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Purification and Crystallization of Human Carboxypeptidase A†

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ABSTRACT: Human carboxypeptidase A has been isolated from activated pancreatic juice by means of affinity chromatography employing the competitive inhibitor benzylsuccinic acid as an affinity ligand. The structural and functional features of the human and bovine enzymes are quite analogous. The molecular weights of human and bovine carboxypeptidases A are virtually identical, their amino acid compositions are similar, both contain 1 g-atom of zinc/mole, and the activities of both are restored by addition of zinc to the apoenzyme. The inhibition of human carboxypeptidase by chelating agents is reversed by either dilution or addition of a metal such as Cu^{2+}. When other metals are substituted for the native zinc, peptidase activity of the human metallocarboxypeptidases follows the order: cobalt > nickel > manganese > cadmium, while the sequence for esterase activities is: manganese > cobalt = cadmium > nickel. The latter sequence differs from that observed for the bovine enzyme. Human carboxypeptidase A crystallizes after dialysis at low ionic strength. Hydrolysis of the dipeptide carbobenzoxyglycyl-L-phenylalanine and of the ester benzoylglycyl-L-α-hydroxy-β-phenyllactate exhibits kinetic anomalies, but that of their longer homologues does not. Chemical modification with tyrosine reagents alters esterase and peptidase activities. The affinity chromatographic method here described should greatly facilitate future studies of this enzyme from human and other sources.

Carboxypeptidase A activity was first identified in bovine pancreatic extract by Walschmidt-Leitz and Purr (1929), and the enzyme was crystallized later by Anson (1937). Since then, the structure and function of this metalloexopeptidase have been studied extensively. It has been isolated and studied from a number of species including the pig (Folk and Schirmer, 1963), Pacific spiny dogfish (Lacko and Neurath, 1970), and, most recently, the white shrimp (Gates and Travis, 1973). While the presence of carboxypeptidase[1] activity in human pancreatic juice has been demonstrated (Keller and Allan, 1967) and preliminary studies have been performed (Ebata and Miyazaki, 1967; Berndt et al., 1968), thus far the human enzyme has not been isolated in a pure form or in quantities sufficient for detailed examination.

We have designed an affinity chromatographic method to isolate carboxypeptidase from pancreatic juice and have now obtained quantities of the human enzyme in crystalline form and adequate for the detailed study of its cardinal compositional and functional properties. Present results demonstrate that in all these respects human carboxypeptidase closely resembles the bovine enzyme. These studies are prerequisite to the elucidation of the structure and function of the human enzyme and should bear on the biochemical basis of human pancreatic physiology and pathology.

Materials and Methods

Activated pancreatic juice, obtained from a patient with a traumatic pancreatic fistula, was collected in sterile containers, refrigerated within 8 h of collection, and then stored at −20 °C.

Sepharose 4B was obtained from Pharmacia. Bovine trypsin, chymotrypsin, porcine carboxypeptidase B, carbonic anhydrase, and lima bean trypsin inhibitor were purchased from Worthington Biochemical Corp. Bovine carboxypeptidase A, AcTyrOEt,[2] and *N*-acetylimidazole were obtained from Sigma, D-arginine, L-arginine, BzGlyArg, and ε-aminocaproic

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[1] Carboxypeptidase referred to in the text will be carboxypeptidase A unless otherwise specified.

[2] Abbreviations used are: AcTyrOEt, acetyl-L-tyrosine ethyl ester; TosArgOMe, tosyl-L-arginine methyl ester; CbzGlyPhe, carbobenzoxyglycyl-L-phenylalanine; BzGlyPhe, benzoylglycyl-L-phenylalanine; BzGlyGlyPhe, benzoylglycylglycyl-L-phenylalanine; BzGlyOPhe, benzoylglycyl-L-α-hydroxy-β-phenyllactate; BzGlyGlyOPhe, benzoylglycylglycyl-L-α-hydroxy-β-phenyllactate; BzGlyArg, benzoylglycyl-L-arginine; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Mes, 4-morpholineethanesulfonic acid.

acid from Cyclo Chemical Co., and D,L-benzylsuccinic acid from Burdick and Jackson Co., Muskegon, Mich. Tetranitromethane was purchased from Aldrich Chemical Co. Solutions of metal ions were prepared from the spectroscopically pure sulfate salts (Johnson-Matthey "specpure" grade). 1,10-Phenanthroline (Aldrich), α,α'-bipyridyl (G. Frederick Smith), and 8-hydroxyquinoline-5-sulfonic acid (Eastman Organic) were used without further purification. The carboxypeptidase A substrates (CbzGlyPhe, BzGlyPhe, BzGlyGlyPhe, BzGlyOPhe, and BzGlyGlyOPhe) were gifts from Dr. David S. Auld. All other chemicals used were of reagent grade.

Assays. In the course of purification, carboxypeptidase activity was monitored both by esterase activity, determined potentiometrically using 5 mM BzGlyOPhe (Petra, 1970), and by peptidase activity, determined spectrophotometrically, with 1 mM CbzGlyPhe (Whitaker et al., 1966). Trypsin and chymotrypsin activities were monitored with 0.01 M TosArgOMe and 0.01 M AcTyrOEt (Wilcox, 1970) by means of a radiometer pH-stat at 25 °C. Carboxypeptidase B activity was measured spectrophotometrically with 1 mM BzGlyArg using the rate of increase in absorbance at 254 nm (Folk and Schirmer, 1963). In Table I, one unit of carboxypeptidase A activity corresponds to the hydrolysis of 1 μmol of CbzGlyPhe/min, and units of carboxypeptidase B activity refer to the percent hydrolysis of BzGlyArg/min. Bovine trypsin, chymotrypsin, and porcine carboxypeptidase B served as standards.

