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Interactions of the Endopeptidase Subunit of Bovine Procarboxypeptidase A-S6*

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ABSTRACT: Activated procarboxypeptidase A-S6 (PCP A-S6) and fraction II, the endopeptidase subunit of PCP A-S6, are both irreversibly inactivated by diisopropyl phosphorofluoridate (DFP), *N-trans*-cinnamoylimidazole (at pH 5.0), and L-(1-carbobenzoxyamido-2-phenyl) ethylchloromethyl ketone (ZPCK). Inhibition by ZPCK occurs considerably more slowly than inhibition of α-chymotrypsin. Quantitative measurements of the incorporation of these inhibitors indicate that the two activated zymogens possess only a fractional value for the number of active sites per molecule, *i.e.*, 0.60 and 0.14, respectively. However, the catalytic activity per mole of "active sites" is the same for both. Isolation of the corresponding [^{32}P]DIP and [^{14}C]ZPCK peptides shows that these reactions are specific for seryl and histidyl residues, respectively, the isolated peptides displaying close similarity to analogous peptides derived from trypsin or chymotrypsin.

Isolation of [^{14}C]ZPCK-labeled fraction II (containing approximately 0.25 active site/molecule) and from [^{14}C]ZPCK–procarboxypeptidase A-S6 (containing 0.59 active site/molecule) suggests that the zymogen contains two endopeptidase subunits of similar if not identical nature.

Bovine pancreatic procarboxypeptidase A-S6 is the zymogen of two enzymatically and physically distinct enzymes: an endopeptidase which resembles chymotrypsin in some of its enzymatic and chemical properties, and the exopeptidase, carboxypeptidase A (Keller *et al.*, 1956, 1958; Brown *et al.*, 1961, 1963). Yamasaki *et al.* (1963) have shown that procarboxypeptidase A is an aggregate of three subunits and that each of the two enzymatic activities, endopeptidase and exopeptidase, is associated with distinct subunits. Brown *et al.* (1963) were able to dissociate procarboxypeptidase A-S6 into three different fractions by incubation in aqueous solution at pH 10.5 and, more recently, Freisheim *et al.* (1967) described the disaggregation of the procarboxypeptidase A complex by succinylation with succinic anhydride and the isolation of viable succinylated fraction I which could be converted by the action of trypsin to succinyl carboxypeptidase A.

Fraction II, the zymogen of the endopeptidase, exhibits both esterase and endopeptidase activity after activation with trypsin. These activities can be generated either in the trimeric precursor (PCP A-S6)[1] or after isolation of the individual subunit(s). A third chromatographically distinguishable protein component, designated fraction III, is of unknown function; no enzymatic activity could be associated with this protein before or after incubation with trypsin (Brown

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[1] The following abbreviations are used: PCP A-S6, bovine procarboxypeptidase A with a sedimentation velocity of approximately 6 S; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; ZPCK, (1-carbobenzoxyamido-2-phenyl)ethyl chloromethyl ketone; DNS-Cl, dimethylaminonaphthalenesulfonyl chloride; DNS-OH, dimethylaminonaphthalenesulfonic acid; DNS-NH_2, dimethylaminonaphthalenesulfonamide; Ser·PO_3^{2-}, *o*-phosphoserine; HPLA, hippuryl-DL-β-phenyllactate.

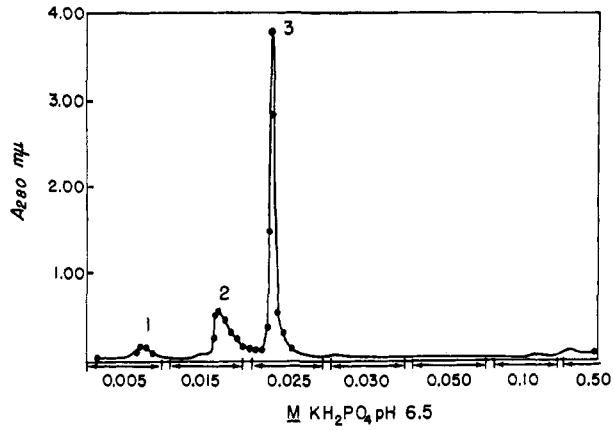

FIGURE 1: Chromatography on hydroxylapatite-cellulose of succinyl fraction II. A solution of succinyl fraction II (20 mg/ml) was applied to a hydroxylapatite-cellulose column (2.5 × 10 cm) previously equilibrated with 0.005 M KH_2PO_4 (pH 6.5). The protein was eluted by stepwise application of the following buffers (100 ml each): 0.005, 0.015, 0.025, 0.030, 0.050, 0.100, and 0.500 M KH_2PO_4, each titrated to pH 6.5 with KOH. The fraction volume was 2.5 ml, and the flow rate was regulated at 18.8 ml/hr. The overall yield of succinyl fraction II (all fractions isolated) was 21%. Activity and *N-trans*-cinnamoylimidazole measurements for peaks 1, 2, and 3 are given in Table I. Larger scale preparations were used for the peptide isolation experiments.

et al., 1963). In the present investigation, certain physical and chemical characteristics of the endopeptidase subunit were determined in order to define more precisely the relationship of this component to the structure and apparent biological function of the zymogen complex, procarboxypeptidase A.

Experimental Section

Materials

Procarboxypeptidase A-S6 was isolated from aqueous extracts of acetone powders of bovine pancreas glands and purified by chromatography on DEAE-cellulose, as described by Brown *et al.* (1963). The procedure was modified by an additional step employing gel filtration on Sephadex G-150 using a column (Pharmacia) equipped with upward flow adaptors from which the zymogen was eluted with 0.05 M KH_2PO_4 (pH 8.0) (R. W. Tye, unpublished results).

Carboxypeptidase A was prepared from procarboxypeptidase A according to the procedure of Cox *et al.* (1964).

Fraction II was prepared by disaggregation of procarboxypeptidase A at pH 10.5 and chromatographically purified according to the method of Brown *et al.* (1963).

Trypsin (salt free, twice crystallized, and lyophilized) and *α-chymotrypsin* (three-times crystallized) were purchased from the Worthington Biochemical Corp., Freehold, N. J.

Succinyl fraction II was obtained as a by-product of the preparation of succinyl fraction I which involves gel filtration on Sephadex G-100 of succinylated PCP A-S6, as described by Freisheim *et al.* (1967). Protein material exhibiting endopeptidase activity after activation was further purified by chromatography on hydroxylapatite-cellulose at 4° (Figure 1). Material eluted by 0.025 M KH_2PO_4 (pH 6.5) (peak 3) was analyzed for amino acid composition and molecular weight. Its composition was similar to fraction II isolated by Brown *et al.* (1963) except for the groups modified by succinylation. No further chemical characterization, however, of this modified protein was attempted. A representative low speed sedimentation equilibrium experiment was indicative of overall homogeneity with weight-average and Z-average molecular weights of 25,200 and 25,390, respectively.

