Tilghman, S. M., Hanson, R. W., & Ballard, F. J. (1976) in *Gluconeogenesis: Its Regulation in Mammalian Species* (Hanson, R. W., & Mehlman, M. A., Eds.) p 47, Wiley, New York.
Umbreit, W. W., Burris, R. H., & Stauffer, J. E. (1964) *Manometric Techniques, 4th Ed.*, p 18, Burgess Publishing Co., Minneapolis, MN.
Utter, M. F., & Kurahashi, K. (1954) *J. Biol. Chem. 207*, 821.
Utter, M. F., & Kolenbrander, H. M. (1972) *Enzymes, 3rd Ed. 6*, 136.
Veneziale, C. M., Walter, P., Kneer, N., & Lardy, H. A. (1967) *Biochemistry 6*, 2129.
Walaas, E. (1958) *Acta Chem. Scand. 12*, 528.
Wilkinson, G. N. (1961) *Biochem. J. 80*, 324.
Williamson, J. R., Browning, E. T., & Olson, M. S. (1968) *Adv. Enzyme Regul. 6*, 67.
Williamson, J. R., Scholz, R., & Browning, E. T. (1969) *J. Biol. Chem. 244*, 4617.
Yoshida, A., Kaplan, B. E., & Kimura, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 486.

Purification and Characterization of Carboxypeptidase A from Rat Skeletal Muscle†

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ABSTRACT: Carboxypeptidase A (EC 3.4.17.1) has been purified 44 000-fold in 33% yield from rat skeletal muscle by a four-step procedure. Purification in the presence of dichlorovinyl dimethyl phosphate conveniently inactivates an accompanying chymotrypsin-like enzyme and other serine protease(s) to ensure isolation of pure carboxypeptidase A free of polypeptide contaminants. The enzyme preparation consists of two components with molecular weights of approximately 39 300 and 37 800. The rat muscle carboxypeptidase is very similar to bovine pancreatic carboxypeptidase A in terms of (1) substrate specificity, (2) kinetics and molecular activity, (3) influence of metal ions on catalysis, (4) interaction with inhibitors, (5) effects of ionic strength on activity, and (6) stability and activity as functions of pH. Both muscle and pancreatic carboxypeptidases exhibit enhanced esterolytic activity when assayed in the presence of a variety of indoles and imidazoles or after incubation at relatively high concentrations of $MnSO_4$. The muscle enzyme is substantially less stable than its pancreatic homologue, and in impure preparations is very much less soluble. The latter property is attributable to a binding substance present in such preparations which renders muscle but not pancreatic carboxypeptidase A insoluble until ionic strength is increased to values near 2 M.

Elevation in the activity of a cytosolic aromatic amino acid esterase in skeletal muscle from mice, hamsters, and chickens with genetic muscular dystrophy has been under study for some time in our laboratory. Besides being elevated in dystrophic muscle, the esterase is increased after denervation of rat muscle (Meyer et al., 1972; Nwizu et al., 1974) and is increased 20-fold in muscle from vitamin E deficient rats (Meyer et al., 1972) and quokkas (W. L. Meyer and B. A. Kakulas, unpublished experiments). Variation in the level of the esterase has been described as a function of muscle development and in preliminary surveys of human neuromuscular disorders (Meyer et al., 1972, 1973, 1974). Esterase activity rapidly decreases in muscle when rats are fasted (Meyer et al., 1972), and its level is strongly influenced by dietary protein (J. R. Feussner and W. L. Meyer, unpublished experiments). In attempting to determine the relationship between levels of the cytosolic aromatic amino acid esterase and tissue and blood amino acid pools, it was observed that free phenylalanine and tyrosine were particularly elevated in the blood and muscle of dystrophic mice and chickens (Meyer et al., 1973). A powerful system for releasing phenylalanine was detected in the insoluble fraction of skeletal muscle (Reed & Meyer, 1974). The spectrum of amino acids released by this system was determined (Reed, 1975), which led to the hypothesis that the system was composed of chymotrypsin-like[1] and carboxypeptidase A-like activities for which evidence was reported (Meyer & Reed, 1975). These two enzymatic components of the phenylalanine releasing enzyme system appear to be located in mast cells scattered through the muscle, and the chymotrypsin-like insoluble aromatic amino acid esterase is entirely distinct from the previously mentioned cytosolic aromatic amino acid esterase for which no proteolytic activity has been detected (W. L. Meyer, M. L. Douglas, J. P. Reed, and D. N. Weinberg, unpublished experiments). We here report on the purification and characterization of the carboxypeptidase A component from rat skeletal muscle. Reports on carboxypeptidase from rat liver (Haas & Heinrich, 1979) and rat peritoneal mast cells (Everitt & Neurath, 1980) have

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* Address correspondence to this author. W.L.M. dedicates his contributions in this work to Joseph S. Fruton, who has been a leader for many years in the field of proteases and whose guidance and support at the onset of my career in biochemistry long have been appreciated.

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[1] Enzymes discussed in this paper are classified (International Union of Biochemistry, 1979) as follows: chymotrypsin, EC 3.4.21.1; carboxypeptidase A, EC 3.4.17.1; lysozyme, EC 3.2.1.17; trypsin, EC 3.4.21.4; aldolase, EC 4.1.2.13; carboxypeptidase B, EC 3.4.17.2.

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appeared while this work was in preparation.

Materials and Methods

Materials. Rats were from an inbred colony maintained with occasional outcrossing by our research group. Although carboxypeptidase A (CPA)[2] activity of muscle increases moderately with age, animals of various ages were used in these studies. Rats were killed with a blow to the head, hind limb muscle was removed, and the muscle was used fresh or after storage for various periods at −20 °C. The following proteins were used (number of times crystallized indicated in parentheses) and, except as noted otherwise, were obtained from Sigma Chemical Co., St. Louis, MO: rabbit hemoglobin (2×), Pentex, Kanakee, IL; crystalline bovine serum albumin, M_r 68 000; hen's egg ovalbumin (5×), M_r 43 000; soybean trypsin inhibitor (3×), M_r 22 000; bovine pancreatic CPA, Allan (2×), M_r 34 500; bovine pancreatic α-chymotrypsin (3×); crystalline porcine pancreatic trypsin, M_r 23 500; egg white lysozyme (2×), M_r 14 300, Worthington Biochemical Corp., Freehold, NJ; crystalline rabbit muscle aldolase. CNBr-activated Sepharose 4B (beaded agarose gel) was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Affi-Gel 501 [*p*-(chloromercuri)benzoate linked to an agarose support through an amino propyl ether spacer arm] and P-100 beaded polyacrylamide gel chromatographic medium were obtained from Bio-Rad Laboratories, Richmond, CA. AcA 54 Ultrogel (5% acrylamide–4% agarose) was a product of LKB Instruments, Rockville, MD. Glycyl-L-tyrosine-azo-benzylsuccinic acid linked to Sepharose 4B through the glycyl nitrogen was bought from Pierce Chemical Co., Rockford, IL. Dichlorvos (Vapona brand of 2,2-dichlorovinyl dimethyl phosphate) was of technical grade or analytical standard obtained from Shell Development Co., Houston, TX. Hippuryl-L-phenyllactate, sodium salt, was purchased from Vega Biochemicals, Tucson, AZ. Other chemicals were of reagent grade obtained from commercial sources.

