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Mechanism of Activation of Bovine Procarboxypeptidase A S_5. Alterations in Primary and Quaternary Structure†

Jack R. Uren‡ and Hans Neurath*

ABSTRACT: A reevaluation of the chemical and physical properties of bovine procarboxypeptidase A S_5 has confirmed the majority of the properties described by Brown *et al.* (Brown, J. R., Yamasaki, M., and Neurath, H. (1963), *Biochemistry 2*, 877). A molecular weight of 63,000 was determined by self-association analysis of high-speed sedimentation equilibrium data and by sodium dodecyl sulfate gel electrophoresis. The effects of various salts on the proteolytic activation demonstrated that calcium ions greatly enhance the rate of activation of the zymogen and broaden the specificity of activation by other proteolytic enzymes. These effects may be related to the observation that calcium ions also promote subunit dissociation. Isolation of the products of activation demonstrated that both trypsin and chymotrypsin generate carboxypeptidase A_α with some of the β form present when trypsin is the activating enzyme. The activation of subunit II was analogous to the formation of π-chymotrypsin and 12 residues of the B chain of subunit II were sequenced and compared to those of other serine proteases.

Bovine pancreatic procarboxypeptidase A exists in two forms which differ from each other in sedimentation coefficient and chromatographic behavior on ion-exchange columns (Brown *et al.*, 1963). One of these, procarboxypeptidase A S_5 (PCP A S_5),[1] has a sedimentation coefficient of 5 S and is composed of two subunits (I and II). Subunit I is the immediate precursor of carboxypeptidase A whereas subunit II is the zymogen of an endopeptidase similar to chymotrypsin (Brown *et al.*, 1963; Peanasky *et al.*, 1969). Procarboxypeptidase A S_6 (PCP A S_6) has a sedimentation coefficient of 6 S and contains, in addition, a third subunit (III) which is believed to be an inactive derivative of subunit II (Behnke *et al.*, 1970).

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‡ Present address: Department of Pharmacology, Sterling Hall of Medicine, Yale University, New Haven, Conn. 06510.

[1] The following abbreviations are used: PCP A S_5, procarboxypeptidase A S_5; PCP A S_6, procarboxypeptidase A S_6; HPLA, D,L-hippuryl-β-phenyllactic acid; CGP, carbobenzoxyglycyl-L-phenylalanine; ATEE, acetyl-L-tyrosine ethyl ester; DFP, diisopropyl phosphorofluoridate.

In contrast to the bovine zymogen, procarboxypeptidases of other species (human, dogfish, and lungfish) are monomeric zymogens composed exclusively of the immediate precursor of carboxypeptidase A (Keller and Allan, 1967; Lacko and Neurath, 1970; Reeck *et al.*, 1970).

The role of the quaternary structure in the activation of bovine procarboxypeptidase A (S_5 or S_6) is not fully understood. Keller *et al.* (1958) have shown that the participation of both trypsin and the activated subunit II are necessary for the generation of carboxypeptidase activity. The unusually slow rate of the activation of the complex (several hours at 37°) as compared to monomeric succinyl subunit I (Freisheim *et al.*, 1967a) or monomeric dogfish procarboxypeptidase A (Lacko and Neurath, 1970) has led to the suggestion that the region of primary peptide bond cleavage in the procarboxypeptidase subunit (I) is masked by the quaternary structure of the complex (Neurath *et al.*, 1970).

There is evidence to suggest that the activation of procarboxypeptidase requires metal ions besides the intrinsic metal (zinc) of the active enzyme. Bovine procarboxypeptidase A contains significant amounts of calcium, iron, nickel, and zinc (Piras and Vallee, 1967). Cox *et al.* (1964) reported a substantial enhancement of the activation rate of the zymogen by the addition of calcium. High levels of calcium were also found necessary for the activation of the dogfish zymogen (Lacko and Neurath, 1970).

It is now well established that the enzymatic cleavage of any one of the three different peptide bonds in the polypeptide chain of subunit I can give rise to enzymatic activity. The resulting enzymes (carboxypeptidases A_α, β, and γ) contain Ala_1, Ser_3, and Asn_7 as amino-terminal residues, respectively (using the numbering system of carboxypeptidase A_α of Bradshaw *et al.*, 1969). The identities of the enzymes which generate these three forms of carboxypeptidase A are not known.

It was the purpose of the present investigation to reevaluate some of the physical and chemical properties of the zymogen and to investigate the role of metal ions and of the quaternary structure on the proteolytic induction of enzymatic activity. Because of the ill-defined nature of subunit III, procarboxypeptidase A S_5, containing subunits I and II only, was chosen for this study. The changes in tertiary structure attending zymogen activation are described elsewhere (J. R. Uren and H. Neurath, in preparation).

Experimental Procedure

Materials

Column Media. DEAE-70 cellulose was obtained from Schleicher and Schuell; DE-52 cellulose from Whatman Reeve Angel; and Sephadex G-25, G-75, and G-100 from Pharmacia Fine Chemicals. *N*-(2-Ethylcellulose)glycyl-D-phenylalanine was synthesized as described by Uren (1971).

Enzymes. Carboxypeptidase A (lot COA-7AC), trypsin (lot TRL IGA), chymotrypsin (lot CDI 8JA), and elastase (lot ESFF 8 DA) were obtained from Worthington Biochemical Corp. Subtilisin (Nagarse) was purchased from Teikoku Chemical Industry Co., Ltd., Osaka, Japan. Porcine trypsin (lot Ki 52097) was a product of Armour Pharmaceutical Co. Pancreatic acetone powder (lot 190-400B-34) was obtained through the courtesy of Eli Lilly Co. and was stored at −20°.

Reagents. Organic reagents were obtained as follows: diisopropyl phosphorofluoridate (DFP), Pierce Chemical Co.; [^{14}C]DFP, New England Nuclear; ethylenimine, Matheson, Coleman & Bell; guanidine-HCl (spectrophotometric grade), Heico Inc.; [^{14}C]iodoacetic acid, New England Nuclear; β-mercaptoethanol, Baker Chemical Co.; 2-(*N*-morpholino)-ethanesulfonic acid, Calbiochem; β-phenylpropionic acid, Eastman Organic Chemicals; sodium dodecyl sulfate, Mallinckrodt Chemical Works; tetraethyl pyrophosphate, American Potash and Chemical Co.; tris (hydroxymethyl)aminomethane, Sigma Chemical Co. All inorganic reagents used were reagent grade.

Substrates. D,L-Hippuryl-β-phenyllactic acid (HPLA) and carbobenzoxyglycyl-L-phenylalanine (CGP) were products of Fox Chemical Co. and acetyl-L-tyrosine ethyl ester (ATEE) came from Cyclo Chemical Co.

Methods

Gel Electrophoresis. Disc gel electrophoresis was performed at pH 8.3 by the method of Davis (1964) except that the separating gel was poured before the concentrating gel. Gels were stained with 1% Amido Black in 7% acetic acid for at least 1 hr and destained with the quick gel destainer of the Canalco Co.

