Studies on Procarboxypeptidase A and Carboxypeptidase A of the Spiny Pacific Dogfish (*Squalus acanthias*)*

A. G. Lacko† and Hans Neurath‡

ABSTRACT: A procedure is described for the purification of procarboxypeptidase A from acetone powder prepared from extracts of pancreas glands of the spiny Pacific dogfish. The apparently homogeneous zymogen is a monomer in contrast to the procarboxypeptidase A complex isolated from the bovine pancreas. Physical and chemical properties of dogfish procarboxypeptidase A were found to be similar to those of succinyl fraction I, the chemically modified, monomeric precursor of bovine carboxypeptidase A. Significant differences, however, exist in the number of sulfur-containing residues in each zymogen. Dogfish procarboxypeptidase A was rapidly activated by bovine trypsin or Nagarse in the presence of Ca^{2+} ions (up to 0.5 M). Activation by chymotrypsin resulted in the generation of only about 18% of the total potential activity of the zymogen. Calcium ions increased neither the efficiency nor the extent of activation by chymotrypsin. Dogfish carboxypeptidase A was isolated following the activation of purified procarboxypeptidase A with bovine trypsin.

The enzyme appeared to be homogeneous in the ultracentrifuge and resembled its bovine counterpart in physical, chemical, and catalytic properties. Dogfish procarboxypeptidase A was also found to have catalytic activity toward ester and peptide substrates. Comparison of the catalytic properties of the enzyme and the zymogen reveals that the product of the activation process probably has a more efficient binding site rather than a more effective catalytic site.

The difficulties accompanying the isolation of a monomeric precursor of carboxypeptidase A have long precluded the study of the properties and activation of this zymogen. Although indications of the existence of a monomeric procarboxypeptidase A have been reported (Yamasaki *et al.*, 1963), no effort has proved successful in isolating it without chemical modification.

The first study on the activation of monomeric procarboxypeptidase was reported by Wintersberger *et al.* (1962) who isolated procarboxypeptidase B from bovine pancreatic extracts. The activation of this zymogen was mediated by trypsin and was accompanied by the elimination of about one-fourth of the molecule. Similarly, Prahl and Neurath (1966) found that a fragment with a molecular weight of about 7000 was split off during the activation of dogfish procarboxypeptidase B by trypsin.

Folk and Schirmer (1965), during their studies on the porcine pancreatic system, found indications of the presence of a monomeric procarboxypeptidase A, while Freisheim *et al.* (1967a,b) reported the isolation of a monomeric zymogen precursor of bovine carboxypeptidase A following the succinylation of the trimeric zymogen. This chemically modified zymogen, succinyl fraction I, proved very useful in characterizing partially the immediate precursor of bovine carboxypeptidase A, but fell somewhat short of providing the ideal model for the study of the activation of procarboxypeptidases. In the present communication, we report the purification and characterization of such a monomeric zymogen from dogfish pancreatic extract and its conversion into the corresponding enzyme.

Experimental Procedure

Materials. *N*-Acetyl-L-tyrosine ethyl ester (ATEE),[1] benzoyl-L-arginine ethyl ester (BAEE), carbobenzoxyglycyl-L-phenylalanine (CGP), and hippuryl-β-DL- and -L-phenyllactic acid (HPLA) were purchased from Cyclo Chemical Corp., Los Angeles, Calif.

Acetylglycyl-L-phenyllactic acid was synthesized by P. Grogg. *p*-Mercuribenzoic acid was the product of Sigma Chemical Corp. and was used without recrystallization. *N*-Acetylimidazole was purchased from K & K Laboratories, Inc., and was recrystallized from isopropenyl acetate before use. The reagent was stored over phosphorus pentoxide and recrystallized at the first sign of moisture.

Diisopropyl phosphorofluoridate (DFP) was obtained from the Merck Chemical Co. and was diluted to 1 M with anhydrous 2-propanol. All chemicals used in this study were of reagent grade and were used without further purification. An exception was urea, which was recrystallized and freshly dissolved before use.

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† Taken in part from a dissertation submitted by A. G. Lacko to the Graduate School of the University of Washington in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address: Albert Einstein Medical Center, Department of Biochemistry, Philadelphia, Pa. 19141.

‡ To whom correspondence should be addressed.

[1] The following abbreviations are used: ATEE, acetyl-L-tyrosine ethyl ester; BAEE, benzoyl-L-arginine ethyl ester; HPLA, hippuryl-DL-β-phenyllactic acid; AGLPA, *N*-acetylglycyl-L-phenyllactic acid; CGP, carbobenzoxyglycyl-L-phenylalanine; pMB, *p*-mercuribenzoic acid.

Bovine carboxypeptidase A, chymotrypsin, and twice-crystallized trypsin (salt-free) were obtained from Worthington Biochemical Corp., Freehold, N. J. Nagarse was purchased from Enzyme Development Corp., New York, N. Y.

Methods. Esterase activity of the various enzymes was determined by automatic titration with approximately 0.1 N standardized sodium hydroxide using the TTT1 Radiometer autotitrator equipped with an Ole Dich (Copenhagen) recorder at 25°. For routine assays a 3-ml aliquot of substrate was used to which 5–10 μl of enzyme was added after equilibration with regard to temperature and pH. BAEE (0.01 M in 0.01 M Tris–0.05 M $CaCl_2$–0.1 M KCl, pH 8.0) was employed as substrate to measure chymotryptic activity. The sodium salt of DL-HPLA (0.01 M in 0.005 M sodium Veronal–0.05 M NaCl, pH 7.5) was used for carboxypeptidase assays. Esterase units are expressed as micromoles of base taken up per minute per milliliter of enzyme solution added to 3 ml of substrate.

Exopeptidase activity was determined by measuring the peptide bond cleavage in CGP (10^{-3} M in 0.005 M Tris–0.1 M NaCl, pH 7.5) as described by Whitaker *et al.* (1966). The decrease in absorbance was followed at 223 nm using the Perkin-Elmer 350 recording spectrophotometer. Peptidase activity is expressed as A_{223} per minute per milligram of enzyme. Alternately, the Gilford 2000 recording spectrophotometer was used between the wavelengths 219 and 233 nm. In these instances, absorption data were converted into rate data by the use of respective extinction coefficients and expressed as micromoles of CGP split per minute.

DEAE-cellulose (~0.9 mequiv/g) was supplied by Carl Schleicher and Schuell Co. Chromatography on DEAE-cellulose was carried out according to the method of Peterson and Sober (1956) using a linear gradient. The exchanger was pretreated and repeatedly regenerated by intermittent acid and base treatment followed by equilibration with the proper buffer.

Gel filtration was carried out in columns of Sephadex G-50 or G-100 (obtained from Pharmacia, Uppsala, Sweden) with minimal or no mixing volume at the bottom. The gel filtration media were packed and equilibrated according to instructions supplied by the manufacturer.