Reagents and Enzymes. [^{32}P]DFP was purchased from the New England Nuclear Corp., Boston, Mass. Ac-L-TyrOEt was purchased from the California Corp. for Biochemical Research. Salt-free, twice-crystallized, lyophilized trypsin was obtained from Worthington Biochemical Corp. Leucine aminopeptidase was also obtained from Worthington Biochemical Corp.

TPCK, ZPCK, and [^{14}C]ZPCK were all kindly provided by Dr. Elliott N. Shaw, Brookhaven National Laboratory, Upton, Long Island, N. Y.

Ethylenimine was purchased from Matheson, Coleman and Bell, Norwood (Cincinnati), Ohio.

DNS derivatives of amino acids as well as DNS-Cl, DNS-OH, and DNS-NH_2 were purchased from Calbiochem, Los Angeles, Calif. All DNS derivatives were found to be chromatographically pure on silica gel G thin-layer plates using solvent systems described by Morse and Horecker (1966).

DEAE-Sephadex A-25 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

DEAE-cellulose (selectocel-DEAE, 0.90 mequiv/g) was obtained from the Brown Co., Berlin, N. H.

AG 50W-X2 (Dowex) ion-exchange resin was purchased from Bio-Rad Laboratories, Richmond, Calif., and was processed according to Schroeder (1967).

Methods

Generation of Endopeptidase Activity from PCP A-S6 and Fraction II. The zymogen (0.96–26 mg/ml) was activated for 1–1.5 hr under specified buffer conditions using a 1:100 weight ratio of trypsin:protein at 0°. Activated PCP A-S6 retains its trimeric aggregate structure under these conditions (Keller *et al.*, 1958). The activity generated remains stable for at least 2.5 hr.

Assay Procedures. The rate of esterase activity was measured as the rate of hydrolysis of 0.01 M Ac-L-TyrOEt and the specific activity expressed as milliequivalents per minute per micromole of protein, K°_{obsd} (Table I). Hydrolysis of Ac-L-TyrOEt was followed according to Yamasaki *et al.* (1963) using a Radiometer G-K 2021C glass electrode in conjunction with a TTT-1 Radiometer autotitrator equipped with an Ole Dich recorder (Copenhagen).

Active-site titrations were performed with *N-trans*-cinnamoylimidazole as described by Schonbaum *et al.* (1961) using 0.10 M sodium acetate buffer at pH 5.05, 25°. Both methods A and B were used. α-Chymotrypsin controls were used as standards in each experimental series. Measurements were made on the Perkin-Elmer Model 350 recording spectrophotometer.

Kinetic analysis of activated PCP A-S6 or fraction II toward Ac-L-TyrOEt was carried out in duplicate in the pH-Stat in solutions containing 0.01 M Tris–0.1 M KCl (pH 8.0). The zymogen was dissolved in 0.01 M Tris·HCl–0.1 M KCl (pH 8.0) and activated with trypsin at 0°. Constant

TABLE I: Calculation of the Number of Active Sites per Mole Using *N-trans*-Cinnamoylimidazole.[a]

Protein	Protein ($\times 10^4$ M)	Ac-L-TyrOEt Act.[b]	N of Active Sites $\times 10^4$	Active Site/Mole	Act./Active Site
α-Chymotrypsin	6.74	5.58	5.09	0.76	7.3
Fraction II (zymogen)	2.93	0.00	0.00	0.00	0.0
Fraction II (activated;	4.64	1.30	0.64	0.14	9.3
four preparations)	4.90	1.28	0.72	0.14	9.2
	5.84	1.21	0.76	0.13	9.4
	10.50	1.32	1.39	0.13	9.8
Succinyl fraction II	(1) 4.1	1.89	0.80	0.19	10.0
(peaks 1, 2, and 3;	(2) 5.2	3.26	1.61	0.31	10.5
Figure 1)	(3) 4.5	1.71	0.81	0.18	9.5
PCP A-S6 (zymogen)	1.19	0.00	0.00	0.00	0.0
PCP A-S6 (activated)	1.04	5.66	0.57	0.55	10.3

[a] The following values were assumed for molecular weight and $E^{0.1\%}_{280nm}$, respectively: α-chymotrypsin, 24,600 and 2.0; fraction II, 25,000 and 1.50; PCP A-S6, 87,000 and 1.9. [b] K_{obsd}^0 mequiv of OH/min per μmole of protein.

activity was assured by assays under standard conditions throughout the experiment. The concentration of Ac-L-TyrOEt was varied within the range 0.01–0.0005 M. The final concentrations of PCP A-S6 and of fraction II were 1×10^{-7} and 7×10^{-7} M, respectively.

Sedimentation velocity experiments were carried out in a Beckman Model E analytical ultracentrifuge equipped with phase-plate schlieren, Rayleigh, and absorption optics. The analyses were performed in either a single- or a double-sector synthetic boundary cell. Photographs of the observed schlieren patterns were taken at regular time intervals during the run and the radial boundary position of the maximum ordinate was measured on the plate negatives by use of a microcomparator at each time interval. The sedimentation rate was calculated from a least-squares slope of a plot of the log of the radial boundary position *vs.* time (Schachman, 1957). Viscosities were measured at 20.00 ± 0.01°. Corrections for buffer density at 20.0° were obtained by pycnometry.

Low-Speed Sedimentation Equilibrium. Sedimentation equilibrium measurements were performed in the analytical ultracentrifuge employing Rayleigh interference optics as described by Richards and Schachman (1959) at speeds ranging from 6004 to 10,222 rpm. A double-sector synthetic boundary cell was used to obtain a value of the initial concentration, C_0. The protein sample to be analyzed was layered over FC 43 (3M Chemicals) at column heights of 2.0–2.5 mm. Hinge-point shift was followed in serial patterns; equilibrium was assumed to be attained when no further shift of the fringes could be detected. The data were plotted as ln C *vs.* x^2 (where C = fringe number and x = the distance from the axis of rotation). The apparent weight-average molecular weight over the volume of the cell was evaluated from the equation of Adams (1964).