Sodium dodecyl sulfate gel electrophoresis was carried out as described by Weber and Osborn (1969) except that the sample was dissolved in hot (100°) 0.01 M Na_2HPO_4 (pH 7.0), containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. Incubation for 10 min in this buffer prior to application of the sample to the gel prevented proteolysis. The gels were cut at the Bromophenol Blue band and stained with Coomassie Blue. Destaining was accomplished with 7.5% acetic acid and 5% methanol using the quick gel destainer. The gels were scanned at 280 nm before staining and at 520 nm after staining using the Model 2411 adapter for the Gilford Model 2000 spectrophotometer. Proteins of known molecular weight were used to establish a standard curve relating mobility to molecular weight.

Microzone Electrophoresis. DFP-inactivated aliquots of the activation mixture were subjected to Microzone electrophoresis (Beckman Model R-101) for 1 hr at 4° and 250 V. A pH 8.6 barbital buffer of 0.075 ionic strength was used and the electrophoresis membrane was stained with Ponceau S.

Determinations of Absorbancy Index and Sedimentation Constant. Ten microliters of 1 M DFP in isopropyl alcohol was added to a sample of PCP A S_5 (~15 mg/ml) which was then dialyzed overnight against 0.05 M imidazole–0.10 M LiCl (pH 6.5). The material was centrifuged and the absorbancy at 280 nm of the clear supernatant was measured in a Cary 16 spectrophotometer; the concentration was determined by a refractometric fringe count procedure (Babul and Stellwagen, 1969) in the Beckman Model E ultracentrifuge using a synthetic boundary cell (3.064 fringes/mg). The sedimentation constant was calculated by least-squares analysis of the peak position using the equation $s = [d(\ln x)/dt]/\omega^2$ (Svedberg and Pedersen, 1940), where ω is the angular velocity of rotation, x the distance from the center of rotation to the schlieren peak position, and t the time of centrifugation. The data were converted to standard conditions using a partial specific volume of 0.7319 calculated from the amino acid composition and the residue specific volume (Schachman, 1957): the solvent viscosity was measured in an Ostwald viscometer at 20.00 ± 0.01° (Model 5901A auto-viscometer of Mechrolab); and the solvent density was measured by pycnometry at 20.0°. The sample was then diluted with the dialysate and the procedure was repeated. Samples containing less than 1 mg/ml were measured using an absorption optical system.

Sedimentation Equilibrium Determination. High-speed sedimentation equilibrium analyses were performed using the six-channel centerpiece of Yphantis (1964). The optimum speed for centrifugation was estimated by eq 12 of Teller *et al.* (1969). Sedimentation equilibrium was reached after 12 hr at 20,000 rpm. Photographs of the Rayleigh interference pattern were taken at equilibrium and the base-line pattern was photographed at 4000 rpm after the sample had been thoroughly mixed. Fringe displacement as a function of position was measured with a modified Nikon microcomparator (Teller, 1967). The data were analyzed by a computer program described by Teller *et al.* (1969) which calculates the molecular weight moments as a function of protein concentration across the cell.

Two zymogen preparations were separately dialyzed overnight against 1 l. of 0.10 M LiCl–0.05 M imidazole (pH 6.5). Sedimentation equilibrium was determined for each preparation at three different protein concentrations. Only experiments with initial protein concentrations greater than 0.4 mg/ml are reported.

Amino Acid Composition. All amino acids except tryptophan were determined after hydrolysis in 6 N HCl at 110° using a Spinco Model 120 amino acid analyzer. Tryptophan was determined spectrophotometrically by the procedure of Edelhoch (1967) and colorimetrically by the procedure of Gaitonde and Dovey (1970). Values for serine and threonine were calculated by least-squares extrapolation to zero time from duplicate points at hydrolysis times of 24, 48, 72, and 96 hr. Isoleucine and valine are reported as the average of the 72- and 96-hr hydrolysates. All other amino acids are reported as the average of all time periods. Cystine was determined after reduction and aminoethylation by the procedure of Cole (1969) with the substitution of 6 M guanidine-HCl for 8 M urea.

Amino-Terminal Analysis. A Beckman Sequencer (Hermodson *et al.*, 1972) was used for the determination of the amino-terminal sequence of the protein. The phenylthiohydantoyl amino acids were identified by gas–liquid chromatography with the exception of histidine and arginine which were identified by spot tests using diazotized *p*-anisidine (Sanger and Tuppy, 1951) and phenanthrenequinone (J. Ohm, personal communication). For the identification of cysteine residues a 15-mg sample of PCP A S_5 was carboxymethylated with [^{14}C]iodoacetic acid under conditions similar to those utilized for aminoethylation (Cole, 1969). Following carboxymethylation, the protein was exhaustively dialyzed against a continuous flow of 5% acetic acid for 3 days and lyophilized.

Enzymatic Assays. Zymogen activation procedures and activity measurements toward the substrates HPLA and ATEE were those of Yamasaki *et al.* (1963). For measurements of peptidase activities toward CGP (1 mM) after activation, a spectrophotometric assay was employed using a Cary 16 recording spectrophotometer equipped with a Leeds and Northrup Flexelect recorder set at 224 nm ($\Delta\epsilon$ = 1190). The substrate was dissolved in 0.475 M KCl–0.05 M Tris (pH 7.5).

Active-Site Determination of Subunit II. A sample of PCP A S_5 (2 mg/ml) was dialyzed overnight against 1 l. of 1 M NaCl–0.05 M Tris (pH 7.5). The sample was then activated by trypsin (1:100 weight ratio of trypsin to zymogen) at 0° for 1 hr. [^{14}C]DFP (10 μl of a 0.2 M solution in 2-propanol) was added to 1 ml of the sample. After 1 hr the inactivated material was desalted on a 1 × 50 cm column of Sephadex G-25 and eluted with 0.1 M KH_2PO_4 (pH 6.5). The specific radioactivity of the reagent was determined by reaction with a sample of purified trypsin of known operational normality (Robinson, 1971). An aliquot of the desalted protein was counted using a Packard Model 3003 Tri-Carb liquid scintillation spectrometer with the efficiency being determined by the addition to the sample of [^{14}C]toluene of known disintegrations per minute. Protein concentration was determined from the absorbancy index of 1.77 at 280 nm for a 0.1% solution and a molecular weight of 63,000 (*vide infra*).

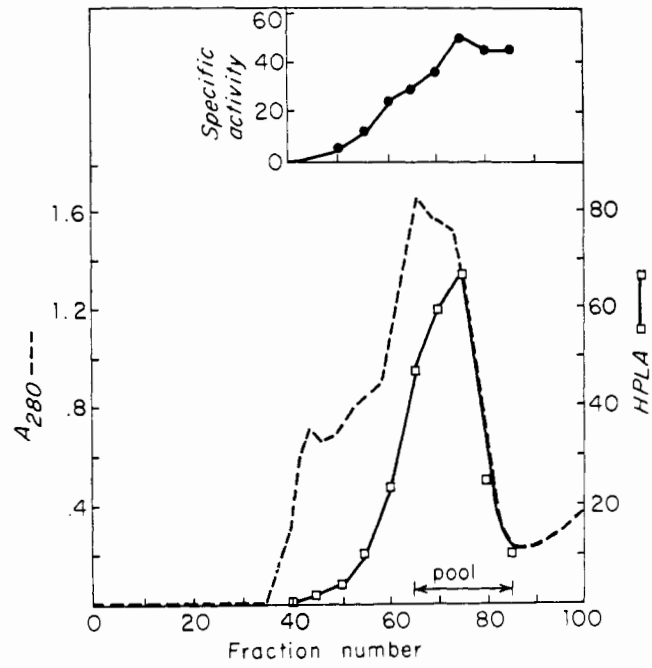

FIGURE 1: Gel filtration on Sephadex G-100 of partially purified PCP A S_5 obtained from chromatography on DEAE-cellulose. The column (5 × 80 cm) was equilibrated with 0.10 M KH_2PO_4 (pH 6.5) and eluted with this buffer at a rate of 1 ml/min at 4°. Fraction volume was 9.6 ml/tube; sample volume was 25 ml. Activity measurements are described in Methods. Units of HPLA activity are μmoles/(min ml); units of specific activity are μmoles/(min mg).