Sedimentation Analysis. Sedimentation analyses were performed in a Spinco Model E analytical ultracentrifuge equipped with phase-plate schlieren optics, Rayleigh fringe optics, and an electronic absorption scanning device. Sedimentation velocity runs were performed in a single or double sector cell at 59,780 rpm at 4–6°. Schlieren patterns on the photographic plates were analyzed by the aid of a microcomparator, establishing the radial boundary position at each time interval. The Svedberg constant was calculated from the least-square plot of the $\log_{10}$ of the radial boundary positions *vs.* time. Buffer density was established by pycnometry, while viscosity of the buffers was determined in a viscometer similar to that described by Frensdorff *et al.* (1953) at 20 ± 0.1°.

Molecular Weight Determinations. For the molecular weight determination, the method of sedimentation equilibrium as described by Richards and Schachman (1959) was used. The sample was layered in a double sector cell over FC43 (3 M Chemicals) at column heights of 2.0–2.5 mm. Attainment of equilibrium was ascertained when no further shift in fringes occurred.

The apparent weight-average molecular weight (M_w) was calculated from the change in concentration across the cell relative to the initial protein concentration using the equation of Lansing and Kramer (1935). The molecular weight was calculated from the slope of the plot ln C *vs.* x^2 (where C is the number of fringes and x is the distance from the axis of rotation).

Molecular weight data were obtained by submitting the observed information to a digital computer (IBM 7040/7094) with the aid of a suitable computer program.

Amino Acid Analysis. Samples for analyses were prepared by the method of Moore and Stein (1963). Amino acid analyses were carried out by the method of Spackman *et al.* (1958) with the Beckman amino acid analyzer, Model 120 (A, B, or C). A mixture of aminoguanidinopropionic acid and thienylalanine was added to samples as an internal standard to correct for variation between the volumes applied to the long and short columns as suggested by Walsh and Brown (1962). The proteins were hydrolyzed for several time periods and the values obtained for serine and threonine were extrapolated to zero time. Valine and isoleucine were adjusted to their maximal values. For the determination of sulfur-containing amino acids, the protein was oxidized with performic acid (Hirs, 1956) prior to hydrolysis. To confirm the half-cystine values, the protein was reduced with mercaptoethanol and subsequently carboxymethylated with iodoacetic acid (Walsh *et al.*, 1962). Tryptophan was determined according to the method of Bencze and Schmid (1957).

Preparation of Pancreatic Acetone Powder. Pancreatic tissue was collected from fish caught by commercial fishermen in the Puget Sound area. After dissection, the glands were frozen as quickly as possible by placing them in Dry Ice.

The frozen tissue was stored at −20° or processed after thawing for 4–5 hr at 4°. Following the removal of blood vessels, the tissue was rinsed with cold acetone and homogenized in a Waring blender for about 30 sec. The homogenized tissue was extracted twice while stirring with acetone for 6 hr in the cold room. Finally the powder was stirred at room temperature (in the fume hood) with a mixture of 50% acetone and 50% ether for 1 hr followed by treatment with pure ether for the same duration. The final product was obtained by vacuum desiccation of the powder at room temperature (Prahl, 1964).

Protein Determinations. Protein concentration of crude preparations was estimated according to Warburg (1957). When the 280:260 nm ratio was less than 1.5, correction for nucleic acid content was made using the Warburg–Christian factors, assuming that the corrected absorbance of 2.0 at 280 nm represented 1 mg/ml of protein. For preparations with purity exceeding 80% procarboxypeptidase A, the extinction coefficient was used to determine protein concentration.

Results

Isolation and Purification of Procarboxypeptidase A. To avoid autoactivation during the purification procedure, an effective tryptic inhibitor had to be found.

For the study of autoactivation, 1-g portions of dogfish pancreatic acetone powder were extracted with 10 ml of distilled water or with 10 ml of solution containing the appropriate tryptic inhibitor. After stirring for 4 hr at 4°, the extract was centrifuged in the cold to remove the particulate

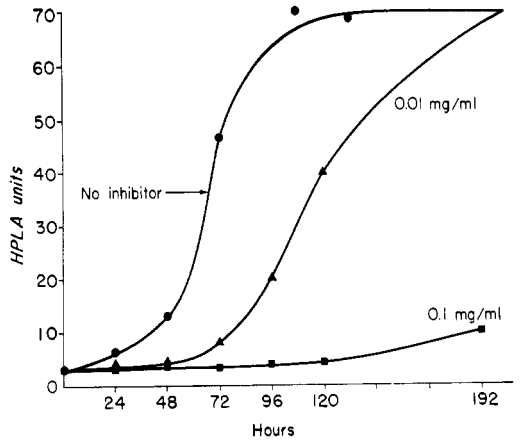

FIGURE 1: The effect of soybean trypsin inhibitor on the activation of procarboxypeptidases in dogfish pancreatic extract. See text for experimental details.

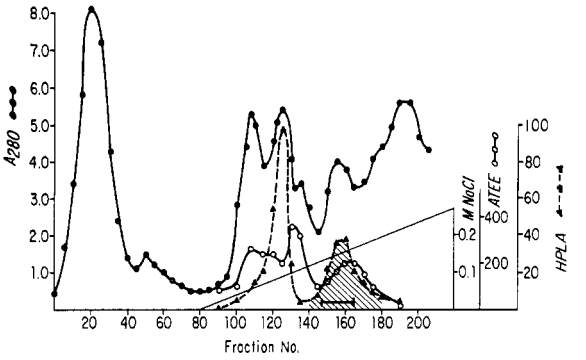

FIGURE 2: The elution profile of dogfish pancreatic proteins on DEAE-cellulose. See text for experimental details.

matter. The clear supernatant of the extract was then placed in the cold room for incubation at 4° and esterase activities were determined using HPLA, ATEE, and BAEE at various time intervals. Benzamidine·HCl, in concentrations of no less than 10^{-2} M, was found to suppress autoactivation up to 48–72 hr.

The inhibitory influence of soybean trypsin inhibitor proved to be more powerful. As is shown in Figure 1, activation of procarboxypeptidases was almost completely inhibited for 3 days in the presence of 0.1 mg/ml of soybean trypsin inhibitor. The inhibition of activation of trypsinogen and chymotrypsinogen was also nearly complete after 3 days. The usefulness of soybean trypsin inhibitor is enhanced by the fact that due to its negative charge at pH 7.0, it will be eluted from DEAE-cellulose considerably later than procarboxypeptidase A.

Preceding column chromatography, the acetone powder was extracted at 4° with distilled water containing soybean trypsin inhibitor, and after the removal of particulate matter by centrifugation (8000*g*), the supernatant was saturated with $(NH_4)_2SO_4$ to separate the proteins from nucleic acids. In a routine preparation, 100 g of acetone powder was extracted with 1 l. of distilled water containing 20 mg of soybean trypsin inhibitor. After 2 hr at 4°, the precipitate in the saturated ammonium sulfate solution was suspended in about 200 ml of 0.01 M sodium phosphate, pH 7.0, and dialyzed against two 6-l. portions of the same buffer containing 10^{-4} M DFP. At the conclusion of the dialysis (16 hr), the extract was pumped onto a DEAE-cellulose column (5 × 60 cm) which had previously been equilibrated with 0.01 M sodium phosphate, pH 7.0. At the conclusion of the chromatography, chymotryptic and carboxypeptidase activities were measured after tryptic activation of the appropriate fractions. From every selected fraction a 0.5-ml sample was activated with 5 μl of 20 mg/ml of bovine trypsin (100 μl) at room temperature. Before assaying for chymotryptic activity, the activation was allowed to proceed for 10 min, while a 1-hr incubation was used preceding the measurement of carboxypeptidase activity.

