments generated by incomplete cleavage of the amino-terminal methionyl residue. Although the results agreed

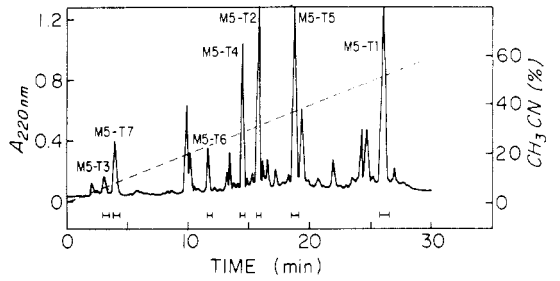

FIGURE 4: Separation of a tryptic digest (100 nmol, at 37 °C, pH 8 for 3 h) from cyanogen bromide fragment M5 on a Synchropak RP-P column by reverse-phase HPLC as described in Figure 2B.

with the amino-terminal sequence of the intact protein (Figure 1), the analysis was not satisfactory to complete the entire fragment. The fragment (40 nmol each) was therefore subdigested with *Staphylococcus aureus* V8 protease (at 37 °C, pH 7.8 for 15 h) or with thermolysin (at 37 °C, pH 8.0 for 2 h), and the digests were separated by reverse-phase HPLC. Analyses of four isolated peptides, M1-E1, M1-E2, M1-E3, and M1-H1, confirmed Asp-22 and Leu-26 and also identified the carboxyl-terminal region of the fragment (Figure 1).

Sequence analysis of intact M2 yielded the amino-terminal sequence of 34 residues including three tentative identifications and two blanks (Figure 1). Analysis of R1, incidentally isolated from a tryptic digest of citraconylated [^{14}C]CM-protein, began with Thr-Met, the last two residues of M1, overlapped the amino-terminal sequence of M2 by 24 residues, and confirmed His-63, tentatively identified in the analysis of M2 (Figure 1). Likewise, analysis of T2 (Figure 3), one of the radiolabeled methionine-containing peptides, yielded a sequence beginning with Glu-66 in the middle of M2, completing the sequence of M2, and overlapping the amino-terminal sequence of M3 by 10 residues (Figure 1).

Sequence analysis of M3 yielded the amino-terminal sequence of 22 residues including one tentative identification (Thr-120) and one unidentified residue 133 (Figure 1). The fragment (125 nmol) was subdigested with staphylococcal protease (at 37 °C, pH 7.8 for 15 h), and five peptides, M3-E1–E5, isolated by reverse-phase HPLC, were subjected to sequence analysis (Figure 1). Analyses of two radiolabeled CM-Cys-containing peptides, R2 and R3 (identical with T3), and a chymotryptic subpeptide T3-C1 of one radiolabeled methionine-containing peptide T3 provided sufficient overlaps for these staphylococcal protease peptides (Figure 1). T3-C1 also provided the overlap for M3 and M4. This overlap is by just one residue, but it appears to be sufficient because M4 is the only cyanogen bromide fragment containing an amino-terminal arginine.

Sequence analysis of M4 yielded the amino-terminal sequence of 20 residues, beginning with Arg-170 (Figure 1), mentioned above. Probably due to its hydrophobic property, the fragment was resistant to staphylococcal protease, trypsin, chymotrypsin, pepsin, subtilisin, and Pronase. Analysis of T4, one of the radiolabeled methionine-containing peptides, identified the carboxyl-terminal region of M4 and also provided the overlap for M4 and M5 (Figure 1). Peptide T4 contained a 30% contaminant of an overlapping peptide (residues 170–230) and a trace of peptide T2. The major sequence of T4 agrees with the sequence of residues 173–188, derived from the analysis of M4, thus extending the sequence to residue 200 by overlapping M5 by four residues (see Figure 1).

Sequence analysis of intact M5 yielded the amino-terminal sequence of 21 residues (Figure 1). The remaining sequence of M5 was determined by analysis of seven tryptic and five staphylococcal protease subpeptides isolated by HPLC (Figure 1). Figure 4 illustrates the separation of a subdigest by trypsin on an HPLC column.

Table II: Degree of Homology among Carboxypeptidases

	crayfish B	bovine B	bovine A	rat A
crayfish B		(134/300)[a] 44.7[b]	(131/298)[a] 44.0[b]	(131/299)[a] 43.8[b]
bovine B	89[c]		(136/304)[a] 48.0[b]	(147/305)[a] 48.2[b]
bovine A	97[c]	85[c]		(243/307)[a] 79.2[b]
rat A	97[c]	84[c]	24.2[c]	

[a] (Identities/possible matches): The number of identical residues between two sequences is compared with the total possible matches between residues using the ALIGN program with the Mutation Data Matrix and a penalty for a gap (break) = 10 (Dayhoff, 1978). [b] Percent identity. [c] Evolutionary distance in *PAM's*[a] (computed from percent differences). PAM = accepted point mutation (rearranged acronym) considered a measure of the amount of evolutionary change (Dayhoff, 1978).