Peptidase activities of purified human carboxypeptidase were measured by an automated ninhydrin method in 0.05 M Tris, 1.0 M NaCl, 25 °C (Auld and Vallee, 1970). Esterase activities were determined by pH titration of the protons released on hydrolysis using a radiometer pH-stat and recorder (Auld and Holmquist, 1974). All titrations were carried out on 3.0 ml of substrate in 0.2 M NaCl, 5 mM Tris, pH 7.5, 25 °C. At substrate concentrations below 1.0 mM, BzGlyOPhe hydrolysis was monitored either spectrophotometrically at 254 nm or by pH titration; the results were identical. Initial velocities were measured in all instances, and substrate concentrations were verified by full hydrolysis of each substrate.

Affinity Chromatography. An affinity resin for carboxypeptidase was prepared from D,L-benzylsuccinic acid, a competitive inhibitor of the enzyme with a K_I of 1.1×10^{-6} M at pH 7.5 (Byers and Wolfenden, 1973). The product of the compound nitrated with nitric and sulfuric acids at 4 °C was dissolved in ethyl acetate and solvent volume was reduced to a minimum by elutrition. A precipitate formed at 4 °C. After 12 h the solvent was removed and the solid was washed with acetone, yielding a yellow product (mp = 119 °C). Hydrogenation (Pd/C) produced aminobenzylsuccinic acid. ε-Aminocaproyl-Sepharose 4B and the active ester were obtained according to the method of Cuatrecasas and Pavikh (1972). Aminobenzylsuccinic acid was added to the active ester-Sepharose 4B complex in 0.1 M sodium acetate, pH 6.4, for 12 h, 4 °C. The resin was then washed thoroughly with 1 M sodium bicarbonate, pH 8.4. The capacity of the column was 25-50 mg of bovine carboxypeptidase A/ml in pH 7.5, 0.02 M Tris, 0.05 M NaCl. The enzyme could be readily eluted from the resin by increasing the concentration of NaCl to 1.0 M.

Lima bean trypsin inhibitor was attached directly to activated Sepharose 4B according to the method of Feinstein et al. (1974); this resin bound both trypsin and chymotrypsin. Addition of D-arginine to the active ester of ε-aminocaproyl-Sepharose 4B under conditions identical with those used for aminobenzylsuccinic acid resulted in an affinity resin for carboxypeptidase B.

Amino Acid Analysis. Protein samples were dialyzed against water and hydrolyzed with 6 N HCl for 24, 48, and 72 h in sealed tubes at 110 °C, and analyses were performed with a Durrum Model D-500 amino acid analyzer. Performic acid oxidation was carried out to determine the half-cystine content as cysteic acid. Human carboxypeptidase A was reduced with dithiothreitol in the presence of nitrogen and 6 M guanidine and then alkylated with iodoacetate. Tryptophan was determined by magnetic circular dichroism according to the method of Holmquist and Vallee (1973).

Disc Gel Electrophoresis. Polyacrylamide gel electrophoresis with sodium dodecyl sulfate was performed according to the method of Weber and Osborn (1969); 10% acrylamide was used and the gels were stained with Coomassie brilliant blue. Samples of human carboxypeptidase A applied to these gels contained 10-50 μg of protein.

Ultracentrifuge. High speed sedimentation equilibrium experiments were carried out in a Spinco Model E ultracentrifuge using protein concentrations of 0.4 mg/ml (Yphantis, 1964). All runs were performed at room temperature in 0.02 M Tris, pH 7.5, 0.1 M NaCl.

Metal Studies. Metal analyses were performed both by atomic absorption spectrometry and emission spectroscopy with apparatus described previously (Fuwa and Vallee, 1963; Fuwa et al., 1964). All buffers were extracted with freshly dissolved 0.01% dithizone in CCl_4. Spectroscopically pure metal salts (Johnson-Matthey) were dissolved in metal-free distilled water and diluted with metal-free buffer to result in the pH and molarity desired for metal reconstitution of the apoenzyme.

Apoenzyme was prepared by suspending the native crystals (5 mg/ml) four successive times in 10 mM 1,10-phenanthroline, 10 mM Mes, pH 7.0, 25 °C, for 15 min followed by four washes with 10 mM Mes, pH 7.0. The crystalline apoenzyme was then reconstituted by addition of a four-fold excess of the desired metal within 3 h after initiating the preparation of the apoenzyme. Apoenzyme obtained in this manner contained less than 1% zinc. In order to preclude dissociation of metallocarboxypeptidase complexes of potentially low stability, dilutions and assays of all metalloenzymes—other than the zinc enzyme—were performed in the presence of 0.1 mM metal ion.

Studies of Heat Inactivation. Experiments were performed by incubating carboxypeptidase in 0.05 M Tris, pH 7.5, 1.0 M NaCl at 50 °C in a Polyscience thermostated circulating water bath. Protein concentrations were 0.2 mg/ml. At time intervals indicated in Figure 1a, 50-μl aliquots were removed for peptidase assays with CbzGlyPhe.

pH Optimum. The effect of pH on peptide hydrolysis was measured with 5 mM BzGlyGlyPhe in 1.0 M NaCl, 25 °C. The buffers used were: pH 5.0-7.0, Mes, 7.5-8.0, Tris, and 9.0-10.0, Ammediol, all 0.05 M; pH was measured before and after each assay.