Figure 2 shows the chromatography of the crude extract on DEAE-cellulose, developed by a sodium chloride gradient. The fractions were collected at the rate of 180 ml/hr. The first peak of HPLA activity (fractions 100–138) represents procarboxypeptidase B, while the second peak (fractions 140–180) corresponds to procarboxypeptidase A. The higher activity of the first peak fraction is due to the more efficient tryptic activation of procarboxypeptidase B as compared with that of the A zymogen under the conditions employed.

Each fraction containing procarboxypeptidase A also contained considerable chymotryptic activity (ATEE), confirming the observations of Prahl and Neurath (1966). This chymotryptic activity, however, did not symmetrically coincide with the carboxypeptidase activity, suggesting that the two zymogens were separate proteins rather than forming an oligomeric complex.

Since these two proteins emerged so closely to each other during ion-exchange chromatography, gel filtration appeared to be the most effective method for their separation.[2] Assuming that the two components resembled their bovine counterparts in size, the corresponding molecular weights would be about 42,000 for procarboxypeptidase A and 25,000 for chymotrypsinogen.

After experimenting with several Sephadex and Bio-Gel filtration media of various porosity, Sephadex G-100 was found to yield the optimal results. The fractions indicated by the shaded area in Figure 2 were pooled, extensively dialyzed against distilled water, and lyophilized. Pooled material from two DEAE-cellulose columns was then combined and taken up in less than 20 ml of 0.1 M Tris, pH 8.0, containing 20 mg of lima bean trypsin inhibitor and 10^{-2} M DFP. (Lima bean inhibitor at this point was substituted for soybean trypsin inhibitor since the latter would interfere with the purification.) The viscous solution then was applied to a Sephadex G-100 column (4.5 × 115 cm) which had previously been equilibrated with 0.01 M sodium phosphate, pH 7.0–0.5 M NaCl.

Figure 3 illustrates the elution pattern of the first gel filtration on Sephadex G-100. The 5-ml fractions were collected at a rate of about 30 ml/hr. Void volume (V_0) of the column turned out to be about 500 ml and the fractions representing procarboxypeptidase A emerged about another 500 ml later. Apart from the separation of potential exopeptidase from the potential endopeptidase activity, this column proved

[2] Further studies by J. Uren showed that dogfish procarboxypeptidase A and two anionic chymotrypsinogens could be resolved on DEAE-cellulose (DE-52) using a linear salt gradient at pH 7.5 (Tris-HCl).

TABLE I: Recovery of Activity during the Purification of Dogfish Procarboxypeptidase A.

Source	A_{280}/A_{260}	Total Protein (mg)	Sp Act. (μmole of CGP/ min per mg of Protein)	Total Act. (μmole of CGP/min)	Recovery (%)
Crude extract	0.64	20,400	0.85	17,400	100
$(NH_4)_2SO_4$ precipitate	1.35	14,600	0.86	12,500	72
Pooled fraction from DEAE-cellulose chromatography	1.54	1,800	3.28	5,900	34
Pooled fraction from first G-100 filtration	1.50	374	7.2	2,700	15.5
Pooled fraction from second G-100 filtration	1.69	174	8.9	1,530	8.8

to be useful in eliminating the colored high molecular weight contaminants that appeared in the breakthrough fractions.

The fractions represented by the shaded area in Figure 3 were pooled, dialyzed, and lyophilized. The gel filtration procedure was then repeated with the results shown in Figure 4. The fractions representing procarboxypeptidase A were completely devoid of potential ATEE activity and were homogeneous by various physical criteria (*vide infra*).

A summary of the purification procedure is given in Table I, which indicates about a tenfold purification and a 10% recovery from the crude extract to the final product. These values are somewhat arbitrary since it would be difficult to assess the contribution of carboxypeptidase B to the total carboxypeptidase activity of the crude extract. In the present experiments, activation was carried out in the presence of 0.5 M $CaCl_2$ to obtain maximal activity (*vide infra*). After the second gel filtration, the purification of dogfish procarboxypeptidase A was considered complete since the zymogen appeared to be homogeneous by several criteria, as will be discussed below.

Sedimentation Analysis. Sedimentation analyses were carried out at pH 8.0 in 0.1 M Tris-HCl–0.01 M $CaCl_2$. At a protein concentration of 17 mg/ml, chosen so as to enhance detection of heterogeneity, the schlieren pattern produced after 175 min at 59,780 rpm was symmetrical, indicative of a high degree of homogeneity. The plot of the Svedberg constant against protein concentration appeared to be linear with a slightly positive slope, indicating a tendency of the protein to aggregate. Extrapolation of the linear part, calculated from least-squares analysis, to zero protein concentration, according to the equation $s_{20,w} = 3.86(1 + 0.0022c)$ where c is the protein concentration in mg/ml, yielded $s_{20,w} = 3.86 \pm 0.03$ S.

Molecular Weight Determination by Sedimentation Equilibrium. Results of the molecular weight determinations confirm the tendency of procarboxypeptidase A to aggregate since the M_b values progressively increase with the protein concentration (Table II). The plots of ln C *vs.* x^2 upon which the data in Table II are based varied from linear to a slight upward concavity. The upswing of the plot was mainly apparent with samples of higher concentration. While considerable differences between the M_m and M_b values are usually interpreted as indications of heterogeneity, this interpretation does not apply to these preparations since their purity was established by independent means (*vide infra*). On the basis of several analyses, an apparent molecular weight of 44,000± was tentatively adopted.

Electrophoresis. Moving boundary electrophoresis was carried out in the Spinco Model H electrophoresis apparatus

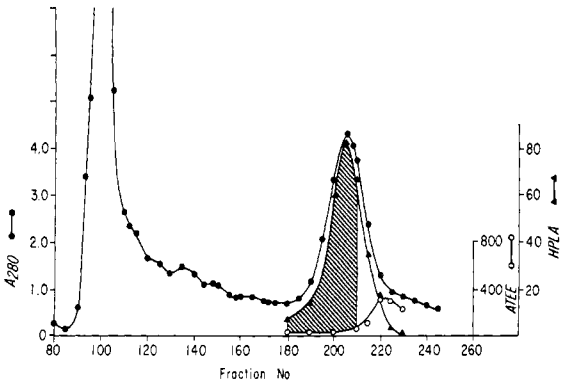

FIGURE 3: Gel filtration of dogfish procarboxypeptidase A on Sephadex G-100. See text for experimental details.