Protein Concentration. Before the physical or enzymatic properties of any sample of PCP A S_5 were measured, 10 μl of 1 M DFP was added and the sample was dialyzed overnight against 1 l. of 0.10 M LiCl–0.05 M imidazole (pH 6.5) to remove phosphate from the lyophilized material. The sample was then centrifuged in a desk-top centrifuge to remove any turbidity or the light scattering was corrected by the equation $(A_{280}) - 1.5(A_{310}) = (A_{280}\ \text{cor})$. All absorbancies were measured with a Zeiss PM QII spectrophotometer. Protein concentrations were calculated from the absorbancy index of 1.77 at 280 nm for a 0.1% solution.

Results

Procedures for the Isolation of PCP A S_5. The conditions for extraction of 100 g of pancreatic acetone powder and procedures for first column chromatography of PCP A S_5 on DEAE-cellulose were those described by Brown *et al.* (1963) except that 0.1% tetraethyl pyrophosphate replaced DFP during the extraction procedure. The leading half of the fractions which contained activity toward HPLA were pooled and precipitated with $(NH_4)_2SO_4$ at 40% saturation. After centrifugation, the precipitate was redissolved in 25 ml of H_2O and applied to a 5 × 80 cm column of Sephadex G-100 which had been previously equilibrated with 0.10 M KH_2PO_4 (pH 6.5). The protein was eluted with the same buffer at a flow rate of 1 ml/min (Figure 1). The fractions of the highest

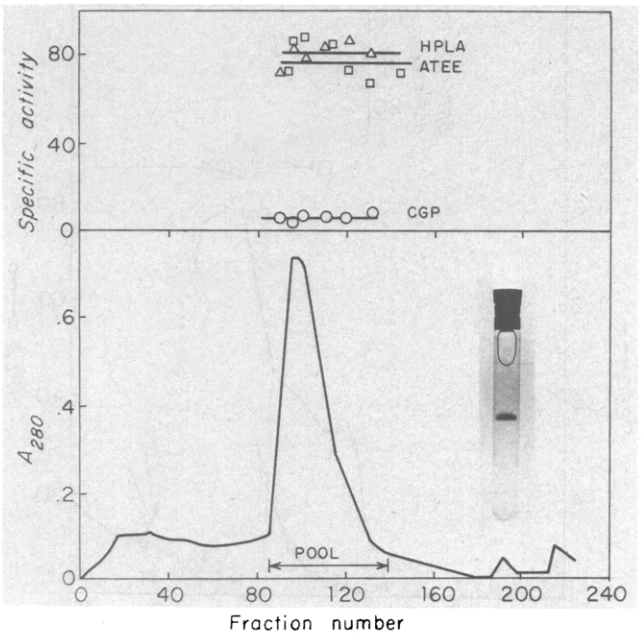

FIGURE 2: Chromatography on DE-52 cellulose of the pooled fractions from the Sephadex G-100 column shown in Figure 1. The column (2.5 × 85 cm) was eluted with a 2 × 2 l. linear gradient of 0.10 M KH_2PO_4 (pH 6.5) at a flow rate of 1.5 ml/min at 4°. Fraction volume was 16.4 ml/tube; activity measurements are described in Methods. Units of specific HPLA and ATEE activities are μmoles/(min mg); units of CGP activity are mmoles/(min mg) × 10^{-2}.

specific activity toward HPLA were pooled and applied directly to a 2.5 × 85 cm column of Whatman DE-52 which had been equilibrated with 0.10 M KH_2PO_4 (pH 6.5). The protein was eluted with a linear gradient of 2 l. each of 0.10 and 0.20 M KH_2PO_4 (pH 6.5) at a flow rate of 1.5 ml/min (Figure 2). The major peak fractions which contained activity toward HPLA, ATEE, and CGP were pooled, dialyzed against 3 l. of H_2O, and lyophilized. This material was then dissolved in 200 ml of H_2O, dialyzed against 800 ml of H_2O, and lyophilized (*vide infra*). A normal yield was 100 mg of PCP A S_5 from 100 g of starting material.

Stability of PCP A S_5. Difficulty was encountered in developing suitable conditions for storage of the material. It was found that either freezing the material after the final chromatography or extended dialysis against H_2O or 0.01 M KH_2PO_4 (pH 6.5) followed by lyophilization would result in precipitation and a diminution in the activatability by trypsin. When the preparation was first dialyzed against a limited quantity of H_2O to reduce the phosphate concentration to 0.04 M and then lyophilized, the zymogen was stable as judged by the ability to generate carboxypeptidase A activity; the potential chymotryptic activity was, however, impaired by this procedure (*vide infra*).

Purity of PCP A S_5. The behavior of PCP A S_5 on ion-exchange chromatography is shown in Figure 2. Although the major peak was not symmetrical, the specific activities toward ATEE, HPLA, and CGP were constant across the peak. The material migrated as a single boundary during polyacrylamide disc gel electrophoresis (Figure 2). If a sample of this material was subjected to gel electrophoresis in the presence of sodium dodecyl sulfate (Weber and Osborn, 1969), only two components could be observed, in accord with the dimeric nature of the zymogen (*vide infra*).

As another criterion of purity, the number of active sites of

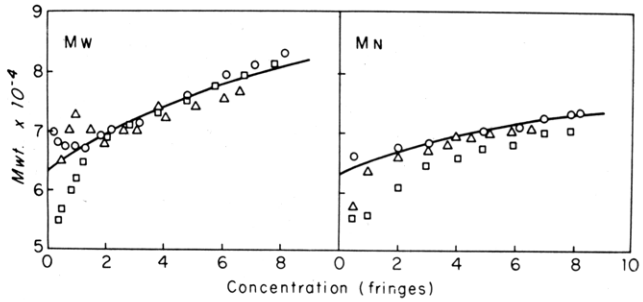

FIGURE 3: High speed sedimentation equilibrium analysis of PCP A S_5. The solvent contained 0.1 M LiCl–0.05 M imidazole (pH 6.5) at 19.2°. Sedimentation was performed at 20,000 rpm for 12 hr. The loading sample concentration was 1.2 mg/ml (○) and 0.9 mg/ml (□) for one preparation and 0.4 mg per ml (△) for another preparation. Calculations are described in Methods. The solid line was calculated assuming a dimerizing system with a monomer molecular weight of 63,000 and an association constant of 8400 ± 1600 l./mole.

activated subunit II was determined by reaction with [^{14}C]-DFP. Preparations that were lyophilized twice during the isolation procedure (*vide supra*) gave values of 0.53 active site/molecule, which are similar to those reported by Behnke *et al.* (1970) for PCP A S_6. When PCP A S_5 was prepared without any lyophilization, using $(NH_4)_2SO_4$ precipitation to concentrate the protein, the active-site values increased to 0.93 ± 0.13. This material, when lyophilized, reacted to a lesser extent with [^{14}C]DFP (0.80 ± 0.005). The data suggest that the unlyophilized material was nearly pure but not stable during lyophilization. Because of the low yields of the $(NH_4)_2SO_4$ precipitation procedure, it was not adopted in subsequent work.