Protein concentrations were calculated from the absorbance at 280 mμ. A value of $E^{0.1\%}_{280nm}$ of 1.9 was used for PCP A-S6 (mol wt 87,000; Yamasaki *et al.*, 1963). Alternatively, protein concentrations were determined from the Rayleigh interference patterns using a synthetic boundary cell in the ultracentrifuge. A refractive index increment of 0.00185 dl g^{-1} at 5464 Å was assumed (Doty and Edsall, 1951). The absorbance index for fraction II was calculated by measuring the optical density at 280 nm and relating to the C_0 values obtained from ultracentrifugation. An average value of $E^{0.1\%}_{280nm}$ of 1.50 ± 0.05 was obtained. Alternatively, using amino acid analysis as a measure of protein concentration as described by Walsh and Brown (1962), and assuming a molecular weight of 25,000 for fraction II, $E^{0.1\%}_{280nm}$ was calculated to be 1.49 ± 0.1. The partial specific volume used for the sedimentation analyses was 0.73 ml/g.

Amino acid analyses were performed according to Spackman *et al.* (1958) using the Beckman amino acid analyzer, Model 120, after acid hydrolysis as described by Freisheim *et al.* (1967).

Radioactivity measurements were carried out in the Packard Tri-Carb liquid scintillation spectrometer Model 3003 using a modification of Bray's solution as the solvent (Bray, 1960). A minimum of 10,000 counts was recorded to provide data with an accuracy of ±1%. The efficiency was calculated to be 74.2% based on internal standards.

Ninhydrin analysis of peptides was carried out according to the procedure of Schroeder *et al.* (1962).

Sequence determination of peptides was carried out according to the Dansyl-Edman procedure outlined by Gray (1967). DNS-amino acids were identified by the procedure of Morse and Horecker (1966).

Results

Physical Characteristics of Fraction II and Succinyl Fraction II. Analytical disc gel electrophoresis of fraction II by the procedure of Ornstein and Davis (1964) exhibited a single band (Figure 2). The protein also sedimented with a single symmetrical boundary in the ultracentrifuge. A plot of the dependence of the sedimentation coefficient upon enzyme concentration exhibited a positive slope, with an intercept value at zero concentration (extrapolated from the linear portion of the plot of $s_{20,w}$ = 2.90 ± 0.05 S) (Figure 3, upper). In contrast, analogous experiments for succinyl fraction II

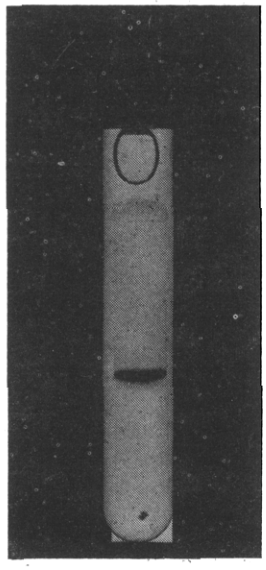

FIGURE 2: Analytical disc gel electrophoresis of fraction II prepared by disaggregation of PCP A-S6 at pH 10.5. The procedure is that reported by Ornstein and Davis (1964).

(Figure 3, lower) yielded a negative slope and a significantly lower intercept value of $s_{20,w} = 2.57 \pm 0.14$ S.

The results of a low speed equilibrium sedimentation of succinyl fraction II are shown in Table II. Point by point weights were calculated from slopes determined by fitting the observed data points with a rational function equation. The discrepancy between the point by point values and the weight-average value is the result of a small error in the value of C_0.

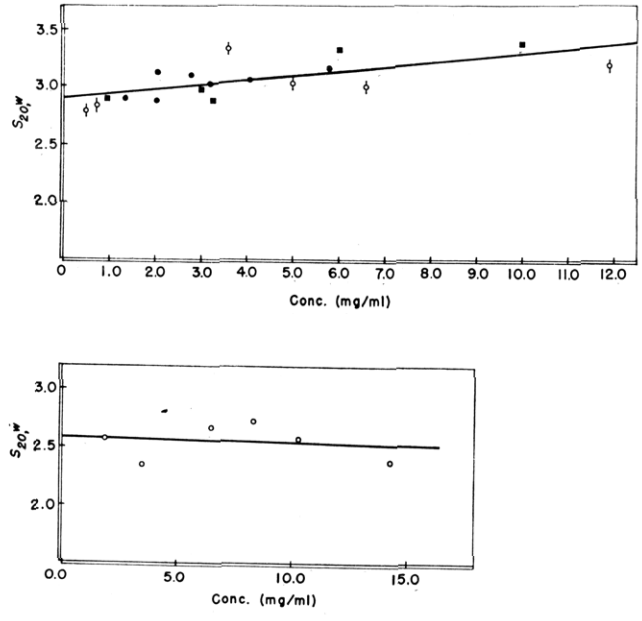

FIGURE 3: $s_{20,w}$ concentration dependencies. Upper: for fraction II. (ϕ) 0.04 M KH_2PO_4, pH 6.5; (○) 0.04 M KH_2PO_4–0.1 M KCl, pH 6.5; (■) 0.04 M KH_2PO_4–0.9 M KCl, pH 6.5. Lower: of succinyl fraction II in 0.038 M KH_2PO_4–0.1 M KCl (pH 7.5).

TABLE II: Molecular Weight of Succinyl Fraction II.[a]

Radius (cm)	Fringes Obsd	Fringes Calcd	Mol Wt (M_w)
6.696	22.226	22.266	24,501
6.734	23.403	23.455	24,539
6.760	24.403	24.330	24,694
6.792	25.403	25.435	24,597
6.814	26.403	26.235	24,619
6.845	27.403	27.375	24,650
6.872	28.403	28.471	24,678
6.895	29.403	29.421	24,702
6.919	30.403	30.440	24,726
6.942	31.403	31.462	24,750
6.964	32,403	32.446	24,772
6.985	33.403	33.468	24,794
7.002	34.403	34.296	24,812
7.010	34.760	34.720	24,820
		Weight average	25,228
		Volume average	24,661
		Z average	25,391

[a] 3.4 mg/ml of succinyl fraction II in 0.038 M KH_2PO_4–0.1 M KCl (pH 7.5), 964-min centrifugation at 8016 rpm, 8.3°.