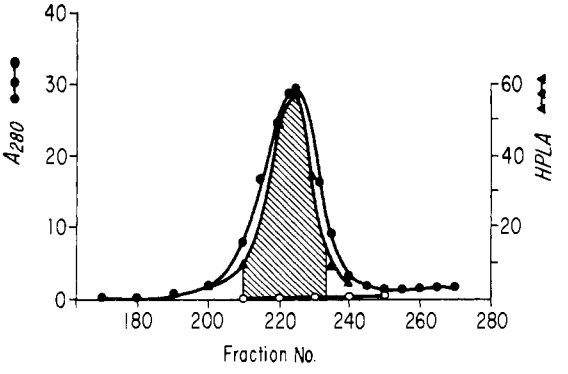

FIGURE 4: Second gel filtration of dogfish procarboxypeptidase A on Sephadex G-100. The open circles represent the activity against ATEE upon tryptic activation. See text for experimental details.

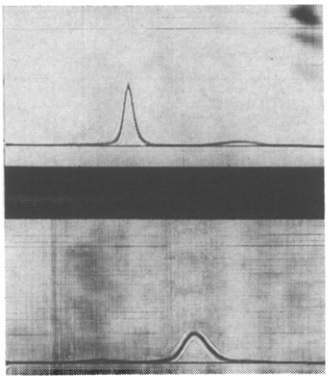

FIGURE 5: Free boundary electrophoresis of dogfish procarboxypeptidase A in 0.1 M Tris-HCl–0.01 M $CaCl_2$, pH 8.0. The pattern was obtained after 315 min in the Spinco Model H apparatus. The upper pattern represents the ascending limb, the lower one the descending limb.

at 1° in 0.1 M Tris–0.01 M $CaCl_2$, pH 8.0. A sample containing 9.5 mg/ml of protein appeared to be homogeneous after 315 min as shown in Figure 5. At pH 8.0 the protein was displaced toward the anode as could be expected from its behavior on DEAE-cellulose. Free boundary electrophoresis was performed at several different pH values to establish the dependence of the electrophoretic mobility of dogfish procarboxypeptidase A on pH. From these data (Figure 6), an isoelectric point of 5.0 was estimated.

To ascertain further the purity of the zymogen, disc gel electrophoresis was also carried out as described by Davis (1964). An electrophoretic pattern developed with the dye amido black is shown in Figure 7. The minor bands in this gel indicate that the last step of purification was necessary to remove these contaminating components.

Absorbancy Index. The absorbancy index was determined by relating A_{280} of several samples to their fringe reading obtained from the Rayleigh interference patterns in the analytical ultracentrifuge. After examining five samples ranging in concentration from 1 to 10 mg/ml, an absorbancy index of 16.5 was obtained for a 1% solution of dogfish procarboxypeptidase A in 0.1 M Tris–0.01 M $CaCl_2$, pH 8.0, at 280 nm.

TABLE II: Molecular Weight Data of Dogfish Procarboxypeptidase A Obtained by Sedimentation Equilibrium.[a]

Protein Concn (mg/ml)	Time (min)	Speed (rpm)	M_w[b]	M_m[c]	M_b[d]
2.17	1000	9013	44,003	39,186	45,852
3.26	1250	9031	42,735	41,927	46,339
5.74	992	9001	45,137	40,836	46,046
7.6	1121	6001	48,801	46,773	50,161
8.24	950	8018	48,524	40,863	67,366

[a] Determinations were carried out in 0.1 M Tris-HCl–0.01 M $CaCl_2$, pH 8.0. [b] M_w = weight-average molecular weight. [c] M_m = molecular weight at the meniscus. [d] M_b = molecular weight at the bottom of the cell.

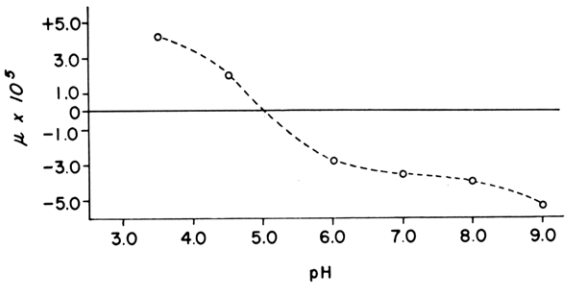

FIGURE 6: Dependence of the electrophoretic mobility of dogfish procarboxypeptidase A upon pH.

In these calculations, a refractive index increment of 0.00185 has been assumed (Perlmann and Longsworth, 1948).

Amino Acid Composition. Amino acid analyses were evaluated on the basis of the molecular weight of 44,000 obtained by sedimentation equilibrium measurements.

The results in Table III show that the dogfish and bovine zymogens are quite similar in amino acid composition, half-cystines and methionines being notable exceptions. The analyses were carried out using norleucine as an internal standard and since the initial protein concentration was established by spectrophotometry, the nearly quantitative recovery of amino acids confirmed the value of 16.5 for the absorbancy index ($A_{280}^{1\%}$).

Sulfur-Containing Amino Acids. Amino acid analysis preceded by performic acid oxidation (Hirs, 1956) revealed the presence of 4 half-cystines (as cysteic acid) and 14 methionines (as methionine sulfone). The number of half-cystines was confirmed by reduction with mercaptoethanol followed by alkylation with iodoacetic acid (Walsh *et al.*, 1962). In the absence of a reducing agent, no carboxymethylcysteine could be demonstrated, even when 10^{-3} M 1,10-phenanthroline and 8 M urea were included in the reaction mixture. Such observations suggest that dogfish procarboxypeptidase A contains four disulfide-linked half-cystines or one cystine and two unreactive cysteines. Since such a feature would have considerable evolutionary and structural significance, an independent method was required to confirm the presence of disulfides in the dogfish zymogen.

This method of choice appeared to be that developed by

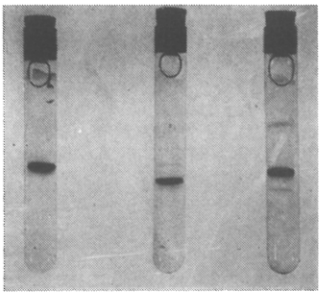

FIGURE 7: Disc gel electrophoresis of dogfish procarboxypeptidase A in 0.005 M Tris-HCl, pH 7.2: (a) dialyzed sample of pure procarboxypeptidase A before lyophilization; (b) same as a, but after lyophilization; (c) procarboxypeptidase A after first gel filtration (Figure 3).

TABLE III: Amino Acid Composition of Dogfish Procarboxypeptidase A and Bovine Procarboxypeptidase A (Fraction I).[a]

	Dogfish			Bovine
Amino Acid	Residues/ 44,000 g of Protein	Nearest Integer/ 44,000 g	Integral No. × Mol Wt of Residue	Nearest Integer/ 44,000 g
Lysine	15.5	16	2,051	17
Histidine	8.6	9	1,234	9
Arginine	15.7	16	2,499	14
Tryptophan	11.0	11	2,048	8
Aspartic acid	35.2	35	4,029	33
Threonine	25.6	26	2,629	25
Serine	31.0	31	2,700	31
Glutamic acid	48.8	49	6,326	40
Proline	16.9	17	1,651	15
Glycine	32.5	33	1,884	27
Alanine	21.3	21	1,493	24
Half-cystine	3.9	4	413	2
Valine	22.9	23	2,280	22
Methionine	13.6	14	1,837	4
Isoleucine	25.5	26	2,943	22
Leucine	23.6	24	2,717	30
Tyrosine	21.7	22	3,590	17
Phenylalanine	13.8	14	2,061	18
			44,385	

[a] Data taken from Freisheim *et al.* (1967a,b).