Physical Properties of PCP A S_5. ABSORBANCY INDEX. The absorbancy index of PCP A S_5 was obtained by relating absorbancy at 280 nm to protein concentration determined refractometrically in the ultracentrifuge by the fringe count procedure (see Methods). A value of $A_{280}^{0.1\%} = 1.77 \pm 0.01$ was obtained at three different concentrations. This value differs from that of 1.9 used by Brown *et al.* (1963) which was assumed to be identical with that of PCP A S_6.

SEDIMENTATION VELOCITY. Within the concentration range of 0.5–8 mg/ml, the sedimentation coefficient of PCP A S_5 varied linearly with protein concentration with a negative slope of 0.011 S ml/mg. The sedimentation coefficient, extrapolated to infinite dilution, was 5.24 ± 0.05 S as compared to 5.0 S reported by Brown *et al.* (1963).

SEDIMENTATION EQUILIBRIUM. High-speed sedimentation equilibrium by the procedure of Teller *et al.* (1969) was used to determine the molecular weight (see Methods). The concentration distribution of molecular weight for two separate zymogen preparations at three different protein concentrations is shown in Figure 3. The weight-average molecular weight increased with increasing concentration from 63,000 to 83,000. A possible explanation for this behavior is self-association of the S_5 protein.

If it is assumed that the results shown in Figure 3 are due to self-association, the two-species equation of Roark and Yphantis (1969) becomes

$$n = \frac{M_{ni}(M_{wi} - M_1)}{M_1(M_{ni} - M_i)}$$

$$n = \frac{M_{wi}(M_{zi} - M_1)}{M_1(M_{wi} - M_1)}$$

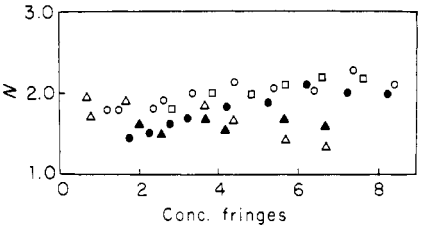

FIGURE 4: Two-species plot based on an assumption of self-association. Equations are described in the text. Open symbols refer to values calculated from M_w and M_z and closed symbols to values calculated from M_n and M_w. The symbols are explained in the legend to Figure 3.

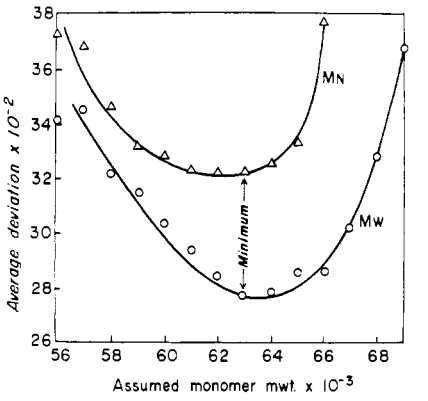

FIGURE 5: Best monomer molecular weight for PCP A S_5 based on fitting the data of Figure 3 to a dimerizing system with an assumed monomer molecular weight. For details, see the text.

where n is indicative of the type of association, *i.e.*, dimerization, trimerization, etc., M_{wi}, M_{ni}, and M_{zi} are the weight-, number-, and z-average molecular weight at each position (i) in the centrifuge cell, and M_1 is the monomer molecular weight. The value of n should be a constant at each position (i) in the cell. Figure 4 shows the plot of the data assuming M_1 = 63,000. As can be seen from Figure 4, dimerization best fits the observed data.

Knowing the most probable form of association, *i.e.*, dimerization, it was then possible to calculate the monomer molecular weight which best fits the data. A computer program of Harris *et al.* (1969) was utilized which iterates the assumed monomer molecular weight from 56,000 to 69,000 with 1000 increments and back-calculates the error in the molecular weight from the fit of the assumed molecular weight to the data based on a dimerizing system. The data shown in Figure 5 demonstrate that 63,000 does best fit the observed data with a minimum error for the monomer molecular weight.

The solid line in Figure 3 was calculated for a dimerizing system with a monomer molecular weight of 63,000 and an association constant of 8400 ± 1600 l./mole. While this model fits the data, other models involving mixed dimers, trimers, and tetramers of the PCP A S_5 subunits or contamination involving more than two species might also fit but are too complex to be tested.

SODIUM DODECYL SULFATE GEL ELECTROPHORESIS. This method was applied to confirm independently the molecular weight of the protein (see Methods). Under these conditions the dimer of PCP A S_5 dissociates into its subunits having average molecular weights of 41,500 for subunit I and 22,250 for subunit II. When these gels were scanned at 280 nm before staining with Coomassie Blue and at 520 nm after staining, an equal distribution of each of the two protein subunits was observed, confirming the dimeric nature of the zymogen. Consequently the PCP A S_5 dimer must have a molecular weight of the sum of each subunit or 63,750.

Chemical Properties of PCP A S_5. AMINO ACID COMPOSITION. The amino acid composition of PCP A S_5 is shown in Table I. These data were calculated on the basis of 39 residues of alanine which gives a composition molecular weight of 63,151, in agreement with the molecular weight of 63,000 (*vide supra*) determined in the ultracentrifuge. With the exception of methionine, phenylalanine, and tryptophan, the present data agree within experimental error with the values reported by Brown *et al.* (1963). When the content of methionine and phenylalanine was calculated from the sum of fraction II (Brown *et al.*, 1963) and succinyl fraction I (Freisheim *et al.*, 1967b), the values obtained (5 methionine and 25.2 phenylalanine residues) were closer to the present data than those of Brown *et al.* (1963) for PCP A S_5. The two techniques used in the present experiments for the determination of tryptophan (spectra in 6 M guanidine-HCl described by Edelhoch (1967) and the colorimetric procedure of Gaitonde and Dovey, 1970) both gave values of 13.7 ± 0.1 residues per molecule of PCP A S_5.

AMINO-TERMINAL RESIDUES. For the determination of the amino-terminal residues in the Beckman Sequencer, the zymogen was first reduced with β-mercaptoethanol and alkylated with [^{14}C]bromoacetate in 6 M guanidine-HCl. Because of the large size of the protein, sequenator analysis did not proceed very well and an overlap occurred after the second turn. The first turn released ^{14}C-labeled cysteine and showed lysine on gas–liquid chromatography. There was no evidence for any major amino-terminal heterogeneity; only the expected end groups of subunits I and II reported by Brown *et al.* (1963) were observed (Table II). The reported sequence of the A chain of subunit II (Peanasky *et al.*, 1969)

TABLE I: Amino Acid Composition of PCP A S_5.