Potato CPA Inhibitor Affinity Chromatography Medium. CPA inhibitor was purified from russet Burbank potatoes by the method of Ryan et al. (1974). The inhibitor was attached to CNBr-activated Sepharose 4B by the method of March et al. (1974). The efficiency of coupling determined by recovery of material absorbing at 280 nm in gel washes was 80%. One milligram of inhibitor was incorporated per milliliter of settled gel volume.

Molecular Sieve Chromatography. AcA 54 Ultrogel was equilibrated with 2 M NaCl–5 mM Hepes, pH 7.5. A 1.6 × 115 cm column was used for molecular weight determinations, and a 2.5 × 58 cm column was used in the experiment described in Table II and Figure 3. Fraction volumes were measured by weighing, elution positions relative to that of blue dextran (M_r 2 000 000) were determined, and the data were analyzed according to Andrews (1965).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed with solutions and protein samples prepared according to Laemmli (1970), except that the separating and stacking gel buffers were devoid of the detergent and the cathode buffer contained 0.03% $NaDodSO_4$. Electrophoresis was carried out with an apparatus contstructed as described by Driedger & Blumberg (1978); 0.5-mm shims were used to increase sensitivity. An 8–15% acrylamide [acrylamide/bis(acrylamide) 19:1] gradient separating gel was employed in conjunction with a 5% acrylamide [acrylamide/bis(acrylamide) 99:1] stacking gel. The bottom of sample wells was 5 mm from the top of the separating gel. Electrophoresis proceeded at 200 V until 30 min after the bromphenol blue tracking dye moved off the gel.

For the removal of NaCl from muscle CPA samples, 2–20 EU of esterase activity was precipitated by a sufficient amount of 50% trichloroacetic acid to give a final concentration of 10%. After 10 min at 0 °C, the sample was centrifuged at 1000*g* for 10 min. The tube and pellet were washed with acetone, and centrifugation was repeated. The washed pellet was dissolved in 50 μL of sample buffer (Laemmli, 1970) and was placed at 100 °C for 2 min, and the entire sample was then delivered to the sample well in the electrophoresis apparatus. Gels were stained with 0.12% Coomassie brilliant blue G–50% methanol–10% acetic acid for 3–4 h and were destained overnight in 5% methanol–10% acetic acid. Destained gels were immersed in 70% methanol–15% glycerol for 3 min, were covered on both sides with dialysis membrane, and were pressed between several layers of filter paper for 4 h. Final drying was done for 4 h in a vacuum desiccator attached to an aspirator. Single channels were cut from the dried gels for scanning on a Photovolt Densicord recording electrophoresis densitometer by using a red filter and a 0.2 × 5 mm slit. In the experiment of Table II, the areas of two adjoining peaks were estimated by multiplying each peak height by twice the horizontal distance from the outside boundary of the peak to the vertical line drawn through the center of the peak from its apex to the base line; this horizontal distance was measured halfway between the apex and base line.

Protein. Protein was determined by a dye-binding method (Bradford, 1976) when in excess of 50 μg/mL and by reaction with fluorescamine (Udenfriend et al., 1972) when greater sensitivity was needed. Bovine serum albumin was the reference standard.

Enzyme Assays. All esterase, protease, and aldolase activities are expressed in enzyme units.[2] The standard assay for CPA was performed at 30 °C by using a Metrohm recording titrator operated in the pH-stat mode to maintain the pH at 6.8 with approximately 4 mM KOH titrant. The initial substrate concentration was 1 mM hippuryl-L-phenyllactate. Unless stated otherwise, muscle CPA was assayed at an initial NaCl concentration of 1.5 M; pancreatic CPA was measured in 0.3 M NaCl. Activity against *N*-acetyl-L-phenylalanine ethyl ester was assayed at initial concentrations of 10 mM substrate and 0.5 M NaCl. Activity against N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester was determined at initial concentrations of 1 mM substrate and 10 mM $CaCl_2$. The KOH titrant was standardized against potassium acid phthalate. Activities are based on the initial rates of ester hydrolysis observed.

Peptide substrates were prepared by incubating 200 mg of bovine serum albumin/mL or 100 mg of rabbit hemoglobin/mL with 0.01 their weights of α-chymotrypsin for 12 h at 37 °C in 0.1 M Tris adjusted to pH 8.5 with HCl. The chymotrypsin then was inactivated by the addition of dichlorvos to a final concentration of 20 mM.

Exopeptidase assays were performed in 5 mM Hepes, pH 7.5, at 21 °C. Activities were measured against 2 mM hippuryl-L-phenylalanine in 1.5 M NaCl, 1 mM hippuryl-L-arginine in 2.0 M NaCl, or chymotrypsin-treated hemoglobin or bovine serum albumin at concentrations of 10 mg/mL of 1.5 M NaCl. For assay, enzyme preparations were mixed with

[2] Abbreviations used: CPA, carboxypeptidase A; dichlorvos, 2,2-dichlorovinyl dimethyl phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; EU, enzyme unit, defined (International Union of Biochemistry, 1973) as that amount of enzyme which catalyzes the transformation of 1 μmol of substrate/min.

Table I: Purification of Muscle Carboxypeptidase A[a]

fraction	recovery of volume (mL)	activity (EU)	activity (%)	protein[b] (mg)	sp act. (EU/mg of protein)	purification (x-fold)
homogenate	935	365	100	18900	0.019	1
NaCl extract	95	295	80	107	2.8	146
$(NH_4)_2SO_4$	7.3	253	69	10.9	23	1220
mercurial-affinity[c]	5.0	121	33	0.145	840	44000

[a] Starting material was 103 g of fresh rat muscle, the purification procedure is described under Results and Discussion, and data are corrected for sampling losses. [b] Protein concentration was determined by the dye-binding method except for the last step where the fluorescamine method was employed. [c] See Figure 1 for elution profile.

substrate at 0 °C, and aliquots of 200 μL were removed. These aliquots were mixed with 200 μL of 10% trichloroacetic acid or were incubated at 21 °C for 10 min before addition of acid. Following centrifugation at 1000*g* for 10 min, 50-μL aliquots of supernates were assayed for phenylalanine (McCaman & Robins, 1962) or arginine α-amino groups (Udenfriend et al., 1972). Exopeptidase activity against the chymotrypsin-treated hemoglobin and albumin was assayed by measurement of the release of free phenylalanine. Aldolase was measured by the coupled assay described by Bergmeyer et al. (1974).