Discussion

The present determination of the amino acid sequence of crayfish carboxypeptidase B is based on the analysis of cyanogen bromide fragments generated from the reduced and S-carboxymethylated protein and of tryptic peptides obtained before and after citraconylation of lysine residues. Most of this proof is unambiguous and relies on replicate or overlapping sequence analyses. Although some of the identifications are derived from single analyses, in each case the sequence data are consistent with the composition of small peptides derived from the corresponding regions. The weakest points in the present proof of structure appear to be the overlaps between fragments M1 and M2 by only two residues and between M3 and M4 by only one residue. These overlaps should be sufficient, however, because M1 is the only cyanogen bromide fragment with a carboxyl-terminal Thr-Hse and M4 is the only fragment with an amino-terminal Arg. Another weak point is the sequence around Trp-195 derived from the analysis of peptide T4 which contained two minor peptides.

A major incentive for the present investigation was the analysis of changes in molecular structure that accompany the evolution of carboxypeptidase. A prerequisite for such an analysis is the knowledge of the enzymatic specificity, amino acid sequence, and three-dimensional structure of bovine pancreatic carboxypeptidases A and B which serve as standards of comparison for the crayfish enzyme.

Most of our current knowledge of bovine carboxypeptidase A has accumulated during the past 30 years and has recently been summarized by Vallee et al. (1983). Despite the long-term effort, involving a great variety of experimental approaches, some of the most basic questions pertaining to the details of the mode of action of this enzyme are still being debated (Vallee et al., 1983; Lipscomb, 1983). These relate in particular to the nature of the transition state complexes during catalysis since these are beyond recognition by sequence analysis and are not necessarily resolved by X-ray analysis of the enzyme alone or of inactive enzyme–inhibitor complexes. The analysis of a phylogenetic variant of carboxypeptidase, which differs in more than half of the amino acid residues from the bovine enzyme (Table II) yet shows clear evidence of homology (Figure 5), offers a test for the compatibility of putative catalytic intermediates with the amino acid replacements in the variant.

It was previously shown that the enzymatic specificity of crayfish carboxypeptidase is analogous to that of bovine carboxypeptidase B but, unlike the bovine enzyme, does not