Effect of Salt Concentration on Peptide Hydrolysis. CbzGlyPhe, 20 mM, and BzGlyGlyPhe, 5 mM, were used in 0.02 M Tris, pH 7.5, to study the effect of ionic strength on peptidase activity.

Inhibition Studies. The enzyme was incubated with chelating agents for 1 h, 4 °C. The temperature was raised to 25 °C and the reaction initiated by addition of CbzGlyPhe, 20 mM, or BzGlyOPhe, 5 mM. Instantaneous inhibition by L-benzylsuccinate was examined with BcGlyGlyPhe, 1.0 M NaCl, 50 mM Tris, pH 7.5, 25 °C.

Acetylation. A 300-fold molar excess of *N*-acetylimidazole

TABLE I: Purification of Human Carboxypeptidase.

		Carboxypeptidase A[a]				Carboxypeptidase B[b]			
Step	Total Protein (mg[c])	Total Act. (Units)	Sp Act. (Units/mg)	Recovery (%)	Purifn (-fold)	Total Act. (Units)	Sp Act. (Units/mg)	Recovery (%)	Purifn (-fold)
Pancreatic Juice	10 700	3220	0.3	100	1	105 000	9.8	100	1
pH 6.0 5 mM Mes 0.5 M NaCl	10 400	0				0			
0.1 M L-Arg pH 6.0 5 mM Mes 0.5 M NaCl	127	0				61 500	490	60	47
pH 7.5 0.05 M Tris	162	3400	21	105	70				

[a] Substrate CbzGlyPhe. [b] Substrate BzGlyArg. [c] Based on $E^{1\%}_{280} = 20$.

was added to human carboxypeptidase, 0.0145 mM, in 0.02 M Veronal buffer, pH 7.5, 23 °C. To stop the reaction, aliquots were removed at varying time intervals and added to 0.05 M Tris, 1.0 M NaCl, pH 7.5. Changes in esterase activity, BzGlyOPhe, 5 mM, and peptidase activity, CbzGlyPhe, 20 mM, were measured under standard conditions (Simpson et al., 1963).

Nitration. Human carboxypeptidase crystals, 5 mg/ml, were suspended in 0.05 M Tris, pH 8.0, 23 °C, and a 20-fold molar excess of tetranitromethane was added in the dark. The reaction was stopped after 45 min by centrifugation (Riordan and Muszynska, 1974). Changes in peptidase and esterase activity were measured using the same substrates.

Results

Enzyme Purification

Frozen pancreatic juice is thawed and a small amount of white precipitate removed by centrifugation at 10 000*g* for 20 min. Two different affinity and chromatographic procedures are used to isolate the enzyme.

Method I involves three successive affinity resins at pH 7.5. Trypsin and chymotrypsin are removed first by passage over a lima bean trypsin inhibitor resin. Unbound protein is collected and dialyzed against 0.02 M Tris, pH 7.5, 0.05 M NaCl, and passed over the aminobenzylsuccinic acid affinity column. After washing with starting buffer, both carboxypeptidases A and B are eluted by increasing the salt concentration to 2.0 M. Passage over the D-arginine affinity column removes carboxypeptidase B. An 80% yield of carboxypeptidase A can be obtained from 1000 ml of pancreatic juice and 100 ml of aminobenzylsuccinic acid resin with a 40-fold purification.

Method II (Table I) employs the aminobenzylsuccinic acid–Sepharose 4B column at pH 6.0, where carboxypeptidase A and B remain bound to the affinity resin even in the presence of 2.0 M NaCl. Chymotrypsin, on the other hand, can be eluted with 0.5 M NaCl at this pH. Subsequent washing of the aminobenzylsuccinic acid resin with 0.1 M L-arginine removes carboxypeptidase B, leaving only carboxypeptidase A which is then removed with 2.0 M NaCl at pH 7.5. The overall purification and yield are 70-fold and 105%, respectively (Table I).

Carboxypeptidase A prepared by either method contains less than 1% of either carboxypeptidase B or chymotrypsin activity, as measured by hydrolysis of BzGlyArg or AcTyrOEt and using the bovine or porcine enzymes as the basis of comparison. The specific activity and purity of carboxypeptidase A prepared by either method is the same. The method at pH 6 requires only a single chromatographic column and fewer elution steps, results in a better yield, and simultaneously provides partially purified carboxypeptidase B.

Protein Characterization

Disc Gel Electrophoresis. Human carboxypeptidase A obtained in the above manner migrates in the presence of sodium dodecyl sulfate as a single band at pH 7.0, similar in appearance to that of the bovine enzyme. Under these conditions and at the highest protein concentrations, 50 μg/gel, the trailing edge of the resulting enzyme spreads to some extent but the leading edge remains distinct. Human carboxypeptidase has a molecular weight of 35 000 when its mobility is compared to that of other proteins of known molecular weight (liver alcohol dehydrogenase, carbonic anhydrase, myoglobin, and chymotrypsin).

Ultracentrifugation. The Yphantis high-speed sedimentation equilibrium method gives linear plots of ln c vs. r^2 at 31 410 rpm, consistent with homogeneity. The molecular weight is 34 710 based on a partial specific volume of 0.73, determined from the amino acid composition (see below).

Amino Acid Composition. Table II shows the recovery of amino acid residues from human carboxypeptidase analyzed in triplicate, after various times of hydrolysis. The number of residues are calculated based on the assumption that both human and bovine carboxypeptidase contain 307 residues, since their molecular weights, determined by sodium dodecyl sulfate gel electrophoresis and ultracentrifugal analysis, are virtually the same. This assumption results in appropriate and nearly integral numbers for the residues which occur in small amounts, e.g., methionine and half-cystine. For all residues other than lysine (0.35) and proline (0.68) the standard deviation is less than 0.3 (Table I). Values for threonine and serine are corrected by extrapolation to zero time. The values for valine and isoleucine are taken at 72 h.