Enzymatic Properties of the Endopeptidase Moiety of PCP A-S6. Kinetics of the hydrolysis of Ac-L-TyrOEt by activated PCP A-S6 and fraction II were investigated as described in Methods. Plots of the initial velocities at specific substrate concentrations were made according to the equation of Eadie *et al.* (1949) and were found to be linear within the range of substrate concentrations employed. For activated PCP A-S6, $V_{max} = 12.1 \pm 0.4$ mequiv of OH^-/min per μmole of protein and $K_m = 9.8 \pm 0.5$ mM; for activated fraction II under the same conditions, $V_{max} = 1.9 \pm 0.1$ mequiv of OH^-/min per μmole of protein and $K_m = 10.7 \pm 0.8$ mM. A comparison of these values reveals a large discrepancy in V_{max} but comparable values of K_m. In view of these findings, the operational normality of the various enzymes was determined.

Spectrophotometric Titration with N-trans-Cinnamoylimidazole and Calculation of the Number of Active Sites per Mole. The operational normality of a given preparation of α-chymotrypsin can be determined from its reaction with *N-trans*-cinnamoylimidazole at pH 5.05 (Schonbaum *et al.*, 1961) which forms a relatively stable acyl-enzyme intermediate (*trans*-cinnamoyl-α-chymotrypsin). The procedure was applied to the determination of the number of active sites per molecule of activated PCP A-S6, fraction II, and succinyl fraction II. Preliminary experiments indicated that the acylation of the endopeptidase takes place rapidly and quantitatively with no evidence of turnover of *N-trans*-cinnamoylimidazole (initial and final slopes representing the water hydrolysis of *N-trans*-cinnamoylimidazole were identical). As with chymotrypsin, inactive protein does not appear to interfere with the reaction nor does the presence of trypsin. All determinations were carried out in triplicate. The data are presented in Table I using constants for α-chymotrypsin given by

Deranleau and Neurath (1966). The significance of these results will be considered in the Discussion.

Reaction of Activated PCP A-S6 and Fraction II with TPCK. The reaction of activated PCP A-S6 with TPCK is presented in Figure 4. Activity declined to a value which was 10% of the control after 200 min. A first-order plot of the reaction remained linear for 2 hr yielding an apparent first-order rate constant of $1.00 \pm 0.03 \times 10^{-4}$ sec^{-1}. Analogous experiments for activated fraction II yielded the same apparent rate constant of $1.02 \pm 0.04 \times 10^{-4}$ sec^{-1}. Similar results were obtained in each case using as low as a 10-fold molar excess of inhibitor in a pH range of 6.5–7.5. Since the reaction rate with PCP A-S6 or with fraction II is much slower than with chymotrypsin, the more effective inhibitor ZPCK was also examined. However, the results were consistent with those determined by use of TPCK.

A Second Calculation of Operational Normality: Labeling with [^{14}C]ZPCK. PCP A-S6 (8.2×10^{-5} M in 0.05 M KH_2PO_4, pH 6.5) was treated with a 10-fold molar excess of [^{14}C]ZPCK (67,500 cpm/mg) at 25 ± 0.1° for a period of 2.5 hr *prior* to activation. The reaction mixture was cooled to 0° and placed on a Sephadex G-25 column equilibrated with 0.05 M KH_2PO_4 (pH 6.5), 0–4°. Protein emerged in the breakthrough fractions well separated from excess inhibitor. Only a relatively small amount of radioactive material migrated with the protein peak (6.6% of the total amount of label incorporated in activated PCP A-S6). This was presumably due in part to some activation during the 2.5-hr incubation period. The zymogen was then activated and the product treated with a 10-fold molar excess of [^{14}C]ZPCK at 25 ± 0.1° as in the zymogen control. After 3 hr the final esterase activity was found to be 1.5% of the initial value. The mixture was separated on Sephadex as above. From the protein concentration (measured by differential refractometry) and the specific radioactivity of [^{14}C]ZPCK, it was calculated that 0.59 mole of the inhibitor was incorporated per mole of activated PCP A-S6. This corresponds closely to the value of 0.55 active site/mole of activated PCP A-S6 determined by titration with *N-trans*-cinnamoylimidazole (*vide supra*).

Chemical Verification of [^{14}C]ZPCK Incorporation into Activated PCP A-S6. Formation of 3-CM-His from [^{14}C]-ZPCK-PCP A-S6. [^{14}C]ZPCK-labeled PCP A-S6 was oxidized with performic acid according to the method of Stevenson and Smillie (1965). This treatment is reported to yield 3-CM-His by a rearrangement in which the carbobenzoxy-2-phenylethyl group migrates to form an ester which upon acid hydrolysis yields 3-CM-His. [^{14}C]ZPCK-labeled PCP A-S6 yielded 0.089 μmole of 3-CM-His/0.148 μmole of protein (both determined by amino acid analysis) corresponding to 0.60 mole of the derivative/mole of PCP A-S6. This value is in agreement with those obtained above.

These same procedures were used to label activated fraction II. In this case, 0.13 mole of [^{14}C]ZPCK/mole of activated fraction II was found to be incorporated, a value quite close to that resulting from *N-trans*-cinnamoylimidazole titration (0.14).

Labeling of Activated PCP A-S6 with [^{32}P]DFP: A Third Calculation of the Number of Active Sites per Mole. Activated PCP A-S6 (15.7 mg/ml) in 0.05 M KH_2PO_4 (pH 6.5) was treated with 2.5-fold molar excess of [^{32}P]DFP (1 mg/ml in ethylene glycol) at 0°. After incubation for 1 hr, no activity against Ac-L-TyrOEt could be detected. The reaction mixture

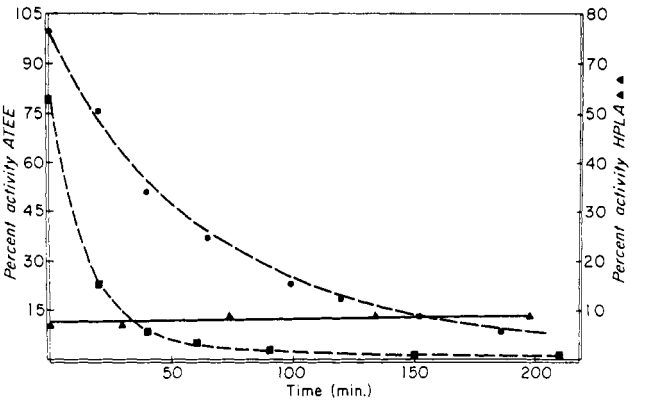

FIGURE 4: Reaction of activated PCP A-S6 with TPCK at 25 ± 0.1°. Protein concentration = 0.96 mg/ml; buffer = 0.05 M KH_2PO_4 (pH 7.5). Sufficient TPCK in methanol (in no case was the concentration of methanol higher than 4%) was added to bring the inhibitor concentration to a 50-fold molar excess over the amount of enzyme. The inactivation was followed using the standard Ac-L-TyrOEt assay. (●- - -●- - -●) Per cent Ac-L-TyrOEt activity compared to control. (■- - - -■- - - -■) Per cent Ac-L-TyrOEt activity for a similarly treated control solution of α-chymotrypsin. (▲- - - -▲- - - -▲) Per cent HPLA activity of PCP A-S6 assayed as defined by Yamasaki *et al.* (1963).

was extensively dialyzed against water and lyophilized. The protein was redissolved in 0.038 M KH_2PO_4 (pH 7.5) and equilibrated against the same buffer by dialysis. Fifty microliters of a solution (8.55 mg/ml) was counted and the data were corrected for radioactive decay. The calculated specific activity was 0.64 residue of [^{32}P]DIP/molecule as compared to the value of 0.59 reported by Brown *et al.* (1963). Thus three different methods of titration all give 0.5–0.6 active site of endopeptidase/mole of activated PCP A-S6.