Kinetic Studies. Lineweaver–Burk plots (Segel, 1975) were used to obtain K_m and V_{max} values. Values were determined from the line obtained by linear regression of the data over the range of 0.1–0.3 mM for hippuryl-L-phenyllactate in 0.9 M NaCl and over a range of 1–5 mM for hippuryl-L-phenylalanine in 1.5 M NaCl. The rate constant k_{cat} was calculated with the expression $V_{max} = k_{cat}E_t$ where E_t is the number of moles of enzyme, using molecular weights of 39 300 for muscle CPA and 34 500 for pancreatic CPA.

Inhibition constants for 3-phenylpropionate were determined from Dixon plots (Segel, 1975) over the range of 0–1 mM 3-phenylpropionate at 2 and 4 mM hippuryl-L-phenylalanine. Linear regression was used to obtain best fits to the data.

Preparation of (Cd^{2+})*CPA.* A 1-nmol sample of muscle or pancreatic CPA was dialyzed against 1 mM $CdCl_2$–2 mM 8-hydroxyquinoline 5-sulfonate–1.0 M NaCl–5 mM Hepes, pH 7.5, for 12 h at 0 °C. The resulting (Cd^{2+})CPA samples were assayed with hippuryl-L-phenyllactate and hippuryl-L-phenylalanine by using the standard assay protocols.

Results and Discussion

Purification. Muscle from the hind legs of freshly killed rats was processed as rapidly as possible. All steps were at 0 °C except for the chromatography which was done at 4 °C. Muscle was homogenized in 8 volumes (v/w) of 400 mM KCl–10 mM dichlorvos–3 M urea–15 mM Hepes, pH 7.5, with a Waring blender operated at high speed for 2 min. After the solution was stirred for 10 min with a motor-driven impeller, the homogenate was centrifuged at 10600*g* for 25 min. The pellet was suspended in another 8 volumes of buffer by using a Tekmar Tissumizer at full speed for 2 min and was stirred and centrifuged as just described. The washed pellet was extracted with 0.75 volume (volume/original weight of muscle) of 2 M NaCl–20 mM dichlorvos–5 mM Hepes, pH 7.5, by using the Tissumizer at maximum speed for 2 min followed by rapid stirring for 1 h. After centrifugation as described above, the supernate was decanted through glass wool to yield the NaCl extract.

Solid $(NH_4)_2SO_4$ was added to the extract to a final concentration of 55% of saturation at 0 °C, and after the mixture was stirred for 1 h, it was centrifuged at 8000*g* for 30 min. The resulting pellet was extracted by rapid stirring for 1 h with enough NaCl-dichlorvos–Hepes buffer to give an enzyme concentration of 30 EU/mL. The ammonium sulfate fraction is the supernate obtained after centrifugation for 20 min at 19600*g* and decantation through glass wool.

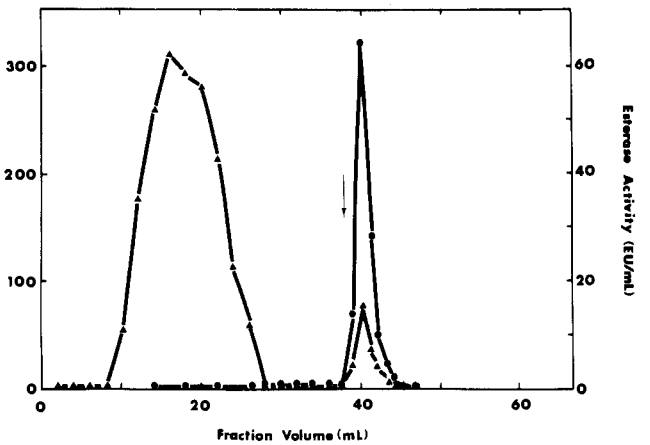

FIGURE 1: Purification of muscle carboxypeptidase A on coupled columns of organic mercurial and potato inhibitor. The $(NH_4)_2SO_4$ fraction of Table I was treated by the coupled-column procedures as described under Materials and Methods. Muscle CPA (253 EU) was applied, the mercurial column was disconnected after 30 mL of fractions was collected, and basic buffer was applied to the inhibitor column as the fraction represented by the arrow was collected. Protein (▲) was measured by the dye-binding method for the left-hand peak and by the fluorescamine method for the right-hand peak. CPA activity (●) was measured by the standard esterase assay. Although the leading edge of the left-hand peak was not assayed for CPA in this experiment, it was assayed in other experiments, and no activity was ever detected.

The ammmonium sulfate fraction was applied to a column containing the organic mercurial Bio-Rad Affi-Gel 501 with a bed volume of 10 mL; the effluent from the mercurial column was run into a column containing potato CPA inhibitor bound to Sepharose 4B with a bed volume of 1.5 mL. The coupled columns were washed with 30 mL of 2 M NaCl–5 mM Hepes, pH 7.5, the mercurial column was disconnected, and the inhibitor column was washed with 5–10 mL of the same buffer. Muscle CPA was eluted from the inhibitor column with 2 M NaCl–10 mM Na_2CO_3, pH 11.2. The eluate was collected in tubes containing 50 μL of 2 M NaCl–1 M Hepes, pH 7.5, per mL of eluate. The elution procedure is modified from that of Ager & Hass (1977). Figure 1 shows the results of a typical chromatographic run.

Typical results for the entire purification procedure are presented in Table I. Muscle CPA was purified 44 000-fold with 33% recovery to a final specific activity of 836 EU/mg of protein. The results for many trials of this procedure corresponded closely to these values.

Purification was always completed in 1 day because of the instability of the enzyme in crude fractions. Once purified, the enzyme lost 10% of its activity after storage for a month at −20 °C. In contrast, storage at the $(NH_4)_2SO_4$ fraction stage resulted in 20–35% loss of activity overnight at −20 °C.