Boyer (1964) as modified by Wintersberger *et al.* (1965), involving the reduction of disulfides with sodium sulfite. Titration with pMB was carried out according to Coombs *et al.* (1964) and readings at 255 nm were taken on the Gilford 2000 spectrophotometer before and after the addition of Na_2SO_3 to measure the reaction between pMB and the free sulfhydryl group. The increase in absorbance took place over a long period of time. Figure 8 shows the time course

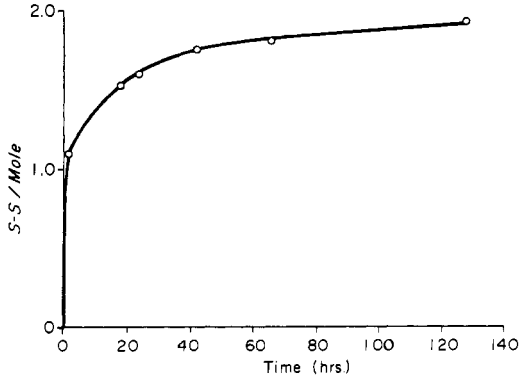

FIGURE 8: Reaction of procarboxypeptidase A with *p*-mercuribenzoate in the presence of 15-fold excess Na_2SO_3. The reaction was carried out in 1 M Tris-HCl–1 M perchlorate–8 M urea, pH 7.5. See text for experimental details.

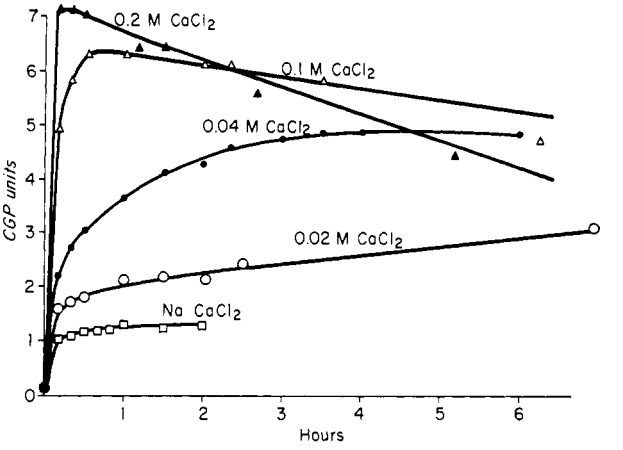

FIGURE 9: The effect of different concentrations of Ca^{2+} ions on the activation of dogfish procarboxypeptidase A. Activation was carried out with a high trypsin to zymogen ratio (1:10) at 25°, in a buffer solution of 0.1 M Tris-HCl, pH 8.0, containing the indicated concentrations of $CaCl_2$.

of the reaction described above. While one disulfide was split rather rapidly (1 hr), the second one required over 120 hr for complete reduction. In a control experiment, bovine carboxypeptidase A gave 0.9 disulfide/mole after about 24 hr and this value did not increase further with time. Since the bovine enzyme contains two half-cystines in disulfide linkage (Lipscomb *et al.*, 1968; Walsh *et al.*, 1970) which react slowly, dogfish procarboxypeptidase A, possessing two disulfides, has one normally reacting and one "slow reacting" disulfide.

Activation. EFFECT OF DIVALENT METAL IONS ON ACTIVATION. A number of difficulties were encountered during the initial attempts to activate dogfish procarboxypeptidase A. No activation occurred in 0.1 M Tris, pH 8.0, at 25° at a zymogen:trypsin weight ratio of 100:1. Even at a 10:1 ratio, only about 18% of the total potential carboxypeptidase was generated after 12 hr. The addition of calcium ions greatly enhanced the rate and extent of activation. Figures 9 and 10

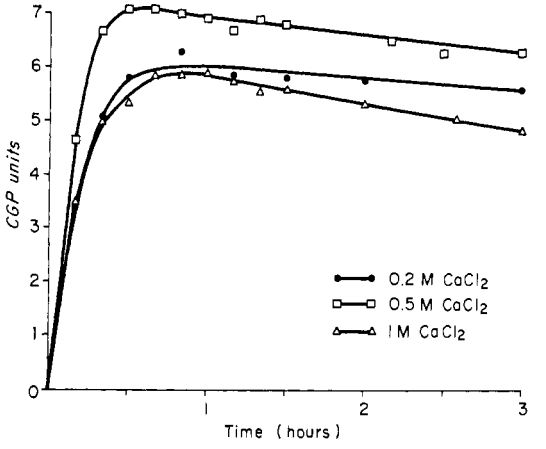

FIGURE 10: Activation of dogfish procarboxypeptidase A at low (1:100) trypsin to zymogen ratio. Activation was carried out at 25° in 0.1 M Tris-HCl, pH 8.0, containing the indicated concentration of $CaCl_2$.

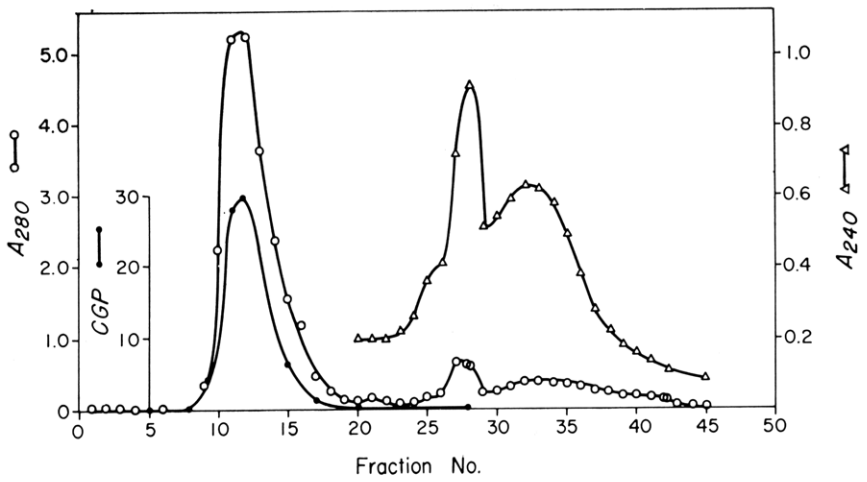

FIGURE 11: Gel filtration of the activation mixture of dogfish procarboxypeptidase A on Sephadex G-50 in 0.005 M Tris-HCl–0.5 M NaCl, pH 8.0. The fractions contained 5 ml each and were eluted at the rate of 30 ml/hr. Exopeptidase activity is expressed as μmoles of CGP split per minute per ml of effluent.

show the magnitude of the effect of calcium ions on the activation process. It appears that 0.5 M $CaCl_2$ in the activation mixture was required to obtain most rapid activation and maximum activity.