Active-Site Distribution of [^{14}C]ZPCK-Substituted PCP A-S6. In an attempt to define more precisely the chemical relationship between endopeptidase as it exists in PCP A-S6 and fraction II, experiments were designed to determine the fate of the labeled site(s) of endopeptidase activity following disaggregation of PCP A-S6.

PCP A-S6 (141 mg) was dissolved in 10.5 ml of 0.038 M KH_2PO_4 (pH 6.5) and activated under standard conditions. The enzyme was then fully inactivated by incubation with a 35-fold molar excess of [^{14}C]ZPCK at 25 ± 0.1° for 2.5 hr and the inactive protein was disaggregated by succinylation with succinic anhydride (Freisheim *et al.*, 1967). Isolation of [^{14}C]ZPCK-labeled succinyl fraction II was carried out as specified for the isolation of succinyl fraction II. The elution profile is shown in Figure 5. The fraction was resolved into two major and two trace peaks; the major ones contained only 1% activity of carboxypeptidase A toward HPLA (activation conditions as specified by Freisheim *et al.*, 1967). Both major peak fractions (1 and 2) were homogeneous in the ultracentrifuge and exhibited identical s values (*i.e.*, $s_{20}' = 2.5$ S) (Table III) and amino acid compositions (see Table IV). The data thus indicate that the material eluted in peaks 1 and 2 was characteristic of succinyl fraction II.

Furthermore, approximately 0.25 mole of [^{14}C]ZPCK was incorporated per mole of succinyl fraction II as compared to 0.59 mole/mole originally incorporated in [^{14}C]ZPCK PCP A-S6.

TABLE III: Comparison of Certain Properties of Two Chromatographic Subfractions of [^{14}C]ZPCK-succinyl Fraction II.

Hydroxylapatite-cellulose Chromatography	HPLA Act. (mequiv/min mg × 10^3)	s'_{20} (S)	$A^{0.1\%}_{280m\mu}$ (Amino Acid Anal.)	$A^{0.1\%}_{280m\mu}$ (Fringe Count)	Sp Radioact. (Amino Acid Anal.; Mole of [^{14}C]ZPCK/Mole of Protein)	Sp Radioact. by Fringe Count
Peak 1	1.43	2.50	1.21	1.36	0.240	0.214
Peak 2	3.30	2.50	2.18	2.27	0.259	0.250

Structural Features of the Active Site of Fraction II. In order to confirm a specific reaction of activated fraction II with [^{14}C]ZPCK and [^{32}P]DFP, peptides containing functional seryl and histidyl residues of the endopeptidase were isolated using succinyl fraction II as the starting material. Succinyl fraction II (781 mg) was dissolved in 30 ml of 0.038 M KH_2PO_4–0.1 M KCl (pH 7.5) and activated with trypsin as described for PCP A-S6 and fraction II. The activity was completely abolished after incubation with an equimolar amount of [^{32}P]DFP for 1 hr at 0°. Excess [^{32}P]DFP was removed by dialysis against water followed by lyophilization.

In order to provide additional sites for proteolytic degradation the *S*-aminoethyl derivative of [^{32}P]DIP-succinyl fraction II was prepared using the procedure of Cole and Raftery (1966). The reaction of [^{32}P]DIP-succinyl fraction II with ethylenimine was complete as indicated by the lack of cystine in the acid hydrolysate and by the formation of nearly stoichiometric amounts of *S*-aminoethylcysteine.

Isolation of the [^{32}P]DIP Peptide. *S*-Aminoethyl-[^{32}P]DIP-succinyl fraction II, prepared as described above, was dissolved in 100 ml of water and digested with α-chymotrypsin (enzyme–substrate, 1:50, w/w) at pH 8.0 at 25° for 24 hr. After digestion the solution was lyophilized, dissolved in 14 ml of 1.0 M acetic acid, and applied to a Sephadex G-25 column (2.5 × 100 cm) previously equilibrated with 1 M acetic acid (pH 2.4). Suitable aliquots were taken for radioactivity measurements and for ninhydrin analysis according to Schroeder *et al.* (1962). The elution profile is presented in Figure 6. The peptide material in peak 1 (peak 2 was later found to contain several peptides, some of which were radioactive but present in low yield) was collected, dried using a cold air stream, and redissolved in 2.0 ml of 0.1 M ammonium acetate (pH 8.0). The mixture was then digested with subtilisin (subtilisin–peptide material, 1:50, w/w) at 25° for 16 hr. After digestion, the mixture was brought to pH 2.0 by the addition of HCl and placed on an AG 50W-X2 column (0.9 × 100 cm) equilibrated at 37° with 0.20 M pyridine–acetate buffer (pH 3.1). The progress of the chromatography was followed by ninhydrin (A_{570nm}) and radio-

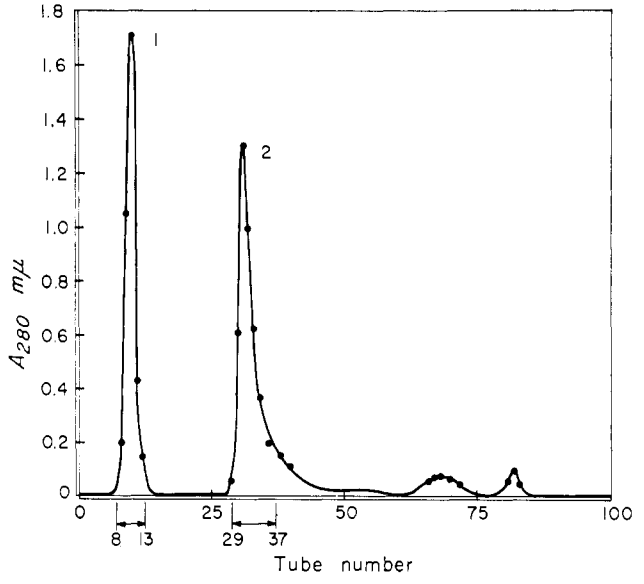

FIGURE 5: Column chromatography on hydroxylapatite-cellulose of [^{14}C]ZPCK succinyl fraction II. The protein in peak 1 was eluted with 0.005 M KH_2PO_4 (pH 6.5), 4°. The protein in peak 2 was eluted with 0.025 M KH_2PO_4 (pH 6.5). Flow rate = 0.8 ml/min; tube volume = 2.5 ml.