Amino Acid	Residues/ Molecule	Integer	Brown *et al.* (1963)
Aspartic acid	55.8 ± 1.5	56	54
Threonine	40.5 ± 1.5	40	39
Serine	46.0 ± 0.9	46	43
Glutamic acid	61.9 ± 1.4	62	60
Proline	22.9 ± 0.5	23	24
Glycine	46.2 ± 0.6	46	44
Alanine	39	39	39
Valine	39.6 ± 0.8	40	40
Methionine	5.0 ± 0.3	5	6
Isoleucine	31.7 ± 0.7	32	33
Leucine	47.3 ± 0.9	47	47
Tyrosine	23.1 ± 0.7	23	24
Phenylalanine	23.9 ± 0.5	24	21
Tryptophan	13.7 ± 0.1	14	19
Lysine	22.7 ± 1.1	23	24
S-Aminoethylcysteine	10.0 ± 0.2	10	10
Histidine	14.2 ± 0.7	14	14
Arginine	23.6 ± 1.3	24	24
Molecular weight		63,151	

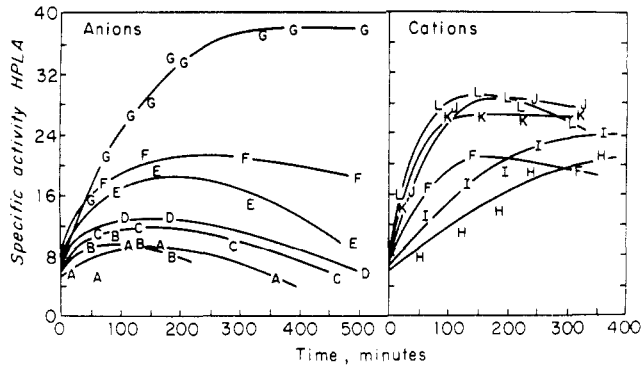

FIGURE 6: Salt effect on the activation of PCP A S_5. The zymogen (0.076 mg/ml) was activated with trypsin (0.01 mg/ml) at 38.6° in a buffer containing 0.05 M imidazole (pH 6.5). The following salts were also included: (A) 0.1 M Na_2SO_4, $I = 0.30$; (B) 0.1 M Na_2CrO_4, $I = 30$; (C) 0.1 M NaSCN, $I = 0.10$; (D) 0.1 M $NaClO_4$, $I = 0.10$; (E) 0.1 M NaI, $I = 0.10$; (F) 0.1 M NaCl, $I = 0.10$; (G) 0.1 M NaH_2PO_4, $I = 0.165$; (H) 0.1 M LiCl, $I = 0.10$; (I) 0.3M NaCl, $I = 0.30$; (J) 0.1 M $MgCl_2$, $I = 0.30$; (K) 0.1 M $BaCl_2$, $I = 0.30$; (L) 0.1 M $CaCl_2$, $I = 0.30$. HPLA activities were measured as described in Methods. Units of HPLA activity were μmoles/(min mg). I is ionic strength.

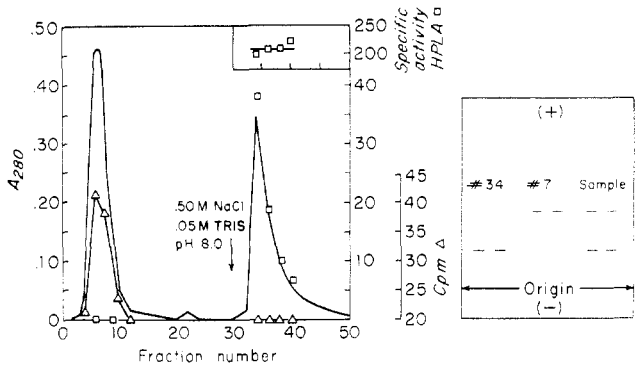

FIGURE 7: Gel filtration of DIP-PCP A S_5 on Sephadex G-75 in 1 M $CaCl_2$–0.05 M imidazole (pH 6.5). (A) DIP-PCP A S_5 (5 ml of 0.85 mg/ml) was applied to an equilibrated column of Sephadex G-75 (1.5 × 85 cm) and eluted with the above buffer at 4° at a flow rate of 0.16 ml/min. Activity toward HPLA was measured after activation with trypsin at room temperature for 1 hr. The curve was analyzed with a DuPont 310 curve resolver. (B) The major peak, pool A, was concentrated to 5 ml by Diaflo filtration and fractionated as described above. Microzone electrophoresis was performed on the original sample, on concentrated pools A and B, and on carboxypeptidase A (see Methods). Units of specific activity toward HPLA were μmoles/(min A_{280}).

enabled the assignment of the amino-terminal sequence of subunit I by difference.

Salt Effects on the Activation Rate. The effects of various anions of the Hofmeister or lyotropic series (Nagy and Jencks, 1965) on activation rates are shown in Figure 6. The Hofmeister series ($SCN > I > ClO_4 > Cl > PO_4 > SO_4$) showed no relationship to the observed order ($PO_4 > Cl > I > ClO_4 > SCN > SO_4$) in enhancing activation rates. Among the cations, those of group IIa of the Periodic Table were most effective. Calcium had the greatest effect and enhanced activation rates at all concentrations tested although above 1 M the activity subsequently declined. Hence all activation experiments were conducted in buffers containing 1 M $CaCl_2$.

Effects of $CaCl_2$ on the Quaternary Structure of PCP A S_5. In order to evaluate the mechanism of the calcium effect on the enhancement of the activation rate, the quaternary structure of the zymogen was examined by sedimentation velocity and gel filtration in the presence and absence of calcium ions. The sedimentation constants of PCP A S_5 and DIP–PCP A S_5 in buffers containing 0.10 M LiCl–0.05 M imidazole (pH 6.5) and 1 M $CaCl_2$–0.05 M imidazole (pH 6.5), respectively, are given in Table III. Within the experimental error the $s_{20,w}$ values of the zymogen and its DIP derivative extrapolated to zero protein concentration are 5.2 S in 0.10 M LiCl and 4.2 S in 1 M $CaCl_2$. Since $s^0_{20,w}$ of succinylated subunit I is 4.06 S (Freisheim *et al.*, 1967b) and that of succinylated subunit II is 2.57 S (Behnke *et al.*, 1970), complete subunit dissociation of PCP A S_5 would result in a weight-average sedimentation constant of 3.51 S. The calcium effect was reversible and solutions which gave the lower sedimentation constants in $CaCl_2$ returned to the higher values when dialyzed against 0.10 M LiCl. It was not apparent whether the reduced sedimentation constants in 1 M $CaCl_2$ were due to subunit dissociation, solvent binding, or changes in protein shape.

To evaluate further the nature of the calcium effect, DIP–PCP A S_5 and PCP A S_5 were subjected to gel filtration on Sephadex G-75 in 1 M $CaCl_2$. Two components could be observed (Figure 7). The major component represents 78% of the sample and displayed activity toward HPLA after activation. The minor component represents 22% of the sample and was devoid of potential activity. When the fractions containing the major component were pooled (pool A), concentrated by Diaflo ultrafiltration, and refractionated on the same column, the elution pattern shown in the lower diagram of Figure 7 was obtained. Pool A contained only 6% of the minor component as judged by the location of the pooled fractions. Consequently, the minor component (19%) observed in Figure 7 must have arisen by further subunit dissociation. Microzone electrophoresis, shown in Figure 7, was also consistent with subunit dissociation. The original sample migrated as a single component, characteristic of the DIP–PCP A S_5 dimer. Pool A contained two components, the major

TABLE II: Amino-Terminal Sequence of Subunits I and II of PCP A S_5.

Step	Subunit I[a]	Subunit II[a]	Trace
1	Lys	Cys	Thr
2	Glu	Gly	
3	Asp	Ala	Glu
4	Phe	Pro	Asp
5	Val	Ile	Phe, Pro
6	Gly	Phe	Val

[a] Sequence assignment by difference from subunit II (Peanasky *et al.*, 1969).

