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Characterization of Bovine Carboxypeptidase A(Allan)*

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ABSTRACT: Bovine carboxypeptidase A (Allan), prepared by the method of Allan, Keller, and Neurath (*Biochemistry* 3, 40, 1964), is a mixture of active enzymes which differ in their amino-terminal sequences. The predominant component, A_β, has the amino-terminal sequence Ser-Thr-Asn-Thr-Phe-Asn-Tyr-Ala-. Minor components are carboxypeptidase A_α with the amino-terminal sequence Ala-Arg-Ser-Thr-Asn-Thr-Phe-Asn-Tyr-Ala-, and carboxypeptidase A_γ with the amino-terminal sequence Asn-Tyr-Ala-. In the course of this investigation an amide placement in the sequence of carboxypeptidase A has been corrected by demonstrating that Glu_{31} rather than Glu_{28} is amidated.

Three methods of preparation of bovine carboxypeptidase A have been described in the literature and the resulting products denoted according to originators are CPA(Anson), CPA(Cox), and CPA(Allan) (Bargetzi *et al.*, 1964). Each of these preparations contains the α, β, and γ forms of the enzyme in varying proportions. CPA(Cox) contains predominantly the α enzyme and smaller amounts of the β and γ forms, whereas CPA(Anson) is largely in the γ form with β and α present in lesser proportions (Pétra and Neurath, 1969a,b).[1] The structural relationships of the three forms are summarized in Figure 1.

The composition of CPA(Allan) seems less certain. The amino-terminal residue was reported to be the same as that of CPA(Anson), *i.e.*, asparagine (Coombs *et al.*, 1964; Bargetzi *et al.*, 1964). The Allan enzyme, however, had a higher solubility and was more completely reactivated on addition of zinc to the apoenzyme than the Anson preparation (Vallee *et al.*, 1960). CPA(Allan) was therefore assumed to be yet a different molecular species and was called CPA_δ (Bargetzi *et al.*, 1964).

The purpose of the present investigation was to clarify the chemical structure of the Allan enzyme by sequenator analysis and by chromatographic resolution. A batch of CPA(Allan), prepared by Miss Barbara Allan in 1956, was analyzed and compared to two new preparations isolated in 1970 by the procedure of Allan *et al.* (1964). The results described herein indicate that typical preparations of CPA(Allan) contain predominantly the β form of the enzyme and that there is no experimental basis for the existence of CPA_δ as an independent entity.

Experimental Section

Materials. Two preparations of CPA(Allan) were isolated from acetone powders of bovine pancreas glands by the method of Allan *et al.* (1964). A third preparation, isolated by Miss Barbara Allan in this laboratory in 1956, had been stored as a suspension of crystals at 4° for the intervening 15 years. CPA(Anson) was a twice-crystallized product of Worthington Biochemical Corp. and was prepared by the method of Anson (1937) as modified by Putnam and Neurath (1946). CPA(Cox) was prepared from acetone powders of bovine pancreas glands by the method of Cox *et al.* (1964). Microgranular DEAE-cellulose (DE-52) was obtained from Reeve Angel Co.

Methods. The various preparations of CPA were analyzed by examining ten turns in the Beckman sequencer using a

* From the Department of Biochemistry, University of Washington, Seattle, Washington 98105. *Received July 2, 1971.* This work has been supported by research grants from the National Institutes of Health (GM 15731) and the American Cancer Society (P-79).

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[1] Each of these three activation products occurs in either of two allotypic forms, and Val type and the Leu type. The nomenclature of the six species of enzyme is defined by Pétra and Neurath (1969a).

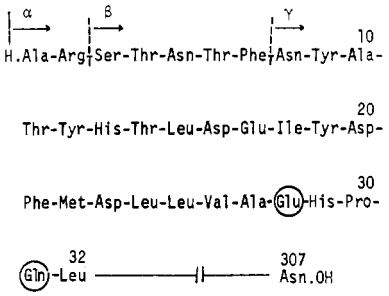

FIGURE 1: N-Terminal sequence of carboxypeptidase A (taken from Bradshaw *et al.*, 1969). The sites of activation yielding the α, β, and γ forms are indicated by dashed lines. The two circles residues are corrections of the original sequence.