The half-cystine content, determined as cysteic acid, is 2.13 ± 0.04 mol/mol of protein and that of bovine carboxypeptidase, determined in parallel, is 2.03 mol/mol. The *S*-carboxymethylcysteine content, determined in duplicate samples after reduction with dithiothreitol and alkylation with iodoacetate,

TABLE II: Amino Acid Composition.

Residue	Mean	σ	Integer	Bovine[d] α	Porcine[e] A_2	Dogfish[f]
Asp	29.3	0.24	29	29	32	27
Glu	24.6	0.20	25	25	29	29
Pro	13.5	0.68	13	10	14	17
Gly	22.7	0.18	23	23	26	30
Ala	23.4	0.23	23	20	22	21
Met	3.03	0.03	3	3	3	9
Leu	23.4	0.27	23	23	21	16
Tyr	16.8	0.24	17	19	17	19
Phe	15.8	0.13	16	16	17	11
His	10.1	0.14	10	8	9	7
Lys	16.7	0.35	17	15	13	14
Arg	9.5	0.10	10	11	11	14
Thr			27[a]	26	27	21
Ser			26[a]	32	28	25
Val			13[a]	16	12	17
Ile			23[a]	20	19	20
Cys			2[b]	2	2	4
Trp			8[c]	7	7	11
Total			308	307	309	309
Mol wt			34 617	34 600	34 606	34 973

[a] Calculated from destruction or appearance. [b] Determined after performic acid oxidation. [c] Determined by MCD. [d] From Bradshaw et al. (1969). [e] From Folk (1963). [f] From Lacko and Neurath (1970).

is 2.01 and 2.00.

Human carboxypeptidase A displays an ultraviolet absorption spectrum with a maximum at 278 nm. A molar absorptivity of 6.63×10^4 at 278 nm is determined by measuring the absorbance at this wavelength and then determining the protein concentration by amino acid analysis. Based on this molar absorptivity, tryptophan determination by magnetic circular dichroism (Holmquist and Vallee, 1973) gives a value of 8.0 mol of tryptophan/mol of protein.

Zinc Content. Adventitious metal ions are removed prior to metal analysis by dialysis of the enzyme against metal-free 0.02 M Tris, pH 7.5, 0.1 M NaCl. The zinc content of human carboxypeptidase is 1.01 ± 0.05 g-atoms of zinc/mol of protein by atomic absorption spectrometry. No other metal is present by emission spectrography.

Crystallization. Human carboxypeptidase crystallizes on dialysis against buffers of low ionic strength between pH 6.0 and 8.0. Figure 1 shows these crystals which resemble those of bovine carboxypeptidase A_α. Human carboxypeptidase crystallizes at a salt concentration lower than that of the bovine enzyme when dialyzed at the same protein concentrations, buffer, and pH.

Heat Inactivation. Figure 2A shows the loss of peptidase activity vs. time of incubation of both bovine and human carboxypeptidase at 50 °C. Both enzymes lose almost 90% of their native activity after 50 min, and their rates of inactivation are the same.

pH Optimum. Peptidase activity of human carboxypeptidase is optimal between pH 7.5 and 8.0 (Figure 2B). At pH 5.0 the enzyme is inactive even in the presence of 10^{-6} M zinc.

Ionic Strength. The effect of salt concentration on peptide hydrolysis is shown in Figure 2C. For both substrates, activity falls off markedly below 0.15 M NaCl, while above this concentration a slow increase in activity is evident.

Kinetics

Peptide Hydrolysis. The dipeptides, BzGlyPhe and CbzGlyPhe, were studied over the concentration range of 0.1–80 mM and BzGlyGlyPhe between 0.1 and 10 mM. For BzGlyPhe, double-reciprocal plots demonstrate substrate activation above 2 mM and no apparent substrate inhibition. The plot for CbzGlyPhe is triphasic; at low substrate concentrations activity decreases linearly up to 2 mM at which point v^{-1} begins to decrease more rapidly, reflecting substrate activation. Above 10 mM CbzGlyPhe v^{-1} increases, reflecting substrate inhibition. The plot for the tripeptide substrate, BzGlyGlyPhe, is linear throughout the concentration range examined.

Ester Hydrolysis. The depsipeptides were studied from 0.1 to 10 mM substrate concentration. BzGlyOPhe exhibits substrate inhibition at concentrations greater than 1 mM, while BzGlyGlyOPhe has linear kinetics throughout, similar to its oligopeptide analogue.

Table III compares the kinetic parameters K_m and k_{cat} for human carboxypeptidase, derived from the linear segment of the double reciprocal plots of these ester and peptide substrates, with those obtained previously for the bovine enzyme. The greatest differences between the two enzymes are evident when comparing their behavior toward the dipeptides, CbzGlyPhe and BzGlyPhe; the kinetic parameters for the two ester substrates and the tripeptide, BzGlyGlyPhe, are nearly identical.

Inhibition

Metal Binding Agents. The inhibition of carboxypeptidase by 1,10-phenanthroline is time dependent. After 30 min of incubation at 4 °C with 0.1 mM 1,10-phenanthroline, activity becomes constant at 20% of that of a control incubated without the agent. Either dilution or addition of $CuCl_2$ fully reverses the inhibition; the latter maximally when the Cu:1,10-phenanthroline ratio is 1:3.