TABLE III: Salt Effect on Sedimentation Coefficient of PCP A S_5 and DIP–PCP A S_5.[a]

Zymogen	0.10 M LiCl	1 M $CaCl_2$
PCP A S_5	5.24 ± 0.05 S	4.35 ± 0.15 S
DIP–PCP A S_5	5.10 ± 0.10 S	4.18 ± 0.04 S

[a] Buffer: 0.05 M imidazole (pH 6.5).

TABLE IV: Specific Activities Generated from DIP-PCP A S_5 by Various Proteases in the Presence and Absence of 1 M $CaCl_2$.[a]

Activating Enzyme	Sp Act. (μmoles/(min mg))	
	In 0.1 M LiCl	In 1.0 M $CaCl_2$
Trypsin	11.5	87.7
α-Chymotrypsin	10.1	78.5
Subtilisin	20.1	61.0
Elastase	7.2	20.6

[a] DIP-PCP A S_5 (1.39 mg/ml) was activated for 300 min in 0.05 M imidazole (pH 6.5), containing 0.1 M LiCl or 1.0 M $CaCl_2$ by the enzymes (0.23 ± 0.1 mg/ml) listed in the first column. In the case of elastase, the zymogen concentration was 1.09 mg/ml.

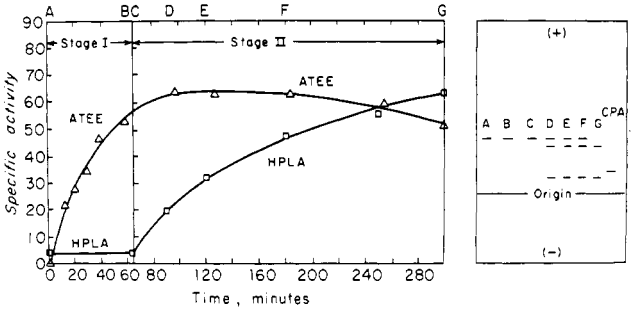

FIGURE 8: Stages of activation of PCP A S_5 by trace amounts of trypsin. Stage I: PCP A S_5 (2.56 mg/ml) was incubated with trypsin (1.8 μg/ml) in 0.10 M LiCl–0.05 M imidazole (pH 6.5) at 0°. Stage II: An equal volume of 2.0 M $CaCl_2$–0.10 M LiCl–0.05 M imidazole (pH 6.5) was added and incubation continued at room temperature. Aliquots (50 μl) were removed at times designated as A, B, C, D, E, F, and G; the activation was stopped by the addition of 10 μl of 10 mM DFP and the samples were subjected to Microzone electrophoresis. A sample of carboxypeptidase A (Anson) was used as a position marker. Activities toward HPLA and ATEE were measured as described in Methods. Units of specific HPLA and ATEE activity are μmoles/(min mg).

component being the dimer, and the smaller component subunit I. Pool B contained besides subunit II a small amount of the dimer. When PCP A S_5 was fractionated on Sephadex G-75 in 1 M $CaCl_2$, a smaller amount of subunit dissociation occurred, *i.e.*, only 15% of subunit II as compared to 22% for DIP-PCP A S_5. The identification of the minor component as subunit II was confirmed by the increased specific activity toward ATEE and loss in specific activity toward HPLA when compared to the major component.

Theoretical considerations of the protein elution patterns observed in Figure 7 enabled the calculations of the dissociation constant of 12.1×10^{-6} mole/l. for DIP-PCP A S_5 and 4.5×10^{-6} mole/l. for PCP A S_5. Knowing the percent removal of subunit II previous to rechromatography of pool A and applying the calculated dissociation constant for DIP-PCP A S_5, a percentage of subunit II of 18% should be observed in Figure 7. Actually 19% was observed. When subunit interconversions are of a sufficiently slow nature, correspondence between sedimentation and fractionation experiments can be realized. When the weight-average sedimentation constants of DIP-PCP A S_5 and of PCP A S_5 were calculated, based on their dissociation constants and on individual subunit sedimentation constants, values of 4.19 and 4.32 S were obtained. These may be compared to values of 4.18 and 4.32 S given in Table III.

Stages of the Activation Process. The activation of PCP A S_5 could be separated into a two-stage process. Stage I proceeded with rapid activation of subunit II (chymotrypsin activity) while subunit I (carboxypeptidase A precursor) remained unaffected. The conditions for stage I activation were 1000:1 weight ratio of PCP A to trypsin at 0° in 0.10 M LiCl–0.05 M imidazole (pH 6.5) (see Figure 8). Figure 8 also shows Microzone electrophoresis patterns of the activation mixture after the activation was stopped by the addition of DFP. At the end of stage I, DIP-PCP A S_5 showed the same electrophoretic mobility as the native zymogen. This was also confirmed by polyacrylamide disc gel electrophoresis and by gel electrophoresis in the presence of sodium dodecyl sulfate. Both electrophoretic techniques showed DIP-PCP A S_5 to be indistinguishable from native PCP A S_5. This unchanged mobility indicates that the proteolytic activation did not significantly alter the size or charge of subunit II. Since the sodium dodecyl sulfate gel procedure would separate the individual chains of α- or γ-chymotrypsin, it can be concluded that the activation of subunit II was analogous to the formation of π-chymotrypsin from chymotrypsinogen A. The lower esterase activity at the end of the activation as compared with the values reported by Brown *et al.* (1963) may be due to an impairment of chymotryptic sites due to the lyophilization procedure, discussed above.

Stage II of the activation process involved warming the sample to room temperature in the presence of 1 M $CaCl_2$. This procedure caused activation of subunit I with a small loss in chymotryptic activity (Figure 8). The rate of this phase of activation increased with increasing zymogen concentration, suggesting that activation was mediated by active subunit II. Microzone electrophoresis at different times during the activation process demonstrated that the material with the mobility of DIP-PCP A S_5 decreased with increasing concentration of other components; one of these has a mobility somewhat slower than DIP-PCP A S_5 whereas the mobility of the other component was considerably slower and was similar to that of carboxypeptidase A (Anson). No other intermediates could be detected in the process.

Proteolytic Specificity of the Activation of DIP-PCP A S_5 in 1 M $CaCl_2$. To eliminate activation of subunit I by active subunit II, PCP A S_5 was activated to stage I and inactivated with DFP. This inhibited zymogen was then activated by the addition of various proteolytic enzymes in buffers containing 0.1 M LiCl or 1.0 M $CaCl_2$. Table IV shows that none of the enzymes tested could effectively activate the DIP-zymogen in the absence of calcium. This observation is similar to the findings of Keller *et al.* (1958) that the tryptic activation of DIP-PCP A would not occur in the presence of phosphate buffer. In the presence of 1 M $CaCl_2$ all of the enzymes investigated activated the DIP-zymogen. Trypsin was the most effective activating enzyme and elastase the least. Yet even the least effective enzyme was more effective in the presence of calcium than trypsin in the absence of calcium. This broad specificity of activation was similar to the specificity of activation of dogfish PCP A (Lacko and Neurath, 1970) and succinylated bovine subunit I (Freisheim *et al.*, 1967a).