Calcium ions enhance the activation rate but do not significantly increase the activity of carboxypeptidase itself. The acceleration of activation is ion specific, calcium being somewhat more effective than magnesium ions, whereas sodium ions did not materially enhance the acceleration by suboptimal concentrations of calcium.

SPECIFICITY OF ACTIVATING ENZYMES. The activation of dogfish procarboxypeptidase A can be mediated by different enzymes (Lacko and Neurath, 1967). While the rates of activation by bovine trypsin and Nagarse follow similar paths, activation by bovine α-chymotrypsin proceeds at a much slower rate and results in the generation of only about 10% of the potential activity. Calcium ions in concentrations of 0.04–0.5 M cause a substantial increase in the activation rate by both trypsin and Nagarse, while the activation by chymotrypsin appears to be unaffected. These findings suggest that during activation, chymotrypsin splits different bonds than trypsin and Nagarse and thus produces largely nonproductive proteolysis of the zymogen.

Isolation of Dogfish Carboxypeptidase A. For purposes of enzyme isolation, 100 mg of procarboxypeptidase A was dissolved in about 10 ml of 0.1 M Tris-HCl–0.5 M $CaCl_2$, pH 8.0, and incubated with 1 mg of bovine trypsin for 30 min at 25°. When enzymatic activity reached a maximum, the reaction mixture was adjusted to 10^{-2} M DFP[3] and was then applied to a Sephadex G-50 column (1.6 × 90 cm) that had been previously equilibrated with 0.005 M Tris-HCl, pH 8.0–0.5 M NaCl.

Figure 11 shows the elution profile obtained. While the enzyme seems to be well separated from the lower molecular weight material, the peptide fractions contained at least three components. The enzymatically active fractions were pooled and adjusted to 30% saturation with ammonium sulfate. After settling in the cold room for 2 hr, the precipitate was collected by low-speed centrifugation (5000*g*) at 0°. The precipitate could be readily dissolved in 0.005 M sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl and was used as such for the preliminary characterization of dogfish carboxypeptidase A.

Physical Characterization. The sedimentation pattern of the enzyme is shown in Figure 12. A plot of $s_{20,w}$ *vs.* protein concentration (Figure 13) indicates a tendency for aggregation which appears to be even stronger than that of the zymogen (Figure 5). Least-square analysis of the linear portion yields a value of $s_{20,w} = 3.18 \pm 0.10$ S at infinite dilution.

Molecular weight determination of the enzyme was carried out using the same methods as described for the zymogen. Table IV shows the results obtained from sedimentation equilibrium experiments in the ultracentrifuge. The apparent molecular weights calculated from the bottom of the cell (M_b) showed a tendency to increase with protein concentration, confirming the aggregation of the enzyme observed during the sedimentation velocity runs. Since detailed association studies were not carried out, the monomer molecular weight of dogfish carboxypeptidase A was assumed to be about 35,000 on the basis of the data available.

The absorbancy index was determined using the same method as described for the zymogen, yielding a value of $A_{280}^{1\%} = 18.5$.

While the enzyme appeared to be homogeneous in the ultracentrifuge, it failed to penetrate the gel columns during

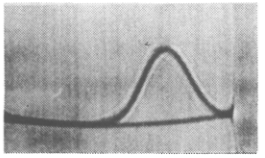

FIGURE 12: Sedimentation of dogfish carboxypeptidase A in 0.005 M PO_4–0.5 M NaCl, pH 8.0; protein concentration = 13.8 mg/ml. The photograph was taken after sedimentation for 80 min at 60,032 rpm at 4°.

[3] DFP (5 M) was used in this experiment to avoid precipitation of protein by excess amounts of 2-propanol.

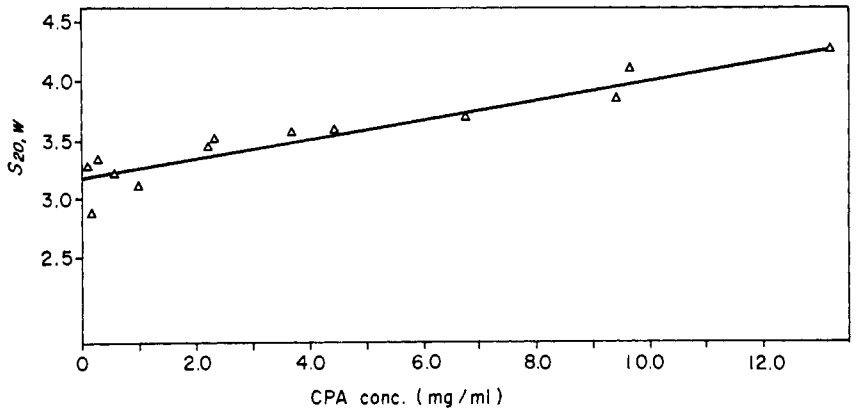

FIGURE 13: The concentration dependence of $s_{20,w}$ of dogfish carboxypeptidase A. Conditions for each run were the same as in Figure 12.

disc gel electrophoresis. However, considering that the enzyme was isolated from an essentially pure zymogen, dogfish carboxypeptidase A was judged adequate to be used for further characterization.

Chemical Characterization. The amino acid analyses were interpreted by assuming 21 residues of alanine per 35,000 molecular weight. This value for alanine was limiting since the same number of residues was found in the zymogen (Table III). Hence the molecular weight of the enzyme could not be higher than the assumed value.

Table V shows the amino acid composition of the enzyme and the composition of the fragment(s) eliminated during activation. As determined by reduction with mercaptoethanol followed by carboxymethylation with iodoacetate (as described for the zymogen), the enzyme contained the same number of half-cystine residues per mole as the zymogen, *i.e.*, 4.0. These residues are probably present in the enzyme in the same state as their counterparts in the zymogen.

Twenty glutamic acid residues and seven aspartic acids or their respective amides are lost during activation, while most of the aromatic amino acids are retained in the enzyme, probably causing the marked reduction in solubility of the enzyme compared with that of the zymogen.

Catalytic Activity. Dogfish carboxypeptidase A appears to be a true exopeptidase, lacking endopeptidase activity. The rate of hydrolysis of several substrates was examined at different concentration levels to compare the steady-state kinetic parameters with those of other exopeptidases (Table VI).

The dogfish enzyme was also found to hydrolyze acetylglycyl-L-phenyllactic acid (AGPLA) but at a considerably lower rate than HPLA. Such an observation indicates the preference of the enzyme for an aromatic blocking function of the NH_2 group of the penultimate residue.

The hydrolysis of CGP appeared to be associated with substrate activation similar to the bovine enzyme (Whitaker *et al.*, 1966). Peptidase activity was diminished by reacting the enzyme with *N*-acetylimidazole (Simpson *et al.*, 1963) while the esterase activity (against 0.01 M DL-HPLA) increased simultaneously (Lacko and Neurath, 1967). β-Phenylpropionate totally prevented the acetylation of the group(s) that are instrumental in bringing about these changes (Lacko and Neurath, 1967).

TABLE IV: Molecular Weight Data of Dogfish Carboxypeptidase A Obtained by Sedimentation Equilibrium.