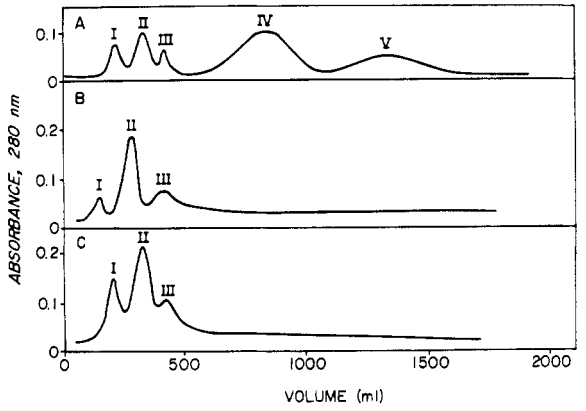

FIGURE 2: Elution patterns of the chromatography (100 mg each) of preparations of bovine carboxypeptidase A on a 1.5 × 90 cm column of DEAE cellulose (DE-52) developed at 34 ml/hr at 4° with a buffer containing 0.05 M β-phenylpropionate, 0.04 M LiCl, 0.05 M Tris-base, adjusted to pH 7.5 with 10 N NaOH. (A) Carboxypeptidase A (Anson): peak I = CPA_{α}^{Val}; peak II = CPA_{α}^{Leu} and CPA_{β}^{Val}; peak III = CPA_{β}^{Leu}; peak IV = CPA_{γ}^{Val}; peak V = CPA_{γ}^{Leu}. (B) Carboxypeptidase A (Allan) prepared according to Allan *et al.* (1964). This preparation corresponds to 1970-I in Table I. (C) Carboxypeptidase A (Allan) prepared by B. J. Allan in 1956 and kept as a crystalline preparation for 15 years at 4°.

modification of the procedure of Edman and Begg (1967). *N*,-*N*-Dimethylbenzylamine (1 M in 40% aqueous pyridine brought to pH 9.2 with trifluoroacetic acid) was employed as the buffer, and 0.4 mM dithioerythritol (Pierce Chemical Co., purified) was added to the chlorobutane to stabilize the sequenator products (Hermodson *et al.*, 1970). The PTH-amino acids were separated by the general procedure of Pisano and Bronzert (1969) and where possible were quantitated with a Beckman GC-5 gas chromatograph.

Chromatographic purifications were performed on a 1.5 × 90 cm column of DEAE cellulose (DE-52) as described by Pétra and Neurath (1969a). The six forms of the enzyme were identified on the basis of their chromatographic elution patterns[2] using CPA(Anson) as the control (Figure 2A).

Results

Characteristic chromatographic elution patterns of the three preparations of CPA(Allan) are shown in Figure 2. Since the two preparations obtained in 1970 behaved identically in this chromatographic system, only one of them (1970-I) is shown in Figure 2B. The major component is peak II containing a mixture of CPA_{β}^{Val} and CPA_{α}^{Leu}. Minor components are CPA_{α}^{Val} and CPA_{β}^{Leu}, whereas the γ form of the enzyme appears to be absent altogether.

TABLE I: Component Distribution in Various Preparations of Bovine Carboxypeptidase A.

Method of Prepn	Prepn No.	% of Component in Prepn Estimated by Sequenator Anal. α	β	γ
Allan	1956	35	65	0
Allan	1970-I	9	73	18
Allan	1970-II	9	70	21
Cox		75	25	0
Anson		10	15	75

For chemical characterization the enzyme preparations were subjected to sequenator analysis and the amino-terminal sequences compared to the sequence of carboxypeptidase A (Bradshaw *et al.*, 1969) as shown in Figure 1. The several forms were readily recognized in each preparation by qualitative examination of the products of the sequential Edman degradation. Since not all of the amino-terminal residues can be accurately quantitated as PTH amino acids by gas–liquid chromatography, the relative amount of each form of CPA was judged by comparison of the residues PTH-Phe and PTH-Tyr in turns 5 and 7. These two derivatives respond well in the flame ionization detector and are quantitatively recovered from the sequenator. Thus the β:γ ratio was determined at turn 5 from the quantity of PTH-Phe derived from the β form and of PTH-Tyr derived from the γ form. Likewise, the β:α ratio was determined at turn 7 from PTH-Phe of the α and PTH-Tyr of the β form. This method obviates correction for incomplete reaction during each cycle of the degradation. However, a small correction must be made for PTH-Tyr background.

The results of these analyses, summarized in Table I, confirm previous observations that CPA(Cox) and CPA(Anson) contain predominantly CPA_{α} and CPA_{γ}, respectively, whereas the major component of CPA(Allan) is CPA_{β}.