Isolation of the Products of Activation. At the end of stage II (Figure 8), the activated material was inactivated with [^{14}C]DFP and dialyzed against two changes of 2 l. of 0.5 M

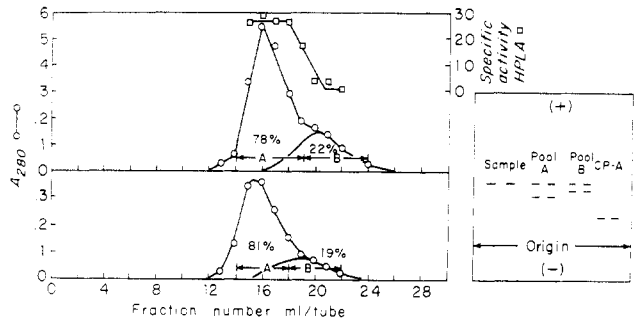

FIGURE 9: Chromatography of the products of activation on *N*-(2-ethylcellulose)glycyl-D-phenylalanine. PCP A S_5 was activated as described in Figure 8. The activation was stopped by the addition of [^{14}C]DFP at the end of stage II. The [^{14}C]DIP–activation mixture was chromatographed on *N*-(2-ethylcellulose)glycyl-D-phenylalanine (1 × 15 cm) under the conditions described in the text. Fractions of 2 ml/tube were collected. Measurements are described in Methods. Aliquots of the original sample and fractions 7 and 34 were subjected to Microzone electrophoresis. Units of HPLA activity are μmoles/(min ml) and those of specific HPLA activity are μmoles/(min mg).

NaCl–0.01 M 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5). After dialysis and centrifugation the specific esterase activity (HPLA) was 102 μmoles/(min mg), which is considerably higher than the value of 64 μmoles/(min mg) at the end of stage II prior to dialysis. The difference was due to the precipitation of inactive protein by the calcium. The maximum level of activation of PCP A S_5, calculated from the turnover rate of HPLA by crystalline carboxypeptidase A (Bargetzi *et al.*, 1963), is 114 μmoles/(min mg). Consequently the specific activity of 102 μmoles/mg of the soluble activation mixture represents 90% of the theoretical value.

These soluble proteins were chromatographed on the affinity adsorbent *N*-(2-ethylcellulose)glycyl-D-phenylalanine (Uren, 1971) which had been equilibrated with 0.50 M NaCl–0.05 M 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5). After six breakthrough volumes of the above buffer had passed through the column, the protein was eluted with 0.50 M NaCl–0.05 M Tris (pH 8.0) (Figure 9). All of the ^{14}C-labeled material eluted in the first peak and all of the fractions active toward HPLA eluted in the second peak. The specific esterase activity of the second peak fraction was 210 μmoles/(min mg), which agrees well with the value of 208 reported by Bargetzi *et al.* (1963) for crystalline carboxypeptidase A. Each peak represented approximately 50% of the total absorbance (91% of the absorbancy at 280 nm was recovered). Microzone electrophoresis of the initial solution and of each of the two peak fractions demonstrated that neither was contaminated by the other (Figure 9).

TABLE V: Amino-Terminal Sequences of Various Serine Proteases.

Step	Bovine Subunit II	Bovine Chymotrypsin B[a]	Bovine Chymotrypsin A[a]	Porcine Elastase[a]	Bovine Trypsin[a]
1	Val	Ile	Ile	*Val*	Ile
2	Val	*Val*	*Val*	*Val*	*Val*
3	Gly	Asn	Asn	*Gly*	*Gly*
4	Gly	*Gly*	*Gly*	*Gly*	*Gly*
5	Glu	*Glu*	*Glu*	Thr	Tyr
6	Asp	*Asp*	Glu	Glu	Thr
7	Ala	*Ala*	*Ala*	*Ala*	Cys
8	Ile	Val	Val	Gln	Gly
9	Pro	*Pro*	*Pro*	Arg	Ala
10		Gly	Gly	Asn	Asn
11		Ser	Ser	Ser	Thr
12	Trp	*Trp*	*Trp*	*Trp*	Val
13	Pro	*Pro*	*Pro*	*Pro*	*Pro*
14	Trp	*Trp*	*Trp*	Ser	Tyr
Total identities		9	8	7	4

[a] Sequences taken from Dayhoff (1969).

TABLE VI: Forms of Carboxypeptidase A Generated from PCP A S_5 Activated in 1 M $CaCl_2$.

Zymogen	Activating Enzyme	Carboxypeptidase (%) A_α[a]	A_β[a]	A_γ[a]
PCP A S_5	Trace trypsin[b] Subunit II	97	3	0
DIP–PCP A S_5	Trypsin[c]	61	39	0
DIP–PCP A S_5	α-Chymotrypsin A[c]	96	4	0

[a] The amino-terminal sequence of subunit I and the bonds cleaved during activation may be represented as follows (Sampath Kumar *et al.*, 1964):

NH_2Lys---X-Ala-Arg-Ser-Thr-Asn-Thr-Phe-Asn---
↑ α ↑ β ↑ γ

[b] Conditions of activation are given in the legend to Figure 8 (stage II, G). [c] Conditions of activation: zymogen:enzyme weight ratio 4:1, 0.05 M imidazole buffer (pH 6.5), containing 1 M $CaCl_2$. Activation for 100 min at room temperature.

Amino-Terminal Sequence of the Activation Products. Each of the isolated components shown in Figure 9 was concentrated by Diaflo ultrafiltration and exhaustively dialyzed for two days against a constant flow of 10 l. of 5% acetic acid. The dialyzed material was lyophilized and 10 mg of each component was subjected to sequence analysis in a Beckman Sequencer (see Methods).

The amino-terminal sequence of the [^{14}C]DFP-reacting component is shown in the first column of Table V. The A chain of this material contained only 13 amino acids (Peanasky *et al.*, 1969). The peptide was too small to stay in the sequencer cup and hence only the amino-terminal sequence of the B chain was observed. The homology to other serine proteases is evident and suggests the following order of decreasing homology to subunit II: chymotrypsin B > chymotrypsin A > elastase > trypsin (Table V).

Carboxypeptidase A isolated from the activation mixture by affinity chromatography (Figure 9) was subjected to amino-terminal sequence analysis. The results are shown in Table VI and demonstrate that regardless of the activating enzyme, carboxypeptidase A_α was the predominant form generated (even when activation was mediated by α-chymotrypsin). The formation of relatively large amounts of carboxypeptidase A_β under the influence of trypsin is consistent with the tryptic cleavage of an Arg_3–Ser_4 bond (Sampath Kumar

et al., 1964) but the simultaneous formation of the α form (61%) is unexpected since the same peptide bond (X–Ala) usually is not hydrolyzed by both trypsin and chymotrypsin.

Discussion

A reevaluation of the chemical and physical properties of bovine PCP A S_5 has confirmed most of the data previously reported by Brown *et al.* (1963). With the exception of methionine, phenylalanine, and tryptophan, the amino acid composition determined in this investigation agrees with the data previously reported. The amino-terminal residues lysine (subunit I) and half-cystine (subunit II) were confirmed and the amino-terminal sequence of subunit I was extended by an additional five residues. The amino-terminal sequence of bovine subunit II agrees with the data previously reported by Peanasky *et al.* (1969); the high degree of homology with bovine chymotrypsins A and B and with other serine proteases is consistent with the characterization of subunit II as the zymogen of a serine protease having the specificity of chymotrypsin. The dimeric nature of this form of the zymogen was confirmed by gel electrophoresis in the presence of sodium dodecyl sulfate. The higher value for $s^0_{20,w}$ of 5.24 ± 0.05 S as compared to 5.0 S determined by Brown *et al.* (1963) may be the result of greater self-association in the solvent used in the present experiments.