Protein Concn (mg/ml)	Time (min)	Speed (rpm)	M_w[a]	M_m[b]	M_b[c]
5.62	1080	8026	36,535	32,417	56,617
2.24	896	7984	34,516	32,721	41,831

[a–c] See footnotes *b–d*, Table II.

TABLE V: Amino Acid Composition of Dogfish Carboxypeptidase A and of the Activation Peptide.

Amino Acid	Residues/ 35,000 g of Protein	Nearest Integer/ 35,000 g of Protein	Integral Number Mol Wt of Res	Composition of Activation Peptide by Difference
Lysine	13.7	14	1,794	2
Histidine	6.9	7	960	2
Arginine	13.8	14	2,187	2
Tryptophan	11.1	11	2,048	
Aspartic acid	26.6	27	3,108	8
Threonine	21.0	21	2,123	5
Serine	24.7	25	2,178	6
Glutamic acid	28.7	29	3,744	20
Proline	17.0	17	1,651	
Glycine	29.8	30	1,713	3
Alanine	21.2	21	1.493	
Half-cystine	4.0	4	413	
Valine	17.2	17	1,586	6
Methionine	9.0	9	1,181	5
Isoleucine	20.1	20	2,263	6
Leucine	16.3	16	1,811	8
Tyrosine	18.8	19	3,101	3
Phenylalanine	11.3	11	1,619	3
			Mol wt 34,973	9037

TABLE VI: Comparison of Steady-State Kinetic Constants of Carboxypeptidases from Different Species and of Dogfish Procarboxypeptidase A.

Enzyme[a]	HPLA		CGP		AGPLA	
	K_m (10^{-3} M)	k_0 (sec^{-1})	K_m (10^{-3} M)	k_0 (sec^{-1})	K_m (10^{-3} M)	k_0 (sec^{-1})
Bovine CPA[b]	0.05	424	5.83	106		
Porcine CPA[c]	0.01	1000	16.6	139		
Dogfish CPA	0.02	635	0.21	14.7	0.44	86
Bovine CPB[d]		126				
Dogfish CPB[e]	2.8	29				
Dogfish pCPA	8.0	106	1.8	1.2	12	24

[a] Abbreviations used are: CPA, carboxypeptidase A; CPB, carboxypeptidase B; pCPA, procarboxypeptidase A. [b] Whitaker *et al.* (1966). [c] Folk and Schirmer (1963). [d] Wintersberger *et al.* (1962). [e] Prahl and Neurath (1966).

TABLE VII: Comparison of the Kinetic Constants of Dogfish Procarboxypeptidase A and Carboxypeptidase A.

Enzyme	HPLA		CGP		AGPLA	
	K_m (10^{-3} M)	k_0 (sec^{-1})	K_m (10^{-3} M)	k_0 (sec^{-1})	K_m (10^{-3} M)	k_0 (sec^{-1})
Carboxypeptidase	0.02	635	0.21	14.7	0.44	86
Procarboxypeptidase	8	106	1.8	1.2	12	24

According to preliminary observations, dogfish carboxypeptidase A appears to be a metalloenzyme since upon dialysis against 10^{-3} M 1,10-phenanthroline, the activity against HPLA was practically abolished. The esterase activity of the zymogen decreased slowly and in about 3 hr approached zero (Lacko and Neurath, 1967). Upon dialysis against 10^{-3} M $CdCl_2$, the esterase activity was diminished. When the "cadmium enzyme" was in turn dialyzed against 10^{-3} M Zn^{2+}, both esterase and peptidase activities approached their initial values, thus indicating that the native enzyme contains zinc.

Inherent Catalytic Activity of Dogfish Procarboxypeptidase A. Bovine procarboxypeptidase A was reported by Yamasaki *et al.* (1963) to display a low level of esterase activity (HPLA), but the possibility that small amounts of active enzyme were present in these preparations could not be excluded.

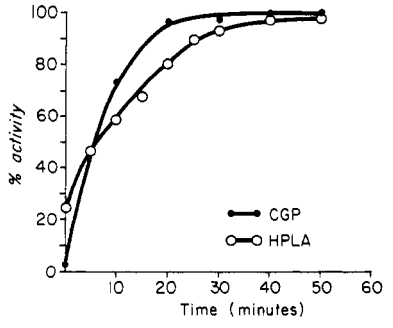

FIGURE 14: Activation of dogfish procarboxypeptidase A (1.5 mg/ml) by bovine trypsin (15 μg/ml) in 0.1 M Tris-HCl–0.5 M $CaCl_2$, pH 8.0. See text for further experimental details.

In the case of dogfish procarboxypeptidase A, the initial esterase activity was as much as 25% of the total potential activity when 0.01 M DL-HPLA was used to monitor activity (Figure 14), while only about 2% initial activity could be observed with 10^{-3} M CGP as substrate. These observations suggested that a true catalytic site was operational in the zymogen and the steady state kinetic constants obtained for both dogfish procarboxypeptidase A and carboxypeptidase A (Table VII) seem to substantiate this point of view. An investigation of the substrate dependence of the esterase activity toward L-HPLA revealed a strong inhibition by the substrate above concentrations of 10^{-3} M. In contrast, no substrate inhibition could be observed with the zymogen up to a substrate concentration of 0.1 M. The two curves illustrating the substrate dependence of both zymogen and enzyme are shown in Figure 15. From these data it is evident that there exist two distinct esterase sites, one in the enzyme and the other one in the zymogen, a different set of kinetic parameters defining their respective mechanisms of action (Table VII). While there is an increase in the turnover number following activation, a much more significant change takes place in the K_m value, indicating considerably tighter binding of HPLA by the enzyme than the zymogen (Table VII). The existence of the esteratic site in the zymogen was confirmed by the use of another substrate, acetylglycyl-L-phenyllactic acid. This substrate was shown to give rise to only moderate substrate inhibition when hydrolyzed by bovine carboxypeptidase A (R. M. MacDonald, unpublished). While there was some substrate inhibition of the dogfish enzyme when

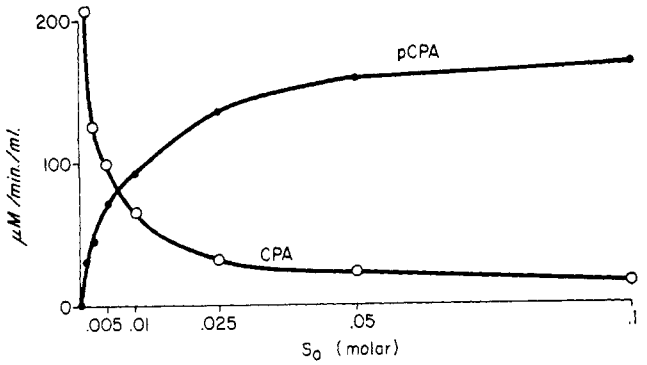

FIGURE 15: Substrate concentration dependence of HPLA hydrolysis by procarboxypeptidase A and carboxypeptidase A.