Continuation of sequenator analyses through 30 turns of CPA(Allan) indicated that the assignments (Bradshaw *et al.*, 1969) of Gln and Glu to positions 28 and 31 in CPA_{α} should be reversed. To confirm this, Edman degradations were performed in the sequenator on both CPA(Anson) and on the fragment of CPA(Anson) containing residues 23–103 obtained by cleavage with cyanogen bromide (Nomoto *et al.*, 1969). The results corroborate the sequence in Figure 1 and establish the correct amide placement in CPA_{γ} as $-Glu_{28}$-His-Pro-Gln_{31} (using CPA_{α} numbering). Assignment of Glu at position 28 is also in agreement with the recent studies of the carboxyl modification of CPA_{γ}^{Val} (Pétra and Neurath, 1971).

Discussion

Since none of the three preparative procedures yields chromatographically homogeneous bovine carboxypeptidase A

[2] In this chromatographic system CPA_{α}^{Leu} and CPA_{β}^{Val} emerge together as peak II.

and each preparation contains different proportions of the α, β, and γ forms, it is important to define the composition of the various preparations in terms of the component forms. This information is of particular significance since each of the three products of isolation, CPA(Cox), CPA(Anson), and CPA(Allan), has been preferentially employed in certain investigations of carboxypeptidase A. Most of the studies on the role of metal ions in enzyme function have been carried out on CPA(Allan) (*e.g.*, Coleman and Vallee, 1960; Coleman *et al.*, 1966), whereas the Anson preparation has been preferentially used for chemical modification experiments (Vallee and Riordan, 1968) and for the determination of the amino acid sequence of this enzyme (Bradshaw *et al.*, 1969). The different responses of the apoenzymes of the Anson and Allan preparations to reactivation by zinc led to the suggestion that these two products also differ in intrinsic structural details (Coombs *et al.*, 1964). CPA(Cox) was the material employed in X-ray diffraction analysis (Lipscomb *et al.*, 1968). In contrast to the Cox and Anson preparations, the chemical nature of CPA(Allan) was only qualitatively defined. The present data indicate that, contrary to earlier reports (Coombs *et al.*, 1964; Bargetzi *et al.*, 1964), the predominant species in CPA(Allan) is the β form (Table I). The accuracy of determination of the species distributions by sequenator analysis is approximately $\pm 5\%$. The minor components are probably overestimated beyond this limit, since after turn 6 the background generated by nonspecific cleavage of the protein becomes a significant fraction of the amount of minor components present in the enzyme. Thus the percentage of the α form in CPA(Anson) may well be 5 instead of 10 given in Table I.

The reliability of the sequenator analysis as a method of characterizing CPA(Anson) is indicated by the agreement between the values obtained by this method and by chromatographic resolution, respectively. Pétra and Neurath (1969a,b) reported ratios of 2:25:70 for α:β:γ in CPA(Anson) and a ratio of 76:27 for α:β in CPA(Cox). These data agree closely with the present sequenator analysis. Although the sequenator data indicate some variation in the minor components of the three preparations of CPA(Allan), all contained approximately 70% of the β form. Chromatographic analysis of two of these preparations (Figure 2) confirms the conclusion that CPA(Allan) is predominantly in the β form. The α and γ forms are present to a lesser and variable extent.

The major amino-terminal residue of the main component of the preparations of CPA(Allan) examined in the present study differs from the identifications previously reported (Coombs *et al.*, 1964; Bargetzi *et al.*, 1964). These investigations yielded Asx rather than Ser as the amino-terminal residue. This discrepancy may reflect variations in the acetone powders of the pancreatic glands or individual variations in the timing of critical stages in the preparation of the enzyme. Alternatively, since the previous methods relied on a direct analysis of either DNP-Ser or PTH-Ser and since both derivatives are less stable than the corresponding derivatives of Asx, the conclusions might have been biased in favor of the latter. Whatever the explanation, there is no evidence in the current analysis to support the existence of a fourth species of the enzyme (CPA_δ) in the preparations of CPA(Allan) that have been examined.

Comparison of the preparation of 1956, the analyses of 1964, and the current analyses makes it clear that the products of these preparations vary in their relative content of the three forms of enzyme. No significant differences have been observed in the enzymatic activity of the three species toward substrates (Pétra, 1970). They do differ, however, in heat stability (Pétra and Neurath, 1969a). Moreover, recent work has shown that in contrast to the β form, the γ enzyme contains subsidiary sites in the amino-terminal region reactive toward the reagent *N*-bromoacetyl-*N*-methyl-L-phenylalanine (Hass and Neurath, 1971). Hence the component distribution of a preparation of carboxypeptidase A must be regarded as a significant variable in the structural and functional analysis of this enzyme.

Acknowledgment

The authors thank Mr. Don Grahn for his excellent technical assistance.

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