The effect of the concentration of a series of metal binding agents on the degree of inhibition of the enzyme was also examined. Esterase and peptidase activities were both measured after incubation of the enzyme with inhibitor (Figure 3). Values of K_I, the concentration of inhibitor needed to achieve 50% inhibition, and $\bar{n}$, the average number of moles of ligand

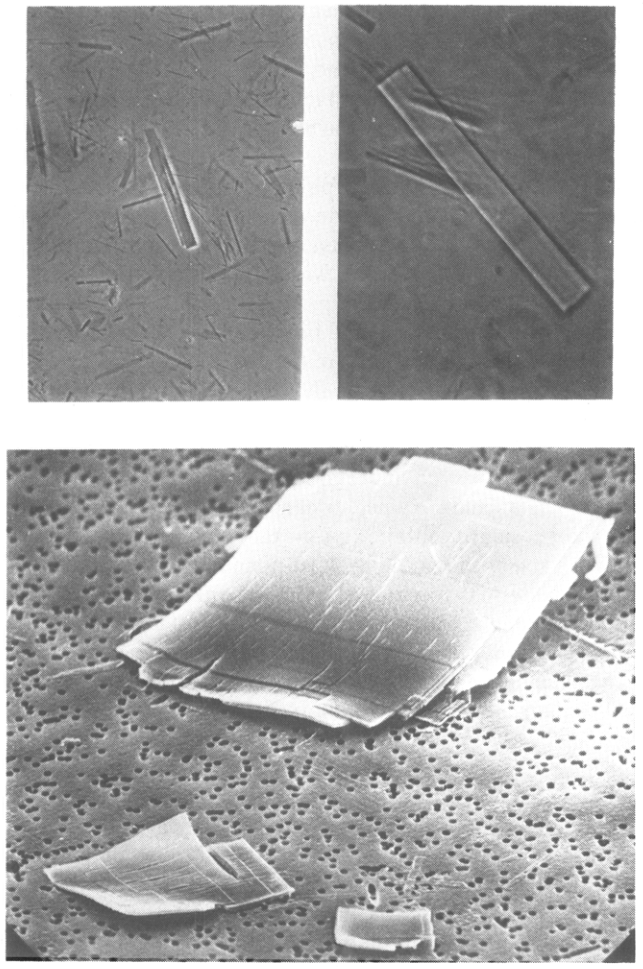

FIGURE 1: (a, top) Human carboxypeptidase crystals. Left approximately 333×. Right approximately 1000×. (b, bottom) Electron micrograph taken with a JEOL JSM-35 scanning electron microscope. The crystals were dehydrated in absolute ethanol and spotted on Nucleopore filters whose pores provide the gauge for comparison of size (pore size = 1 μm). The preparations were air-dried and coated with gold–palladium in a Technics Hummer. The photographs were taken by Dr. Michael Gottesman, Department of Anatomy, Harvard Medical School.

TABLE III: Kinetic Constants, Human and Bovine Carboxypeptidase.

Substrate	$K_m \times 10^4$, M^{-1}		$k_{cat} \times 10^{-3}$, min^{-1}	
	Human	Bovine[a]	Human	Bovine[a]
CbzGlyPhe				
4–40 mM	133	59	6.7	10.1
1–4 mM	30	19	2.9	5.5
BzGlyPhe				
1–10 mM	100	27	4.0	8.7
0.1–0.4 mM	2.3	8.1	0.7	5.5
BzGlyOPhe	2.0	0.8	30.3	28.6
BzGlyGlyPhe	9.0	10	0.8	1.2
BzGlyGlyOPhe	2.0	3.3	46	30

[a] Davies et al. (1968), Auld and Holmquist (1974).

complexed/mole of enzyme, are determined based on $(v_0/v_i) - 1 = K_I I^n$, where v_0 is the control activity of the native enzyme and v_i is the activity in the presence of free inhibitor, I_{free}. Values of K_I and $\bar{n}$ obtained from the linear plots of log $((v_0/v_i) - 1)$ vs. log I_{free} are shown in Figure 3. Values for $\bar{n}$ are all greater than 1. The values of K_I parallel the dissociation constants of the corresponding 1:1 metal–inhibitor complexes (K_L).

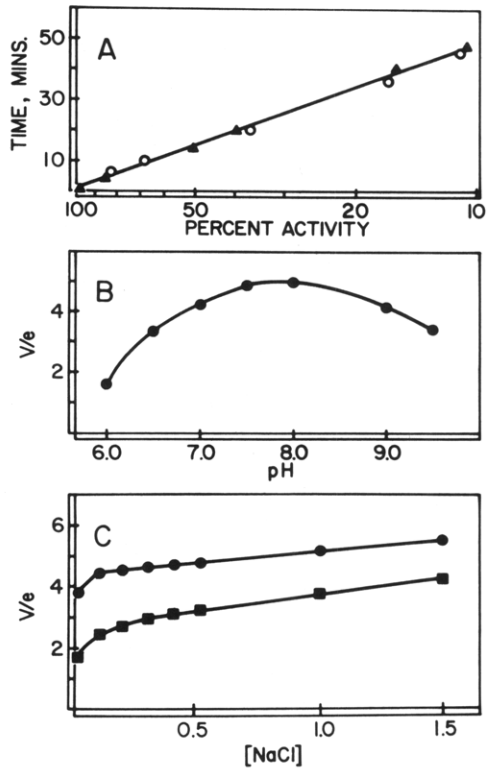

FIGURE 2: Physicochemical properties of human carboxypeptidase. (A) Thermal stability at 50 °C. (▲) Human and (○) bovine enzyme. Protein concentration is 0.2 mg/ml in 0.05 M Tris, pH 7.5, 1.0 M NaCl. Activity measured with 20 mM CbzGlyPhe. (B) Effect of pH. Activity measured with 5 mM BzGlyGlyPhe in 1.0 M NaCl, 25 °C. (C) Effect of ionic strength. (■) 20 mM CbzGlyPhe and (●) 5 mM BzGlyGlyPhe. In 0.02 M Tris, pH 7.5, 25 °C. $V/e \times 10^{-3}$ for CbzGlyPhe and $\times 10^{-2}$ for BzGlyGlyPhe.