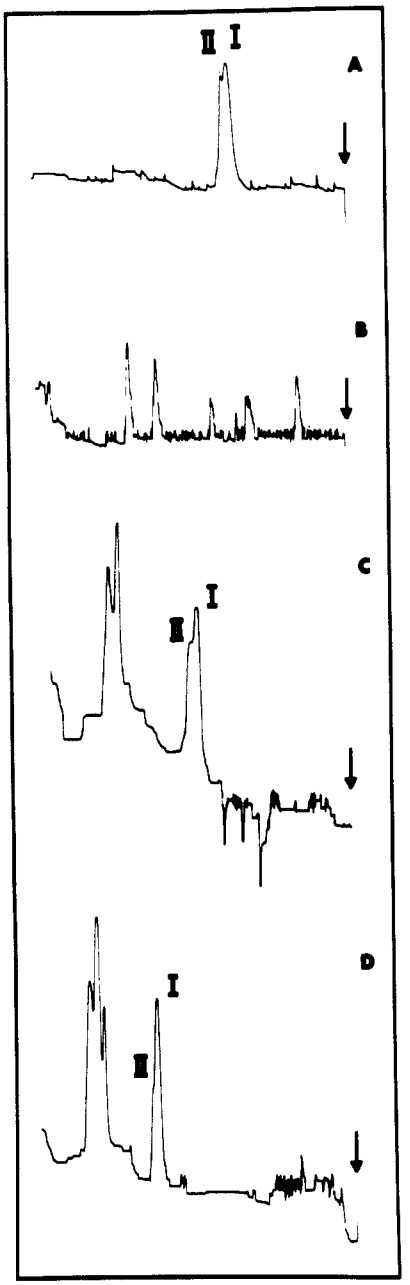

FIGURE 2: Gel electrophoresis of purified muscle carboxypeptidase A under denaturing conditions. The figure shows densitometer tracings of electrophoretograms of the following experiments: (A) 5 μg of muscle CPA purified according to the standard procedure; (B) standard proteins—lysozyme (1 μg), soybean trypsin inhibitor (1 μg), pancreatic CPA (1 μg), ovalbumin (1 μg), and bovine serum albumin (0.5 μg)—run on the same gel as (A); (C and D) two other experiments with 8-μg samples of muscle CPA purified in the absence of dichlorvos. The two peaks of muscle CPA protein are labeled I and II. The origin is indicated by the arrow.

A number of the features in the purification procedure deserve comment. Urea proved useful by preventing gel formation, thus eliminating the need for ultracentrifugation of homogenates and reducing the volume of extractant necessary to solubilize CPA from the pellet. Urea was used without special purification because passage of urea solutions through a mixed ion-exchange medium prior to use did not affect the results. Although 2 M NaCl was eventually chosen to solubilize muscle CPA in the standard preparation, 2 M KCl and 1 M $(NH_4)_2SO_4$ were effective also. An alternative to the potato inhibitor affinity column was one employing glycyl-L-tyrosine-azo-benzylsuccinic acid as the affinity agent [cf. Peterson et al. (1976)]. The results with such an agent

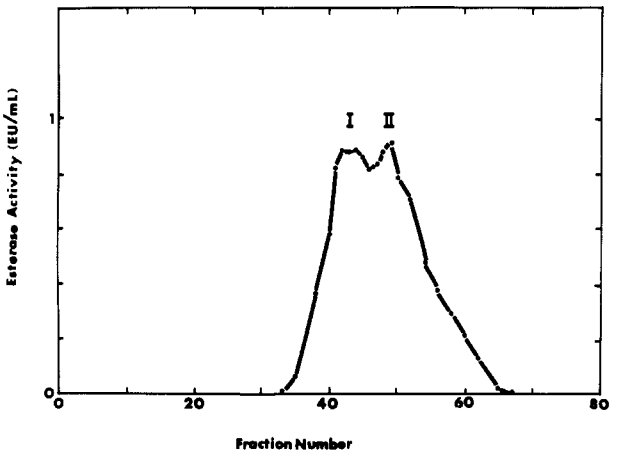

FIGURE 3: Molecular sieve chromatography of purified muscle carboxypeptidase A. A 50-EU aliquot of CPA in 2 mL was chromatographed with a flow rate of 30 mL/h, and 2-mL fractions were collected.

Table II: Partial Separation of Two Molecular Weight Species of Muscle Carboxypeptidase A by Molecular Sieve Chromatography[a]

fractions pooled[b]	13–14	15–16	18–19	20–21
total EU pooled	2.4	12.9	12.7	4.9
EU recovered after affinity concn of pool[c]	2.1	7.6	9.4	3.4
peak I area (mm^2)[d]	45	126	66	7.7
peak II area (mm^2)[d]	9.5	52	80	24.7

[a] A 3-mL aliquot of an $(NH_4)_2SO_4$ fraction of muscle CPA containing 78 EU was chromatographed as described in Figure 3 except that fractions of 5-mL volume were collected. [b] Fraction 17 was the center of the peak and was not pooled. [c] Pooled fractions were concentrated by the potato inhibitor column method used in the standard purification procedure (Table I) except that the mercurial column was not used. Concentrated pools were analyzed by $NaDodSO_4$ polyacrylamide gel electrophoresis as described under Materials and Methods. [d] The area of each peak from gel scans similar to those of Figure 2 was estimated by triangulation as described under Materials and Methods.

were effectively the same as with potato inhibitor, but the latter proved more convenient in use. The conditions for using the benzylsuccinic acid affinity column have been described, including a variety of solutions for eluting adsorbed muscle CPA from such columns (Bodwell, 1980). Treatment of muscle CPA with an organic mercurial column prior to affinity chromatography was important for success in using either type of affinity column. The mercurial column removed yellowish material which otherwise bound to the affinity column, and the mercurial treatment eliminated the appearance of low levels of CPA in the affinity column breakthrough fraction. The yellowish material was removed from the mercurial column by treatment with buffer containing 10 mM 2-mercaptoethanol as part of the column recycling procedure.

Purity. Muscle CPA at the final stage of purification was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2A). The preparation contained two components of similar molecular weight with no other detectable protein. Because of the denaturing conditions, it was not possible to determine whether one or both components represented the enzyme. Muscle CPA was subjected to gel chromatography (Figure 3) to attack this problem. Clearly, there were two peaks of activity. Evidence that the activity peaks thus obtained correspond to the two protein components noted after electrophoresis is presented in Table II. Although substantial losses of activity occurred during gel chromatography and affinity concentration and protein in the gel chromatography peaks of CPA could not be measured accurately, the exper-

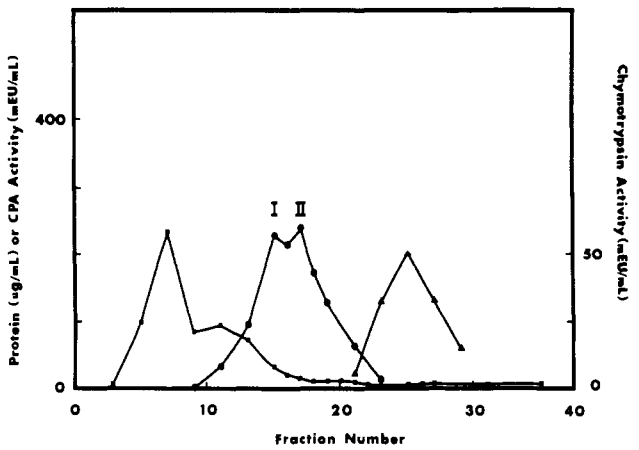

FIGURE 4: Partial separation of muscle carboxypeptidase A from a chymotrypsin-like contaminant by molecular sieve chromatography. An $(NH_4)_2SO_4$ fraction from a preparation made without serine protease inhibitors was used. The chromatographic medium was Bio-Rad P-100 in a 1.6 × 115 cm column with 1.3 M KCl–5 mM Hepes, pH 7.5, at a flow rate of 17 mL/h and with fraction volumes of 3 mL. A sample of 1.9 mL containing 6.6 EU of CPA was applied to the column. CPA (●) and chymotrypsin-like (▲) activities and protein (■) concentration determined by the dye-binding method were measured as described under Materials and Methods.