The molecular weight of 63,000 for PCP A S_5 is in agreement with the literature value derived from the sum of the molecular weights of 40,000 for subunit I (Freisheim *et al.*, 1967b) and of 23,000 for subunit II (Teller, 1970). The present molecular weight was determined by self-association analysis of high-speed equilibrium data and is also in agreement with the behavior of the protein on gel electrophoresis in the presence of sodium dodecyl sulfate and with the value derived from the amino acid composition. On the basis of this molecular weight and an absorbancy index of 1.77, 0.93 active site/molecule was calculated when [^{14}C]DFP was allowed to react with activated PCP A S_5 which had not been exposed to lyophilization. This value is considerably higher than 0.55 previously reported by Behnke *et al.* (1970) for preparations of PCP A S_6 which had been stored as a lyophilized powder.

One of the most significant findings of the present investigation is the effect of calcium on the rate and maximum level of activation. This observation is consistent with the presence of calcium in preparations of the bovine zymogen (Piras and Vallee, 1967) and with the enhancing effects of calcium ions on the activation of dogfish (Lacko and Neurath, 1970) and lungfish (Reeck and Neurath, 1972) procarboxypeptidases. The present gel filtration experiments indicate that calcium affects the quaternary structure of PCP A S_5. While no direct data are available to implicate the site of interaction, it is likely that calcium interacts specifically with carboxyl groups in the region of subunit binding. This conclusion is based on the fact that in high concentrations, salts generally exert a disruptive effect on the quaternary structure of proteins by changing the activity coefficient of groups involved in subunit attraction. The structural components which most likely produce a change in activity are ionic and hydrophobic residues (Jencks, 1969). In the case of hydrophobic interactions, the order of effectiveness of the salts usually parallels the Hofmeister (or lyotropic) series. This is not the case if the rate of activation is considered a measure of subunit dissociation. Alternatively, ionic interactions are the most likely attraction forces involved. The calcium-induced disaggregation of hemoglobin has led Kawahara *et al.* (1965) to a similar

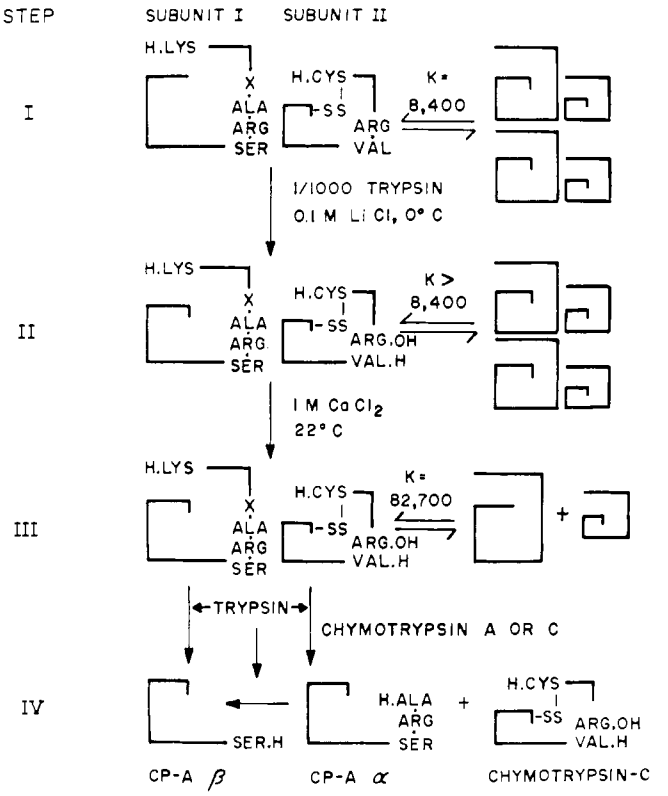

FIGURE 10: Schematic representation of the steps of activation of bovine PCP A S_5. For details, see the text.

conclusion about the role of ionic forces in the structure of hemoglobin. The large preponderance of acidic residues in the activation peptide of subunit I (Freisheim *et al.*, 1967a) and the loss of ability to reassociate when this peptide is removed (Behnke *et al.*, 1970) make this region a highly probable site of subunit association. Consequently, factors which cause subunit dissociation would also cause an enhancement of the activation rate. The rapid activation of monomeric succinylated subunit I (Freisheim *et al.*, 1967a) and of other monomeric procarboxypeptidases is consistent with this proposal.

Consideration of the changes in covalent and quaternary structure which accompany the activation of PCP A S_5 leads to a plausible scheme of the activation of the zymogen, as shown in Figure 10. Intact subunits I and II have a high tendency for association leading to preponderant dimerization with some tetramerization in the absence of calcium ions (step I). The only two amino-terminal residues are lysine (subunit I) and half-cystine (subunit II). As described by Keller *et al.* (1958), trypsin in low concentrations activates subunit II. This process causes cleavage of an Arg–Val bond and converts subunit II into an enzyme (step II) which, like π-chymotrypsin, contains a carboxyl-terminal arginine residue (Peanasky *et al.*, 1969) and migrates as a single band in sodium dodecyl sulfate gel electrophoresis. The addition of calcium ions (1 M) at this stage (step III) enhances subunit dissociation but no changes in covalent structure occur. Incubation of the activation mixture as such or with added trypsin or chymotrypsin A generates carboxypeptidase A from subunit I (step IV). When this activation step is mediated by active subunit II or by α-chymotrypsin, the major product is carboxypeptidase A_α (96–97%) with the β form as a minor component. In the presence of added trypsin, how-

ever, both α and β are formed in a ratio of approximately 3:2 (step IV). It is not certain whether the appearance of the β form is the result of further tryptic attack on carboxypeptidase A_α as described by Sampath Kumar *et al.* (1964) or whether a separate pathway exists from step III to the β form.

These results are difficult to relate to the specificity of the activating enzymes trypsin or chymotrypsin and raise the question of the nature of the amino acid residue preceding the amino-terminal alanine of carboxypeptidase A_α. Since trypsin is six times more efficient than chymotrypsin in the generation of carboxypeptidase activity, it is unlikely that activation was mediated by the intrinsic chymotryptic activity of bovine trypsin. It is also worthy of note that activation of partially purified zymogen always yielded the α or β form of carboxypeptidase but never the γ form. This is also true of the preparations of carboxypeptidase A (Cox) (Pétra and Neurath, 1969b), of carboxypeptidase A (Allan) (Pétra *et al.*, 1971), and of the enzyme prepared from spontaneously activated pancreatic juice (Reeck *et al.*, 1971). Carboxypeptidase A_γ can only be obtained from spontaneously autolyzed pancreas glands (Anson, 1937; Pétra and Neurath, 1969a), suggesting that possibly this form of carboxypeptidase is generated by a specific pancreatic protease which corresponds neither to trypsin nor to chymotrypsin. Taken together, the present data suggest that the activation of PCP A S_5, like that of other zymogens, requires the cleavage of a unique peptide bond (X–Ala) and that the other forms of the enzyme are derivatives of this initial product.

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