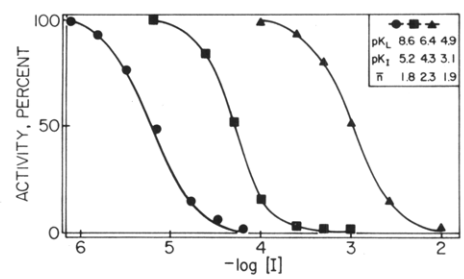

FIGURE 3: Inhibition of human carboxypeptidase by chelating agents. (●) 8-Hydroxyquinoline-5-sulfonic acid, (■) 1,10-phenanthroline, (▲) α,α'-bipyridyl. Peptidase activity (20 mM CbzGlyPhe, 0.05 M Tris, pH 7.5, 1.0 M NaCl) and esterase activity (5 mM BzGlyOPhe, 0.005 M Tris, pH 7.5, 0.2 M NaCl) were identical. Enzyme concentration 0.6 μM.

Benzylsuccinate. A double reciprocal plot shows that L-benzylsuccinate, either 0.4 or 1.0 μM, pH 7.5, competitively inhibits the hydrolysis of BzGlyGlyPhe by human carboxypeptidase with a K_I of 1.6×10^{-6} M.

Metallocarboxypeptidases

Apocarboxypeptidase, the zinc-free enzyme, is essentially inactive when examined by means of either peptide or ester substrates, but addition of zinc to the apoenzyme restores activity to become identical with that of the native enzyme. Reconstitution of the apoenzyme with cobalt, nickel, manganese, and cadmium generates peptidase activities of 148, 44, 15, and 3%, and esterase activities of 70, 60, 100, and 70% of native

activity, respectively. Peptidase activity is measured with CbzGlyPhe and esterase activity with BzGlyOPhe. The relative activities restored by these metal substitutions parallel those of the same metals when substituted for zinc in the bovine enzyme (Vallee et al., 1970). The peptidase activity of the cobalt enzyme is increased, while that of the cadmium enzyme is barely detectable, though the esterase activity of the latter is 70% that of the native enzyme.

Chemical Modifications

Acetylation with *N*-acetylimidazole increases esterase and decreases peptidase activities as a function of time. Under the conditions and concentrations indicated under Methods the activities do not change further after 30 min; at that time peptidase activity is 6% and esterase activity 270% that of the native enzyme when assayed with CbzGlyPhe and BzGlyOPhe, respectively. Deacylation with hydroxylamine restores native esterase and peptidase activities.

Modification of human carboxypeptidase A crystals with tetranitromethane (see Methods) increases esterase and decreases peptidase activities with concomitant appearance of an absorption spectrum characteristic of the nitrated protein. Based on a molar absorptivity of 4.2×10^3 for nitrotyrosine at 428 nm, 2.3 tryosyl residues/mol of protein are nitrated under these conditions. Peptidase activity is 22% and esterase activity 170% that of the native enzyme.

Discussion

Purification of human carboxypeptidase A to homogeneity has been accomplished through the use of highly specific affinity methods employing aminobenzylsuccinate–aminocaproyl-Sepharose 4B. Binding of carboxypeptidase is enhanced markedly at pH 6.0 where the resin seems to act as a true "affinity" resin, while at pH 7.5, ionic strength significantly affects binding, suggesting that ion-exchange properties are important. Benzylsuccinate also inhibits carboxypeptidase B (Zisapel and Sokolovsky, 1974) and, as would be expected, this enzyme also binds to the affinity resin. However, the competitive inhibitor, L-arginine, selectively elutes carboxypeptidase B, leaving carboxypeptidase A to be removed by raising the pH to 7.5 in the presence of 2.0 M NaCl.

Three affinity resins for carboxypeptidase A have been reported previously. Cuatrecasas et al. (1968) coupled the enantiomorphic inhibitor L-tyrosyl-D-tryptophan to agarose and found that carboxypeptidase binds at pH 8.0, 0.3 M NaCl. However, 0.1 M acetic acid, which was required to elute the enzyme from the adsorbant, inactivates the enzyme. Reeck et al. (1971) also used D-tryptophan as an affinity ligand with the ϵ-aminocaproyl group serving as the spacer instead of L-tyrosine. Carboxypeptidase could be eluted from this material with 0.5 M NaCl at pH 7.5 but trypsin, chymotrypsin, and carboxypeptidase B also bound to this resin. Uren (1971) employed D-phenylalanine as the affinity ligand, L-glycine the spacer group, and aminoethylcellulose as the insoluble matrix. The binding of carboxypeptidase to this resin was optimal at pH 6.5; elution occurred on raising the pH to 7.5. Chymotrypsin and carboxypeptidase B also bind to the resin, but use of this adsorbant in separating a mixture of enzymes is not reported. Benzylsuccinate attached to the ϵ-aminocaproyl-Sepharose 4B seems to have more desirable properties as an affinity resin. It allows selective adsorption of carboxypeptidases from a mixture of pancreatic enzymes while permitting complete elution of the enzyme under relatively mild conditions.