TABLE IV: Comparison of Amino Acid Composition of Peaks 1 and 2 of [^{14}C]ZPCK-succinyl Fraction II.[a]

Amino Acid	Residues (Peak 1) 24-hr Hydrolysis	Residues (Peak 2) 24-hr Hydrolysis
Lys	6.3	6.2
His	4.2	4.5
Arg	6.0	6.0
Asp	23.9	23.8
Thr	12.1	13.1
Ser	14.9	15.8
Glu	18.8	21.0
Pro	13.9	13.3
Gly	*23.0*	*23.0*
Ala	16.2	16.7
½-Cys	9.0	9.0
Val	17.5	18.6
Met	1.2	1.1
Ile	10.7	11.1
Leu	16.9	17.8
Tyr	5.8	6.4
Phe	5.9	6.0

[a] The data are expressed as the number of residues relative to glycine (set equal to 23.0). For an explanation of peaks 1 and 2, see text.

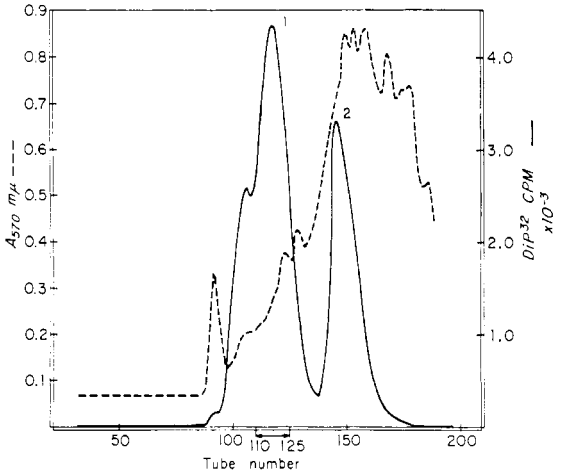

FIGURE 6: Sephadex G-25 gel filtration of an α-chymotrypsin digest of [^{32}P]DIP-*S*-aminoethylsuccinyl fraction II. Eluting solvent = 1 M acetic acid (pH 2.4), 25°. Flow rate = 0.8 ml/min; fraction volume = 2.5 ml.

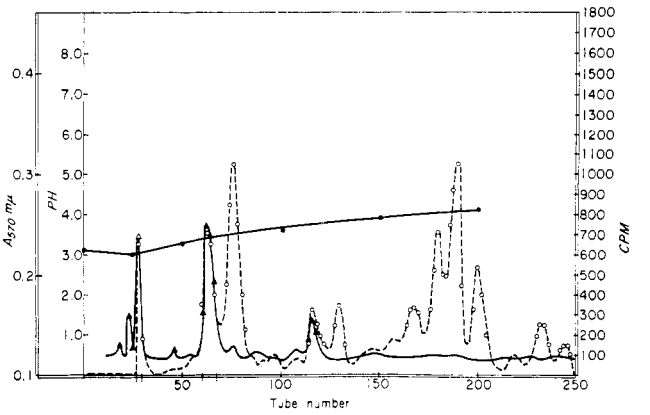

FIGURE 7: AG 50W-X2 column chromatography of a digest (subtilisin) of the [^{32}P]DIP peptide derived from succinyl fraction II. The buffer conditions are those specified by Schroeder (1967). (- - -●- - -) pH gradient; (○- - -○- - -○) absorbance at 570 mμ after base hydrolysis and ninhydrin reaction; (——) radioactivity.

activity measurements (Figure 7). The radioactive peptide in the first major peak was acidic and electrophoretically homogeneous by paper electrophoresis at pH 6.5. Amino acid analysis yielded the following composition (assuming 1.0 proline): Asp, 1.83; Ser, 0.82; Pro, 1.0; Gly, 3.07; Leu, 0.96; Ser·PO_3^{2-}, 1.15.[2]

The second major radioactive peptide from the AG 50W-X2 chromatography was purified on DEAE-Sephadex A-25. The elution profile is presented in Figure 8. The peptide in the major peak was found to be radioactive and homogeneous by electrophoresis and yielded the following composition (assuming 1.0 proline): Asp, 1.81; Ser, 0.83; Pro, 1.0; Gly, 2.95; Leu, 0.98; Ser·PO_3^{2-}, 1.15.[2] It is quite evident that this peptide has a composition identical with that of the first major radioactive peptide (AG 50W-X2 chromatography). The overall yield of two peptides was 29%. They may differ either in the isopropyl groups remaining on the [^{32}P]DIP-seryl moiety or in amide content, thus accounting for their difference in mobility despite identical amino acid compositions. One of the two aspartic acid residues in the latter peptide is probably an asparagine residue as indicated by digestion with leucine aminopeptidase followed by amino acid analysis which yielded the following composition (assuming Asp = 1.0): Ser·PO_3^{2-},[2] 1.15; Asp, 1.0; (Ser + Asn), 1.76; Gly, 1.89. The partial structure of the [^{32}P]DIP peptide (0.07 μmole) was determined by the Dansyl–Edman technique as: Ser-Asx-Asx-Ser·PO_3^{2-}-Gly-Gly(Gly,Pro,Leu).

In the solvent systems employed (Morse and Horecker, 1966), the fourth residue gave two spots. One was DNS-serine, the other was radioactive and had the mobility of DNS-$CySO_3^{2-}$. This was assumed to be α-DNS-Ser·PO_3^{2-}. One of the aspartic acid residues is probably in the amide form (*vide supra*).