iment summarized in Table II is accounted for most readily if peak I and peak II proteins of gel electrophoretograms correspond to peaks I and II of CPA activity from gel chromatography. If this conclusion is valid, purified preparations of muscle CPA contain two CPA species and no other detectable protein. Well over a dozen preparations of muscle CPA were studied, and all contained peak I and II protein in the approximate ratio illustrated in Figure 2A.

Molecular Weight. Determination of molecular weights for four preparations of muscle CPA by $NaDodSO_4$–polyacrylamide gel electrophoresis yielded values of 39 300 ± 400 for component I and 37 800 ± 400 (mean ± standard deviation) for component II. Calculation of molecular weights corresponding to the activity peaks when two preparations of enzyme were studied by molecular sieve chromatography gave values of 39 300 ± 1000 and 35 700 ± 1000 for components I and II, respectively.

Effects of Endogenous Protease Activity. Muscle CPA is rather unstable at all stages of purity. The apparently greater instability of the enzyme in cruder fractions is consistent with a role for endogenous protease activity in the inactivation. CPA is accompanied by large amounts of chymotrypsin-like activity in the earlier stages of purification when dichlorvos is omitted from the procedure (Figure 4). The experiment presented in Figure 4, was not designed to measure molecular weight. Nonetheless, a crude estimate of molecular weight for the chymotrypsin-like activity gives a value in the 20 000 range. CPA and the chymotrypsin-like activity were completely separated in other molecular sieve experiments, and the latter activity was totally absent in experiments in which the preparation was treated with dichlorvos prior to chromatography. A trypsinlike activity was also detected in $(NH_4)_2SO_4$ fractions but was not sufficiently concentrated to be detected in gel chromatography fractions. The yield at the $(NH_4)_2SO_4$ step increases form 30–40% in preparations without dichlorvos to 60–80% when dichlorvos is included in the preparation. Further evidence consistent with proteolytic activity being responsible for loss of CPA is the appearance of protein bands of M_r 10 000–20 000 (compare scans A, C, and D, Figure 2) which invariably occurs when dichlorvos is omitted. Such low molecular weight bands are also seen in

Table III: Comparison of the Activities of Muscle and Pancreatic Carboxypeptidases A against a Variety of Substrates[a]

substrate	pancreatic CPA	muscle CPA
	Sp Act. (EU/mg of Protein[b])	
hippuryl-L-phenyllactate (1 mM)	1440 ± 108	850 ± 117
hippuryl-L-phenylalanine (2 mM)	149 ± 8	25.5 ± 3.4
chymotrypsin-treated bovine serum albumin (10 mg/mL)	12.4 ± 1.7	5.0 ± 0.6
chymotrypsin-treated hemoglobin (10 mg/mL)	15.5 ± 0.5	7.3 ± 0.4
	Loss of Activity (%)	
aldolase[c]	59	67

[a] Assays were as described under Materials and Methods. Results with aldolase are mean values for two digestions with pancreatic CPA and three digestions with muscle CPA. Other results are means ± standard deviations for four replicate assays. [b] Protein concentration was determined by the fluorescamine method. [c] Aldolase was assayed after 150 mEU had been incubated in 1.5 M NaCl–5 mM Hepes, pH 7.5, for 5 min at 21 °C with 2.4 mEU of CPA (measured with hippuryl-L-phenylalanine).

preparations made with rat muscle which is stored 1 month at −20 °C before use. Autodigestions of CPA in addition to serine protease action is a possible factor for the instability. However, no change in the $NaDodSO_4$–polyacrylamide gel electrophoresis pattern and no increase in the fluorescamine-reacting material could be detected when CPA purified by the standard procedure was incubated at room temperature for as long as 12 h. Such incubations did lead to complete loss of activity, pointing to the probability that inherent instability of muscle CPA also is an important mechanism. Preparations of muscle CPA were tested for trypsin-like activity (with N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester) and chymotrypsin-like activity (with *N*-acetyl-L-phenylalanine ethyl ester); neither was detectable at a lower limit of 20 mEU/mL. This represents less than 0.2% contamination (EU/EU) of muscle CPA with either of these types of endoprotease and might be expected in view of the use of dichlorvos in the purification procedure. Carboxypeptidase B-like activity (measured with hippuryl-L-arginine) was not detectable at a lower limit of 1 mEU/mL; thus, it represents less than a 0.01% contaminant (EU/EU) of purified muscle CPA. Carboxypeptidase B was a potential contaminant inasmuch as no carboxypeptidase inhibitors were used during purification, and carboxypeptidase B is known to purify with CPA on potato inhibitor affinity columns (Ager & Hass, 1977).

Substrate Specificity. In characterizing muscle CPA, it was extensively compared with bovine pancreatic CPA obtained commercially and subjected in our laboratory to a potato inhibitor affinity chromatography step identical with the final step in preparing muscle CPA except that the organic mercurial column was not used. The hydrolytic abilities of the muscle and pancreatic enzymes were compared against ester, dipeptide, protein hydrolysate, and protein substrates (Table III). Inactivation of aldolase by pancreatic CPA has been shown to be due to release of carboxyl-terminal tyrosine (Drechsler et al., 1959). Although the presentation in Table III makes it appear that muscle CPA is marginally more effective than the pancreatic enzyme in inactivating aldolase, when the data are recalculated on the basis of milligrams of CPA protein, the pancreatic enzyme appears to be about 5-fold more efficient. Over the range of substrates and conditions used for the experiments of Table III, the pancreatic enzyme appears to be 2–6-fold more efficient as a hydrolase.