Human carboxypeptidase was isolated from pancreatic juice in the fully active form, and no attempt was made to obtain its precursor, procarboxypeptidase. While the bovine zymogen does not bind to this affinity adsorbant, it is conceivable that alteration of the spacer group might allow its isolation, since benzylsuccinate inhibits procarboxypeptidase (T. J. Bazzone, unpublished observations).

This affinity chromatographic procedure is applicable to carboxypeptidases from a wide variety of sources and species (T. J. Bazzone, M. Sokolovsky, and B. L. Vallee, in preparation). The lack of a general, widely applicable method that provides adequate amounts of protein for detailed examination of composition, structure, and function has been a major limitation in comparative studies of the evolution of enzymes. Use of this technique, selective on the basis of function, allows almost complete recovery of highly purified enzyme from tissue of widely divergent evolutionary histories and greatly enhances the feasibility of such undertakings.

Like the bovine enzyme, human carboxypeptidase is a metalloexopeptidase containing 1 g-atom of zinc/mol of protein which is removed readily by 1,10-phenanthroline. The metal atom is essential to catalysis; the apoenzyme is essentially devoid of peptidase and esterase activity, but addition of zinc restores activity identical with that of the native enzyme.

Metal binding agents inhibit activity by removal of the metal rather than by formation of a mixed enzyme–metal–inhibitor complex. The inhibition with 1,10-phenanthroline, while time dependent, is reversible by dilution or by addition of another metal, e.g., copper. In each instance the magnitude of the K_I values for the three chelating agents studied parallels their K_L values. The values derived for $\bar{n}$, the number of inhibitor molecules bound to the metal, exceed 1, consistent with metal ion removal. A value of 1 would be expected only if the mechanism of inhibition involved formation of a ternary complex.

Other metals can substitute for zinc to generate peptidase and esterase activities characteristic of each. The magnitudes of the activities of metal-substituted human carboxypeptidase follow the same order as in the bovine enzyme, i.e., cobalt > zinc > nickel > manganese > cadmium, with significant differences between the various metal substitutions. Esterase activities follow the order: manganese = zinc > cobalt = cadmium > nickel, a sequence somewhat different from that observed for the bovine enzyme. Moreover, the differences observed are less striking than for peptide hydrolysis. Auld and Holmquist (1974) have shown that for peptide hydrolysis metal substitutions of bovine carboxypeptidase primarily affect k_{cat}, while for ester hydrolysis they primarily reflect in K_m with virtually no effect on k_{cat}. The overall activities of the metal-substituted bovine enzyme are very similar to those found here, suggesting analogous roles for the metal atom.

The molecular weights calculated from the amino acid compositions of human and bovine carboxypeptidase are essentially identical (Table II). The greatest variation occurs with serine 26 and 32, respectively, whereas tyrosine, arginine, glutamic acid, and histidine, residues known to be important to the function of the bovine enzyme, are present in similar numbers in both enzymes. The number of sulfur-containing residues is identical, the two half-cystines suggesting the presence of a single disulfide bond. The difference between the number of acidic and basic residues is identical for both carboxypeptidases, although for the human enzyme the degree of amidation is not yet known.

A number of criteria indicate the homogeneity of human carboxypeptidase: it migrates as a single band on disc gel electrophoresis and as a single peak on analytical ultracen-

trifugation, its specific activity is comparable to that of the bovine enzyme, its zinc content of 1 g-atom/mol remains constant on repeated crystallization, and it is free of carboxypeptidase B and chymotrypsin activity.

Three isomers of bovine carboxypeptidase have been recognized—α, β, and γ—dependent on the bond of the direct precursor, subunit I of procarboxypeptidase, which is cleaved on activation. The enzyme isolated from activated bovine pancreatic juice is predominantly the β form (Reeck et al., 1971). Analogous studies of human carboxypeptidase are not on record, though it has been thought that human pancreatic juice might contain from two (Clemente et al., 1972) to four (Kim and White, 1971) different procarboxypeptidases. Activation with bovine trypsin initially results in two or possibly three forms of the active enzyme, but after 18 h of incubation with trypsin a single species of carboxypeptidase is isolated, which Kim and White (1971) refer to as A_3. The present method of isolation leads directly to a highly specific, homogeneous carboxypeptidase. Multiple isomers of the enzyme that may exist need not be apparent under the conditions employed here. Based on the present amino acid composition, studies of the amino-terminal sequence might uncover possible heterogeneity as the result of activation.

Certain physicochemical features of the human enzyme were examined by criteria employed in the characterization of bovine carboxypeptidase. Salt concentration markedly affects the solubility of human carboxypeptidase; crystallization occurs readily at pH 6.0–8.0 when protein concentrations are greater than 1.0 mg/ml and NaCl concentration is less than 0.1 m (see Figure 2C). Salt concentrations greater than 0.3 M NaCl enhance peptidase activity. The effect of temperature on peptidase activity was studied by incubation at 50 °; after 30 min 90% of the initial activity is lost. Peptidase activity is optimal at pH 7.5–8.0; the enzyme is inactive at pH 5.0, and addition of excess zinc does not prevent loss of activity.

The kinetic behavior of human carboxypeptidase, examined with several commonly employed peptide and ester substrates, is similar to that of the bovine enzyme. The shorter substrates (BzGlyPhe, CbzGlyPhe, and BzGlyOPhe) exhibit kinetic anomalies, while the longer substrates (BzGlyGlyPhe and BzGlyGlyOPhe) have linear double-reciprocal plots. Inhibition of BzGlyGlyPhe hydrolysis by L-benzylsuccinate, the ligand used for the affinity resin, is competitive with a K_I of 1.6×10^{-6} M at pH 7.5.