Isolation of the [^{14}C]ZPCK Peptide. [^{14}C]ZPCK-labeled succinyl fraction II (350 mg) was prepared according to procedures described earlier using a 10-fold molar excess of inhibitor at 25 ± 0.1°. After gel filtration and lyophilization, [^{14}C]ZPCK-succinyl fraction II was dissolved in 20 ml of 5% formic acid and digested with pepsin (enzyme–substrate, 1:10, w/w) for 18 hr at 37°. The digest was then placed on a Sephadex G-25 column (2.5 × 100 cm) previously equilibrated with 1 M acetic acid (pH 2.4). Figure 9 illustrates the elution profile. The radioactive portions of the eluent fractions were pooled and subjected to ion-exchange column chromatography on DEAE-Sephadex A-25 as described in Figure 10. The isolated peptide had the amino acid composition listed in Table V. The yield of this peptide was calculated to be 22%. The value for histidine in the composition of this peptide is in doubt because, in the peptide, incomplete conversion of [^{14}C]ZPCK-His to 3-CM-His occurs after

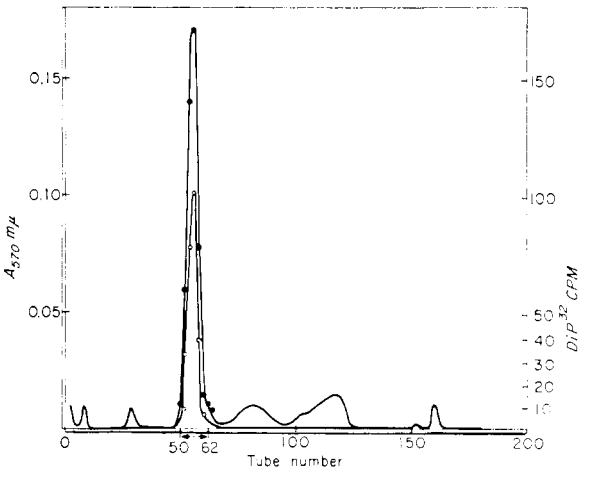

FIGURE 8: DEAE-Sephadex A-25 (0.9 × 30 cm) column chromatography of the peptide(s) in the second major radioactive peak, AG 50W-X2 chromatography. Flow rate = 0.8 ml/min. Fraction volume = 2.0 ml. The column was run at a temperature of 25° using a linear gradient ranging from 0.4 M pyridine–acetate (pH 6.2) to 1.0 M pyridine–acetate (pH 4.4) (200 ml each) (Ong *et al.*, 1965). - - -●- - -, ninhydrin absorbance at 570 mμ; - - -○- - -, radioactivity.

[2] Determined from leucine aminopeptidase digestion and amino acid analysis.

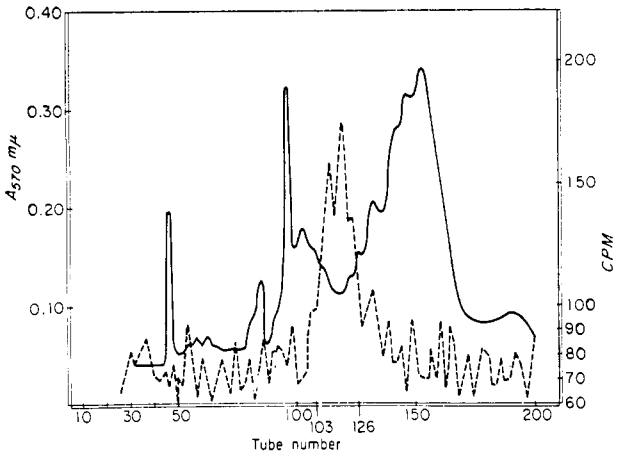

FIGURE 9: Sephadex G-25 gel filtration of a peptic digest of [^{14}C]-ZPCK labeled succinyl fraction II. Eluting solvent = 1.0 M acetic acid (pH 2.4), 25°. Flow rate = 0.8 ml/min; fraction volume = 2.5 ml. (——) Ninhydrin absorbance at 570 mμ; (- - - - - - -) radioactivity measurement.

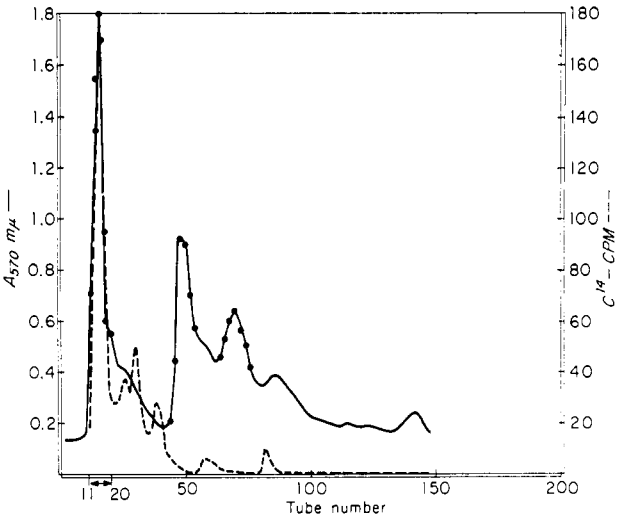

FIGURE 10: DEAE-Sephadex A-25 column chromatography of the peptide material in fractions 103–126 (Sephadex G-25 gel filtration, Figure 9). Conditions are described in the legend to Figure 8.

performic acid oxidation, part of the product reverting to histidine following acid hydrolysis (private communication, R. A. Bradshaw). The possibility that three histidines may be present, one substituted and two unsubstituted, cannot, however, be ruled out. Because of the presence of a disulfide bond connecting two peptides, each turn of the Edman degradation yielded two N-terminal residues in approximately equal concentrations. The first turn of the Edman degradation yielded alanine and aspartic acid (or asparagine). The second turn yielded alanine and serine. No evidence of peptide inhomogeneity could be detected during analysis except for a carry-over of the first N-terminal residue into the second degradation, estimated by fluorescence intensity to be 10%.

TABLE V: Amino Acid Analysis of the [^{14}C]ZPCK Peptide Derived from Succinyl Fraction II.[a]

Amino Acid	24-hr Hydrolysis
His	1.65
Arg	0.97
$CysSO_3^{2-}$ [b]	1.88
Asp	*2.0*
Thr	4.10
Ser	1.07
Gly	2.14
Ala	1.83
3-CM-His	0.16
Ile	0.95
Leu	1.83
[^{14}C]ZPCK	1.13

[a] The data are expressed as the number of residues relative to aspartic acid (assumed to be 2.0). [b] The peptide was oxidized with performic acid prior to analysis according to the method of Moore and Stein (1963).