Kinetics. Kinetic constants for muscle and pancreatic CPA are compared in Table IV. K_m values for the pancreatic

Table IV: Comparison of Kinetic Parameters for Muscle and Pancreatic Carboxypeptidases A[a]

substrate	muscle CPA, this work	pancreatic CPA this work	pancreatic CPA literature
hippuryl-L-phenyllactate[b]			
K_m (μM)	110	190	88[c]
k_{cat} (s^{-1})	933	1100	578[c]
hippuryl-L-phenylalanine[d]			
K_m (mM)	2.7	1.7	2.7[e]
k_{cat} (s^{-1})	40	160	145[e]

[a] K_m and k_{cat} values were determined as described under Materials and Methods by using data from Lineweaver-Burk plots (Bodwell, 1980). [b] In these experiments, both muscle and pancreatic CPA were assayed at pH 6.8 in 0.9 M NaCl; a pH of 7.5 and an ionic strength of 0.5 M were used in the literature experiments. [c] Bender et al. (1965). [d] In these assays, muscle and pancreatic enzymes were analyzed at pH 7.5 in 1.5 M NaCl; 1 M ionic strength and pH 7.5 were used in the literature experiments. [e] Davies et al. (1968).

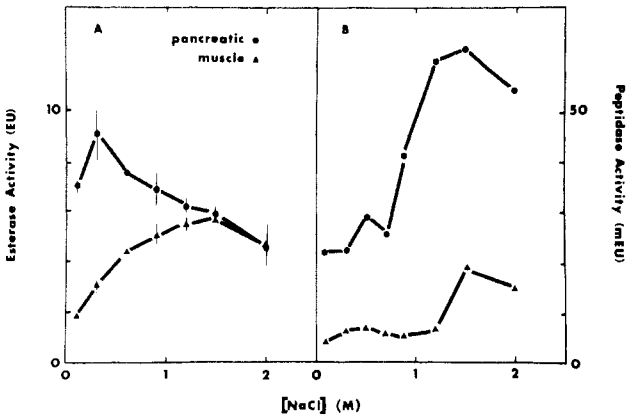

FIGURE 5: Effect of NaCl concentration on pancreatic and muscle carboxypeptidase A activity. A standard amount of muscle (▲) and pancreatic (●) enzymes was assayed with ester (A) and dipeptide (B) substrates as described under Materials and Methods except that NaCl concentration was varied as indicated. Peptidase values are from single determinations; esterase values are averages of duplicate determinations with the range of individual values indicated by the vertical lines where the range is greater than the width of the symbol.

enzyme are in good agreement with the literature although K_m for the ester is somewhat higher. This may be an effect of salt concentration or pH (see next section). The k_{cat} values are somewhat higher than have been reported. This may be due to the affinity column treatment, which did enhance the specific activity of the commercial CPA. The data confirm those of Table III in indicating the superiority of pancreatic enzyme for cleavage of the dipeptide. However, they establish the approximate equivalency of muscle and pancreatic enzymes as esterases. The greater apparent inferiority of muscle CPA as an esterase in the data of Table III is due to differences in assay conditions between Tables III and IV, the somewhat higher molecular weight of muscle enzyme, and the somewhat greater sensitivity of muscle enzyme to substrate inhibition which is a factor at the ester substrate concentrations used for Table III. Both muscle and pancreatic CPA exhibited significant substrate inhibition at ester concentrations of 1 mM and above and some substrate activation at dipeptide concentrations greater than 5 mM (Bodwell, 1980). Both phenomena are known characteristics of pancreatic CPA (Petra, 1970).

Effects of Ionic Strength, pH, and Temperature. The influence of ionic strength on esterase activity was different for muscle and pancreatic CPA (Figure 5A). The optimum for

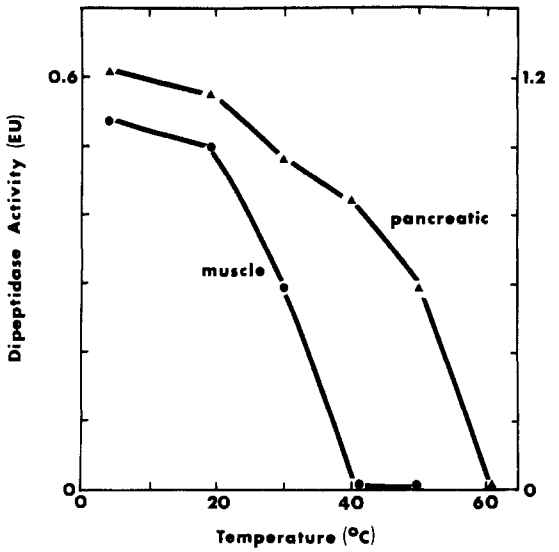

FIGURE 6: Thermal stability of muscle and pancreatic CPA's. Samples of CPA in 2 M NaCl–5 mM Hepes, pH 7.5, were incubated for 10 min at the indicated temperatures and then were placed on ice. Within another minute, samples were assayed by using hippuryl-L-phenylalanine as substrate. The scale for muscle CPA (●) is on the left; that for pancreatic CPA (▲) is to the right.

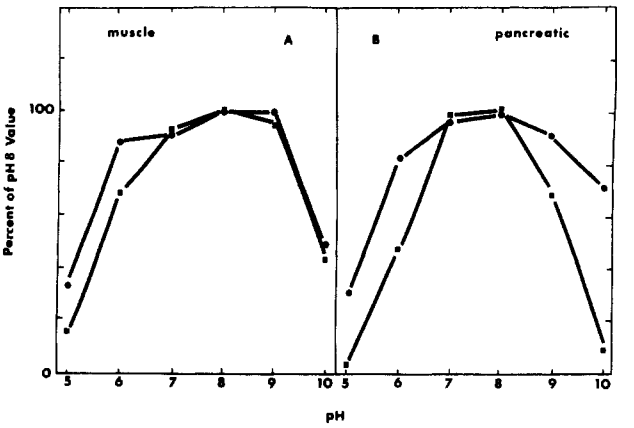

FIGURE 7: Effect of pH on activity and stability of muscle and pancreatic CPA's. For stability studies (●), samples of muscle (A) or pancreatic (B) CPA were mixed with equal volumes of 2 M NaCl–300 mM buffer to give mixtures at final pH values of 5.07 (acetate), 6.09 [2-(*N*-morpholino)ethanesulfonate], 7.04 [3-(*N*-morpholino)propanesulfonate], 7.95 (Hepes), 8.93 (Tris), or 10.0 (glycine). The diluted enzyme was incubated at 21 °C for 10 min, and an aliquot was assayed at pH 7.5 under standard dipeptidase assay conditions except that Hepes buffer was increased to 100 mM. For the determination of pH–activity profiles (■), CPA samples were assayed with hippuryl-L-phenylalanine under standard dipeptidase assay conditions except that 100 mM assay buffers at the indicated final pH values were used: pH 5.0 (acetate), pH 6.0 [2-(*N*-morpholino)ethanesulfonate], pH 7.0 [3-(*N*-morpholino)propanesulfonate], pH 7.0 [3-(*N*-morpholino)propanesulfonate], pH 8.0 and 9.0 (Tris), pH 10.0 (glycine).

pancreatic enzyme was 0.3 M NaCl whereas muscle CPA exhibited an optimum at 1.5 M salt. Effects of ionic strength on dipeptide hydrolysis were more nearly the same, with both enzymes showing optima between 1 and 2 M NaCl (Figure 5B).