The kinetic parameters of human, bovine, porcine, and dogfish carboxypeptidase toward CbzGlyPhe and BzGlyOPhe have been determined (Davies et al., 1968; Auld and Holmquist, 1974; Folk and Schirmer, 1963; Lacko and Neurath, 1970). The human enzyme closely resembles the porcine enzyme especially with respect to CbzGlyPhe, where the values are nearly identical. All four carboxypeptidases share kinetic anomalies toward these two substrates, and the similarity in their kinetic behavior might reflect active site features common to all four. The kinetic parameters of human and bovine carboxypeptidase are nearly identical with respect to the longer substrates, where the kinetics are linear and anomalies are not seen. It is not known if this similarity extends to the dogfish and porcine enzymes.

Thus, human and bovine carboxypeptidase are closely similar in regard to molecular weight and metal content, effects of salt, thermal stability, pH optima, and the kinetic behavior of the substrates studied.

Extensive studies of bovine carboxypeptidase have shown increases in esterase and decreases in peptidase activity upon modification of functional tyrosyl residues, and x-ray crystallography has confirmed the presence of tyrosine at the active site of this enzyme and acetylation of the dogfish enzyme yields similar changes in activity (Lacko and Neurath, 1967). The esterase activity of human carboxypeptidase increases and peptidase activity decreases when modified with both *N*-acetylimidazole and tetranitromethane, an indication that functional tyrosyl residues may also be common features.

Other human pancreatic enzymes that have been isolated include trypsin, chymotrypsin, elastase, carboxypeptidase B, protease E, amylase, phospholipase, and lipase. Studies of these enzymes have concentrated on methods of isolation, isomeric forms, compositional features, reactions with inhibitors, and immunologic identity. While carboxypeptidase has been demonstrated in human pancreatic tissue (Hadorn and Silberberg, 1968), pancreatic juice (Keller and Allan, 1967; Hadorn and Silberberg, 1968; Robinson et al., 1970; Kim and White, 1971; Figarella et al., 1969; Clemente et al., 1972), and duodenal contents (Silberberg and Hadorn, 1968) by enzymatic, chromatographic, electrophoretic, and immunologic techniques, it has now been obtained in homogeneous and crystalline form.

Human pancreatic function is critical to digestion of carbohydrates, lipids, and protein; its disturbance can be serious and often fatal. Though most of the earliest information regarding structure-function relationships of enzymes was obtained largely at the hands of pancreatic enzymes, none of these were obtained from human sources. There is still little understanding of the manner in which their properties may affect human physiology and pathology. In view of the ready availability of bovine enzymes in adequate quantities, these have been studied most extensively and their effects on pancreatic tissue have served to gain understanding of diseases of this organ. Unfortunately, possible differences in the enzymes themselves, in the dietary patterns and the digestive anatomy of the ungulate and human species, have precluded meaningful correlation. Extension of affinity chromatographic techniques, such as those here employed for the isolation of human carboxypeptidase A, to that of other pancreatic enzymes will permit genetic and other comparisons. This, in turn, should also allow both in depth studies of these human enzymes and, further, lead to more complete understanding of human pancreatic physiology and pathology.

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Broad-Line Nuclear Magnetic Resonance Studies of Chloroperoxidase†

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ABSTRACT: Chloroperoxidase, a heme glycoprotein isolated from the mold *Caldariomyces fumago,* was studied by NMR relaxation techniques. Interaction of the chloride ion substrate with the enzyme may be analyzed as consisting of at least three contributions: a weak interaction with the iron atom, nonspecific anion–protein interactions, and a specific interaction generated at low pH. The data indicate that a specific interaction, which develops in parallel with enzyme activity at low pH, does not occur at the iron atom first coordination sphere site. The results are summarized in terms of an enzymatic mechanism not involving chloride ion coordination to the iron atom.

Heme proteins constitute a major class of important metalloenzymes. Many of these enzymes catalyze the oxidation or reduction of various substrates presumably with a corresponding change in the electronic nature of the iron atom of the heme prosthetic group. The electronic processes in these reactions are very rapid and the intermediates short-lived, making direct study of the reaction mechanisms difficult. However, information about the arrangement of the ligands at the fifth and sixth coordination positions of the iron atom can lead indirectly to important conclusions about the catalytic process. Nuclear magnetic resonance relaxation techniques have provided a sensitive and efficient means for investigating metals such as iron in metalloenzymes (Wuthrich, 1970; Mildvan and Cohn, 1970; Ellis et al., 1969, Dweck, 1973).

Chloroperoxidase has a molecular weight of 42 000 and one ferriprotoporphryn IX moiety per monomeric species. This enzyme utilizes hydrogen peroxide to catalyze the oxidation of chloride, bromide, and iodide ions to an enzyme-bound halonium species (Morris and Hager, 1966). Halogenation of various substrates occurs at activated positions such as α to a carbonyl or ortho–para on an aromatic ring. Previous evidence suggests the mechanism is ionic (Brown and Hager, 1967). A possible reaction pathway could involve peroxide and halide ions as ligands at the fifth and sixth ligand position of the iron atom (Hager et al., 1970). Electron transfer could then proceed through the iron to give the oxidized halogen, which could then react with the organic substrate. Recent evidence on the nature of the adduct between hydrogen peroxide and iron, i.e., compound I, indicates that the peroxide does coordinate to the iron atom (Hager et al., 1972); however, the location of a binding site for the halide ion remains uncertain. There are two possibilities: (a) The halide ion could coordinate to the iron atom at a sixth coordination position. (b) The halide ion could

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