Discussion

One of the most characteristic properties of bovine procarboxypeptidase A is its state of aggregation. The three subunits are so firmly bound to each other that it has not yet been possible to separate them from each other in the native form. This tendency for aggregation is also inherent in the isolated fraction II which undergoes a concentration dependent self-association to form higher aggregates. The observation that the slope of the line defining the concentration dependence of the *s* rate for fraction II was positive suggests that higher polymers were being formed as a function of concentration. Quantitative evidence for aggregation by high-speed sedimentation equilibrium is presented elsewhere (Teller, 1970). Succinyl fraction II on the other hand exhibited a negative concentration dependence of the *s* rate, probably due to electrostatic repulsion of the negatively charged molecules.

Low-speed sedimentation analysis of succinyl fraction II revealed that this preparation was homogeneous (in a macromolecular sense) and that the molecular weight of the monomeric unit is close to 25,000. The ultracentrifugal techniques employed, however, were not sufficiently sensitive to detect molecular weight differences among differentially succinylated molecules.

The enzymatic properties of activated PCP A-S6 and activated fraction II toward the ester substrate Ac-L-TyrOEt are similar but seemingly vary in their kinetic parameters. The K_m values are practically identical (10 mM) but V_{max} for activated PCP A-S6 is approximately six times higher than that for activated fraction II. This apparent discrepancy was resolved by active site titrations by three different reagents, all of which indicated that less of the fraction II molecules had active sites than activated PCP A-S6 (Table VI). In fact, there was only 0.14 active site/mole of isolated fraction II in comparison to 0.60 active site/mole of PCP A-S6. Nevertheless, the specific esterase activities of activated PCP A-S6, of fraction II, and of its succinylated derivatives per mole of active site were all quite similar (Table I).

There is at present insufficient information to interpret

these observations unambiguously, but several explanations could account for these results. (1) Partial denaturation may have occurred during the isolation procedure. (2) The conditions for activation of PCP A-S6 and fraction II *in vitro* may be inadequate to generate a full active site. (3) Several species of protein may exist (*e.g.*, allotypes, some of which can be activated while others cannot). If the fractional number of active sites is indeed a property intrinsic to the *in vivo* activation process, for whatever reasons, it is significant that fraction II nevertheless has a fully functional binding site for carboxypeptidase A (Behnke *et al.*, 1970) and presumably also for its precursor, subunit I.

Some of the structural and enzymatic features of activated (succinyl) fraction II are similar to those of α-chymotrypsin and other serine proteases. In particular, the amino acid sequence surrounding the unique seryl residue of α-chymotrypsin is Met-*Gly-Asp-Ser-Gly*-Gly-Pro-Leu. The italicized sequence is common to trypsin, chymotrypsin B, and elastase (Hartley *et al.*, 1965). The similarity of the [^{32}P]DIP peptide from fraction II is obvious and provides structural evidence for the hypothesis that fraction II may be related to chymotrypsin. Additional evidence for this conclusion has been provided by the N-terminal sequence reported by Peanasky *et al.* (1969).

The composition and end group analysis of the [^{14}C]ZPCK-His peptide give additional support to the similarity of fraction II to other serine proteases. The central importance of isolating this peptide, however, is that it confirms that inactivation of activated fraction II by ZPCK results in the substitution of a specific histidyl residue. These same considerations apply to the [^{32}P]DIP-seryl peptide as well. The peptide sequence obtained in this investigation, however, is far too limited to provide evidence for homology between fraction II and trypsin or chymotrypsin, but the similarities of these peptides to the corresponding peptide sequences in the other serine proteases is beyond doubt.

The distribution of [^{14}C]ZPCK in activated PCP A-S6 presents a more difficult problem of interpretation. Succinylation of the labeled trimeric precursor yielded protein material which contained 0.25 mole of [^{14}C]ZPCK/mole protein, a value which is approximately one-half the amount of label present in the aggregated precursor. An argument can be presented that this is the result of a partial activation of both fractions II and III which are similar in the native zymogen. Supporting evidence for their similarity is summarized below.

(1) The amino acid composition of fractions II and III is quite similar (Brown *et al.*, 1963) especially if the data are adjusted to the molecular weight of fraction II as determined in the present investigation. (2) Diagonal peptide maps (Brown and Hartley, 1966) of peptic digests of fractions II and III are qualitatively very similar, including the ninhydrin staining properties of the peptides, their relative distribution, the position of the histidine peptides, and the nature of the disulfide bridges (W. D. Behnke, unpublished observations). The peptide maps, however, were not analyzed quantitatively and further data are needed to support a structural identity of fractions II and III from this point of view. (3) Sedimentation data using the Archibald method yield similar characteristics for fractions II and III. In each case, the molecular weight distribution is indicative of polydispersity. At a concentration of 1.05 mg/ml, M_n for fraction III is 31,600 (J. R. Brown, unpublished data) and for fraction II at this concentration M_n = 31,250. At a concentration of 5.23 mg/ml, the s rate ($s_{20,w}$) = 2.86 S (J. R. Brown, unpublished data) for fraction III whereas for fraction II at this concentration s'_{20} = 2.96 S. (4) The tyrosine:tryptophan ratio of fraction II determined spectrophotometrically is 0.51 compared to a value of 0.50 for fraction III (Brown *et al.*, 1963).

TABLE VI: Determinations of the Number of Active Sites/Mole in Procarboxypeptidase A-S6 and Fraction II.

Enzyme	Moles of Active Site/Mole of Enzyme		
	Cinnamoyl-imidazole	[^{14}C]-ZPCK	[^{32}P]-DFP
Activated PCP A-S6	0.55	0.60	0.64
Activated Fraction II	0.14	0.13	

Since in the original isolation of fraction III care had to be taken to avoid the formation of an autolysis product (fraction III', Brown *et al.*, 1963), it is similarly possible that fraction III is an autolysis product of fraction II and is therefore an artifact of the lengthy disaggregation procedure. Thus PCP A-S6 may indeed contain 2 moles of subunit II/mole, each capable of generating 0.25 active site (endopeptidase)/mole.

It is not clear, however, why fraction II exhibits a rather low value of an active site (0.14 as compared to 0.30 expected; *vide supra*). Only a single fraction, peak 2, in low yield from hydroxylapatite-purified succinyl fraction II attained the full expected value of active sites per mole (see Table I). It is possible that some of the fraction II molecules suffered alteration during their isolation or during activation. It is also possible that the subunit structure of procarboxypeptidase may confer some protection of fraction II toward autolysis during its activation in the trimer.

Another plausible interpretation of the data is that only one subunit in PCP A-S6 is capable of giving rise to endopeptidase activity and that the other one is completely nonfunctional, perhaps as a result of amino acid replacements in regions of the active site. Further work is required for clarification of these issues.

Acknowledgment

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