As the data of Figure 6 indicate, muscle CPA is substantially more heat sensitive than its pancreatic counterpart. The muscle enzyme loses 50% of its original activity in 10 min at 30 °C whereas a temperature of 50 °C is required to induce a similar loss in pancreatic CPA.

The effects of pH on CPA stability and activity are compared for the muscle and pancreatic enzymes in Figure 7. Both enzymes are reasonably stable in the range from pH 6

to 9. The effects of pH on activity are remarkably close to those for stability between pH 7 and 10 for muscle CPA. Such a close correspondence occurs only between pH 7 and 8 for the pancreatic enzyme. The data are consistent with the possibility that the catalytic process for muscle CPA is relatively pH independent over a rather broad range. The pH–activity profile reported here for bovine pancreatic CPA and hippuryl-L-phenylalanine is somewhat broader than that with carbobenzoxyglycyl-L-phenylalanine (Riordan & Vallee, 1963) and is nearly identical with that for CPA of human pancreatic juice acting on hippurylglycylphenylalanine (Peterson et al., 1976).

Inhibition. A classic noncompetitive inhibitor of pancreatic CPA is 3-phenylpropionate, with a reported inhibition constant, K_i, of 190 μM (Auld & Vallee, 1970). We find noncompetitive inhibition constants of 210 and 810 μM, respectively, for pancreatic and muscle CPA [see Materials and Methods and Bodwell (1980)]. Another feature of pancreatic CPA is that benzyl alcohol and other competitive inhibitors of esterase activity act as activators of dipeptidase activity (Vallee & Riordan, 1968). When assayed under our conditions in the presence of 50 mM benzyl alcohol, we found 35% of control rates for esterase activity of both pancreatic and muscle enzymes whereas dipeptidase activity was 231% of control for muscle CPA and 180% of control for its pancreatic homologue (Bodwell, 1980). Vallee & Riordan, using similar but not identical substrates and assay conditions, recorded corresponding values of 35% of control esterase activity and 263% of control dipeptidase activity for pancreatic CPA.

Role of Metal Ions. Pancreatic CPA is a metalloprotein containing 1 mol of Zn^{2+} per mol of enzyme. Chelating agents such as 1,10-phenanthroline and 8-hydroxyquinoline 5-sulfonate will remove the metal ion to produce the apoenzyme. Inactivation curves for pancreatic and muscle CPA in 1.8 M NaCl at pH 7.5 were very similar and gave essentially complete inactivation at 0.5 and 1.0 mM 1,10-phenanthroline (Bodwell, 1980). These results are quantitatively similar to those reported for pancreatic CPA by Coombs et al. (1962).

Various metal ions upon substitution in pancreatic CPA produce substantial effects on esterase and dipeptidase activities. In experiments comparing partially purified muscle CPA with commercial pancreatic enzyme (W. L. Meyer, M. L. Douglas, J. P. Reed, and D. N. Weinberg, unpublished experiments), we found close correspondence in the effects of various metal ions on the activities of both CPA species (Meyer & Reed, 1975) and good agreement with published data on the pancreatic enzyme (Coleman & Vallee, 1961). Because the apoenzyme from muscle is very labile, we were forced to substitute metals by chelator-assisted exchange dialysis. In work with purified muscle CPA, we chose substitution with Cd^{2+} because its effect is particularly characteristic (Coleman & Vallee, 1961). When treated with Cd^{2+} as outlined under Materials and Methods, pancreatic CPA lost 98% of its dipeptidase activity, and esterase activity was 116% of its original value. The esterase to dipeptidase ratio, therefore, was increased from 7.2 to 435. Muscle enzyme, on the other hand, lost 48% of its esterase and 98% of its dipeptidase activity. Thus, its esterase/dipeptidase ratio rose from 15.6 to 370. The loss of esterase activity is probably due to dialysis alone inasmuch as dialysis for 12 h under a variety of conditions generally results in a 40–60% decrease in muscle CPA activity.

Activation. During development of the purification procedure, it was noted that certain substances enhanced the esterolytic activity of CPA. These preliminary observations are summarized in Tables V and VI. Substances containing

Table V: Stimulation of Carboxypeptidase A Esterolytic Activity by Indoles and Imidazoles[a]

effector	effector concn (mM)	activity[b] (% of control)
imidazole	0.01	110
imidazole	0.1	121
imidazole	1.0	140
imidazole	10.0	192
imidazole	1.0	122[c]
1-methylimidazole	1.0	96
2-methylimidazole	1.0	131
L-histidine	1.0	114
histamine	1.0	128
L-tryptophan	1.0	131
5-hydroxytryptamine	1.0	132
glycine	1.0	97
L-phenylalanine	1.0	98
4-aminobutyrate	1.0	98
2-aminoethanol	1.0	84
heparin	1.0[d]	102

[a] Except as noted, muscle CPA at the $(NH_4)_2SO_4$ step of purification was assayed in the presence of the effectors listed. [b] The standard esterase assay was performed except at 0.9 M NaCl and 5 mM hippuryl-L-phenyllactate concentrations. [c] With commercial pancreatic CPA without further treatment. [d] mg/mL.

Table VI: Stimulation of Carboxypeptidase A Esterolytic Activity by Incubation with $MnSO_4$[a]

Mn^{2+} concn (M)	activity (mEU/mL)[b] at incubation time (h)		
	0	1	2
0	814		516
0.05	619		1660
0.25	780		2609
0.5	907		2526
1.0	938		2646
1.5[c]	893		942
1.0	903	2486	
1.0[d]	1164	2127	

[a] Except as noted, muscle CPA was used. The muscle enzyme was prepared from 3 mL (94 EU) of the $(NH_4)_2SO_4$ fraction by gel filtration as described in Figure 3. The resulting preparation (1345 mEU/mL) was mixed with an equal volume of $MnSO_4$ solution (except as noted) or 2 M NaCl–5 mM Hepes, pH 7.5, for the 0 M Mn^{2+} point. The mixtures were incubated at 19 °C, and samples were assayed at the times indicated. [b] Activity was determined with the standard esterase assay except at 0.9 M NaCl and 5 mM hippuryl-L-phenyllactate and is expressed in terms of mEU/mL of incubation mixture. [c] Substituting $MnCl_2$ for $MnSO_4$. [d] Commercial pancreatic CPA used without further treatment.

the imidazole or indole nucleus in which the ring nitrogen(s) is (are) unsubstituted generally increase activity by 10–90% when incorporated into the assay. Such substances include histidine and tryptophan and their derivatives, histamine and 5-hydroxytryptamine (serotonin), which occur at appreciable levels in mast cells. No other aromatic or aliphatic amino acid or amine tested activates CPA esterolytic activity, and heparin, another mast cell constituent, likewise has no activating effect. Pancreatic as well as muscle CPA is susceptible to activation. Neither imidazole (1 mM in the absence of CPA) nor bovine serum albumin (1 mg/mL in the presence of imidazole) effects appreciable hydrolysis of the substrate under the conditions of the assay. Since the enzyme was assayed at a concentration of substrate at which substantial substrate inhibition occurs, it is possible that indoles and imidazoles acted to decrease unproductive substrate binding, thereby reducing inhibition. Some influence to produce an enzyme conformation more favorable for esterase activity is another possible mechanism.