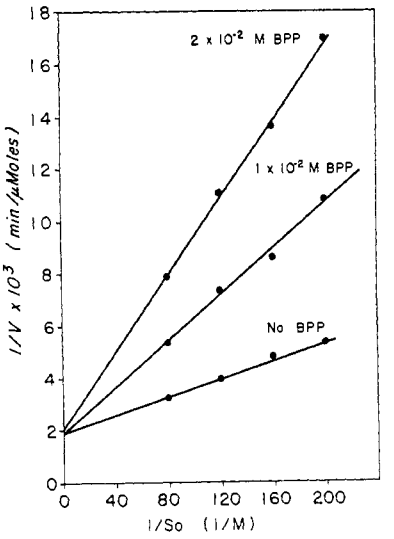

FIGURE 16: The inhibition of HPLA hydrolysis of dogfish procarboxypeptidase A by β-phenylpropionate; $K_i = 6 \times 10^{-3}$. Assays were carried out by adding 5 μl of zymogen solution (3.63 mg/ml) to the reaction mixture (3 ml).

the substrate concentration exceeded 2.5×10^{-4} M, no such inhibition was noted with the zymogen up to substrate levels of 10^{-1} M.

Dogfish procarboxypeptidase A was also found to hydrolyze the peptide substrate CGP. The hydrolysis of this substrate by dogfish carboxypeptidase A is subject to substrate activation while no such effect was found with the zymogen.

The zymogen is competitively inhibited by β-phenylpropionate when the esterase activity is measured with HPLA (Figure 16). The value obtained for K_i is almost identical with the K_m value, *i.e.*, 6.10^{-3} M as compared with 8×10^{-3} M.

The intrinsic activity of the dogfish zymogen is inhibited by 1,10-phananthroline but apparently unaffected by reaction with *N*-acetylimidazole. After addition of a 600-fold excess of the reagent, no change in esterase activity was noted while the acetylated zymogen after tryptic activation showed a 2-fold increase in esterase activity (0.01 M DL-HPLA). These latter findings suggest that, in analogy to the succinylated bovine zymogen, some of the functional tyrosine residues are available for acetylation in the dogfish zymogen. The esterase activity was inhibited by D-HPLA which serves neither as a substrate nor as an inhibitor for carboxypeptidase A (McClure *et al.*, 1964). The evidence in favor of a true catalytic site in dogfish procarboxypeptidase A prior to activation appears to be overwhelming. The elements of the active center prior to activation remain to be identified.

Discussion

Dogfish procarboxypeptidase, like its porcine counterpart, showed a strong tendency to accompany the anionic chymotrypsinogen fractions during chromatography, but could be isolated by the combined techniques of ion-exchange chromatography and gel filtration using DEAE-cellulose and Sephadex G-100. The purified zymogen showed remarkable similarity to bovine succinyl fraction I in physical and chemical characteristics. The major differences in amino acid composition appear to be in the sulfur-containing residues where the dogfish protein contains two more half-cystines and ten more methionines than its bovine analog.

Dogfish procarboxypeptidase A constitutes an ideal model for studying the zymogen to enzyme conversion since its activation appeared to be a rapid and specific process catalyzed by relatively low concentrations of trypsin. To achieve efficient activation, approximately 0.1 M $CaCl_2$ had to be included in the reaction mixture. While Mg^{2+} ions could substitute for the Ca^{2+} requirement, Na^+ ions could not, indicating a specific need for divalent cations to promote the activation process. An explanation for the Ca^{2+} requirement for activation is offered by the presence of the 27 glutamic and aspartic acid residues contributing an overwhelming excess of negative charges to the N-terminal region of the molecule. While at least some of these dibasic amino acids are probably present as amides, their contribution to the total charge of the molecule must be significant since the zymogen is strongly retained by DEAE-cellulose while the enzyme invariably appears in the breakthrough fractions. A similar situation exists in the case of bovine and porcine trypsinogen where the presence of Ca^{2+} ions increases the rate of activation while it decreases the amounts of inert proteins formed during the activation process (Radhakrishnan *et al.*, 1967, 1969; Abita *et al.*, 1969).

Dogfish carboxypeptidase A appears to be a true exopeptidase, lacking endopeptidase activity. Steady-state kinetic parameters for the hydrolysis of esterase substrates by the dogfish enzyme are very similar to those obtained for bovine and porcine carboxypeptidase A.

Of the peptidase substrates only CGP was investigated. The differences in kinetic parameters between dogfish carboxypeptidase A and carboxypeptidases from other species may be at least partially attributable to the substrate activation by carbobenzoxyglycine (Whitaker *et al.*, 1966) which most likely modifies these values.

The modulation of enzymatic activity by acetylimidazole parallels the pattern exhibited by the bovine enzyme (Simpson *et al.*, 1963) although a larger excess of the reagent was needed for maximal effect in the case of the dogfish enzyme.

Dogfish carboxypeptidase A appears to be a metalloenzyme since dialysis against 1,10-phenanthroline resulted in loss of activity. These preliminary findings were confirmed by metal determinations carried out in the laboratory of

Dr. B. L. Vallee in Boston, Mass. Spectrographic analysis of samples of varying purity revealed the presence of significant quantities of zinc in preparations of procarboxypeptidase A. The metalloprotein nature of the zymogen and the enzyme is under further study.

Unequivocal evidence has been presented to show the existence of a true active site in dogfish procarboxypeptidase A, defined by kinetic parameters. While substrate and inhibitor binding occurs in the zymogen, the K_m and K_i values are much higher for dogfish procarboxypeptidase A than for carboxypeptidase A (Table VII). Although the physical meaning of K_m is difficult to interpret precisely, some evidence is available to suggest that in the case of bovine carboxypeptidase A the Michaelis constant is indeed a measure of the affinity of the enzyme for a particular substrate. Thus Lumry *et al.* (1951) concluded that in the case of bovine carboxypeptidase A, K_m represented a catalytic constant, but their argument was based on data obtained from steady-state kinetics, a method which alone is unable to define catalytic mechanisms. Coleman and Vallee (1961), on the other hand, found that apocarboxypeptidase A was binding CGP with a constant (K_s) that was nearly identical with the K_m value obtained for this substrate.

In view of the extensive similarity in the catalytic properties of bovine and dogfish enzymes, one may assume that the K_m values of dogfish carboxypeptidase A closely parallel their respective binding constants. The changes upon activation in K_m for HPLA and AGPLA are more than two orders of magnitude, suggesting that the activation process creates a binding site that is much more favorable for the splitting of these substrates. At the same time (since the zymogen can function as a catalyst), the bond-breaking mechanism may be contrasted to that existing in other zymogens such as bovine chymotrypsinogen A, which is capable of substrate binding (Vaslow and Doherty, 1953; Deranleau and Neurath, 1966) but appears to be totally devoid of catalytic activity prior to activation. Recent work of Freer *et al.* (1970) seems to suggest, however, that during the activation of chymotrypsinogen, the major changes occur in the region of the molecule that relates to the specificity of the enzyme whereas the catalytic site seems largely preformed in the enzyme precursor. The behavior of procarboxypeptidase is thus consistent with the conclusions derived from the crystallographic comparison of chymotrypsinogen and α-chymotrypsin.

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