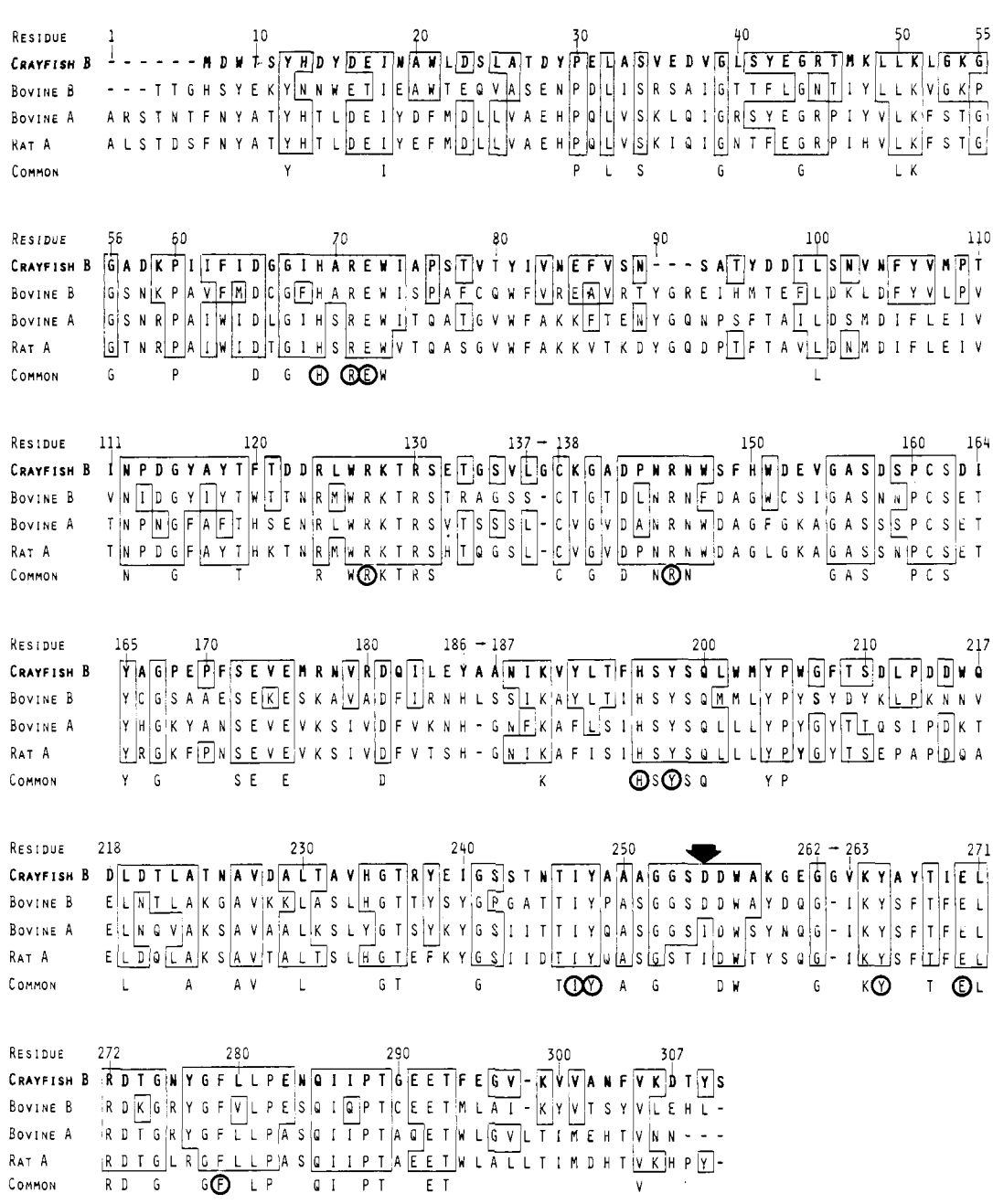

FIGURE 5: Comparison of sequences of bovine carboxypeptidases A and B (Titani et al., 1975), rat carboxypeptidase A (Quinto et al., 1982), and crayfish carboxypeptidase B (the present paper). The alignment is based on the scoring scheme of Dayhoff (1978). Enclosing lines indicate residues identical with the amino acid sequence of crayfish carboxypeptidase B. Boxes around single residues inside the above enclosures indicate nonidentical residues. The residues associated with biological function in bovine carboxypeptidases A and B (Lipscomb, 1968; Schmid & Herriott, 1976) are circled. Arrow indicates the primary substrate binding site. The bovine carboxypeptidase A numbering system is used.

cross-react with substrates of carboxypeptides A (Zwilling et al., 1979). This observation is consistent with the identification of the primary substrate binding site as Asp-255, as in bovine carboxypeptidase B, and in contrast to Ile-255 in bovine carboxypeptidase A (Figure 5). Unlike the mammalian enzymes, the crayfish enzyme does not have a detectable zymogen form (Zwilling et al., 1979). It would be interesting to test whether the amino-terminal methionine is the initiation methionine because such a finding would not only verify the absence of a zymogen form but also indicate the absence of a secretory signal peptide.

Certain features of the crayfish enzyme are unique when compared to the sequence of the other three homologous enzymes (Table II), i.e., one deletion, two insertions, and 35 amino acid replacements (Figure 5). All of these appear to be compatible with the model of the three-dimensional structure of bovine carboxypeptidase B (Schmid & Herriott, 1976). The three-residue deletion (residues 90–92) coincides with a surface loop in carboxypeptidases A and B. The insertion of glycine in position 137a occurs in a surface loop in both bovine enzymes, but the insertion of glycine in position 262a might influence a reverse turn, also at the surface of the molecule. With the following exceptions, all other bovine/crayfish substitutions are conservative, affect only surface residues, and are thus compatible with the crystal structure of bovine carboxypeptidase B: I/V-38, I/M-47, A/I-61, F/I-82, D/N-104, V/T-110, E/D-163, T/I-164, L/M-203, Y/W-206, S/A-251, I/V-263, and F/Y-267. All of these substitutions of internal residues can be accommodated without doing violence to models of the bovine enzymes. There is a

mutally compensating change of I/F-195 and F/I-269 that is also compatible with the structure of the bovine enzyme. The Y/W-206 replacement occurs in a position of one of the three *cis* peptide bonds in carboxypeptidase A. Four of the five cysteine residues of bovine carboxypeptidase B are exposed on the surface, and hence, the substitution in the crayfish enzyme has no structural consequences. The fifth, Cys-290, is partly buried, but its replacement in the crayfish enzyme by glycine is likewise compatible with the known structure. The two Cys residues, Cys-138 and Cys-161, must be in disulfide linkage since there is no free cysteine. This conclusion is fully compatible with the position of the same disulfide bond in bovine carboxypeptidase B. On the basis of all these considerations, the crystal structure of the crayfish enzyme may be predicted to be similar to that of bovine carboxypeptidase B.

We have recently reported that crayfish and bovine trypsins are homologous enzymes which show 43.6% sequence identity, corresponding to 111.2 PAM's (accepted point mutations). The crayfish and bovine carboxypeptidases B are similarly related (Table II), i.e., 44.7% sequence identity or 89 PAM's. This coincidence is sufficiently close to suggest that these two enzymes have evolved together at practically identical rates. Since the crayfish hepatopancreas, unlike the mammalian pancreas, is devoid of detectable activities of chymotrypsin or carboxypeptidase A, one may argue that carboxypeptidase B is an evolutionary precursor of carboxypeptidase A. It is also possible, however, that the crayfish is unique and that chymotrypsin and carboxypeptidase A have been replaced by the recently discovered *Astacus* protease which represents a protease operating by a novel, yet unresolved, mechanism (Zwilling et al., 1981).

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Registry No. Carboxypeptidase B, 9025-24-5; carboxypeptidase B (*Astacus fluviatilis* reduced), 88841-43-4; carboxypeptidase A, 11075-17-5.

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