Table VII: Solubility Studies of Muscle and Pancreatic Carboxypeptidases A[a]

sample	supernate activity (EU/mL)	pellet activity (EU/mL)	recovery of initial activity (%)
pancreatic enzyme	4.37	0.36	80
muscle enzyme	2.75	0.68	41
NaCl extract	0	0.89	86
pancreatic enzyme plus NaCl extract	2.47	0.72	99
muscle enzyme plus NaCl extract	0	3.3	86

[a] Purified pancreatic CPA (5.4 EU/mL), purified muscle CPA (6.6 EU/mL), muscle CPA in the NaCl extract fraction (1.0 EU/mL), and 1:1 mixtures of pancreatic or muscle CPA with the NaCl extract were dialyzed against 1000 volumes of 100 mM NaCl–5 mM Hepes, pH 7.5, for 5 h at 0 °C. The dialyzed preparations were centrifuged at 25000*g* for 20 min, and the supernates and pellets (dissolved in 2 M NaCl–5 mM Hepes, pH 7.5, to the volume of the sample before dialysis) were assayed for CPA activity.

A different kind of enhancement of the esterolytic activity of CPA is caused by $MnSO_4$. Unlike the imidazole effect, which is modest, is evidenced immediately in the assay, and is unaffected by incubation of CPA with imidazole prior to substrate addition, the effect of $MnSO_4$ is to cause up to a 3-fold increase in CPA esterase activity but only after a period of incubation of enzyme with the salt (Table VI). The effect of $MnSO_4$ is relatively specific. No increase of esterase activity is noted upon incubation of muscle CPA in 1.0 M $MgSO_4$, 1.5 M $MgCl_2$, or 0.5 M $ZnSO_4$. Little if any esterase enhancement is noted with $MnCl_2$ (Table VI). As indicated in the table, pancreatic as well as muscle CPA is stimulated substantially by $MnSO_4$. Substitution of manganous ion into holoenzyme or apoenzyme seems an unlikely explanation of the results for a number of reasons. The manganous form of pancreatic CPA has only 35% of the esterase activity of the native enzyme (Coleman & Vallee, 1961), and a corresponding value of 19% was found for muscle CPA (W. L. Meyer, unpublished experiments). The preparation used in these experiments represented 60% of the activity of the homogenate; however, the activity enhancement with $MnSO_4$ was far higher than could be expected for restoration of activity to the 40% of original activity lost during preparation. Furthermore, neither the failure of $MnCl_2$ to enhance activity nor the high concentrations of $MnSO_4$ required for maximal activation are readily explained by simple substitution into the active site of CPA. A time-dependent change of the enzyme into some more active conformation could account for the substantial enhancement of activity noted.

Solubility and Muscle Carboxypeptidase A Binding Factor. As indicated in the introduction, the insolubility of CPA in muscle preparations is an outstanding characteristic which both limits the procedures that can be applied to its purification and contributes to the efficiency of the purification protocol described herein. Purified muscle CPA, however, is found to have a much greater solubility than expected from its behavior in cruder fractions. For the exploration of this phenomenon, experiments like that of Table VII were conducted. At the extract stage, muscle CPA was practically insoluble under conditions where the purified enzyme was soluble to the extent of more than 2 EU/mL. Pancreatic CPA solubility was more than 4 EU/mL. Purified muscle enzyme again became insoluble in low salt when mixed with the NaCl extract. The data are consistent with the presence of a substance in muscle preparations which renders muscle CPA insoluble. Behavior during purifiction suggests that at high ionic strength muscle CPA and a presumed binding substance dissociate. Appreciable amounts of this substance appear to contaminate all but the final fraction in the purification protocol. The binding substance exhibits little, if any, insoluble complex formation with pancreatic enzyme. The data of Table VII indicate 85–100% recovery of CPA activity from dialyses in the presence of extract, whereas when purified pancreatic and muscle enzymes were dialyzed in the absence of extract losses of 20% and almost 60%, respectively, occurred.

General Discussion

This study reports the purification of carboxypeptidase A from muscle by a four-step, high-yield procedure (Table I). From the data, it is calculated that rat muscle contains 4–5 mg of CPA/kg of tissue. Rat muscle CPA is remarkably similar to bovine pancreatic CPA in the majority of its properties. These include the following: (a) apparent metal ion requirement and the effects of metal ions on catalytic specificity (see Results and Discussion), (b) substrate specificity for esters, peptides, and proteins (Table III), (c) kinetic interaction with substrates and molecular activity (Table IV), (d) interaction with inhibitors as diverse as metal complexing agents, substrate analogues, and the proteinaceous inhibitor of carboxypeptidases from potato (Table I, Figure 1), (e) interaction, generally, with ester and peptide substrates as a fucntion of ionic strength (Figure 5), (f) stability and activity as a function of pH (Figure 7), (g) molecular weight range (Figures 2–4), and (h) the enhancement of esterolytic activity by imidazoles, indoles, and $MnSO_4$ (Tables V and VI).

Although carboxypeptidases A have been purified from the pancreatic tissue of a number of mammalian (Anson, 1937; Folk & Schirmer, 1963; Peterson et al., 1976; Marinkovic & Marinkovic, 1976) and nonmammalian species (Lacko & Neurath, 1970; Gates & Travis, 1973), there is a dearth of literature on nonpancreatic CPA. Other than our preliminary reports on muscle CPA (Meyer & Reed, 1975; Bodwell & Meyer, 1979), only the recent report of Everitt & Neurath (1980) on peritoneal mast cell enzyme describes what is clearly a mammalian CPA of nonpancreatic origin. The "novel SH-type carboxypeptidase in the inner membrane of rat-liver mitochondria" described by Haas & Heinrich (1979) is very likely, however, also a tissue CPA similar to, if not identical with, that described here.

Muscle CPA appears to be largely, if not exclusively, a component of muscle mast cells rather than of myocytes. This conclusion is based on a number of lines of evidence (Douglas, 1980), including the following: (a) its disappearance from the muscle of rats treated with mast cell degranulating agents, (b) its solubility and sedimentation behavior, (c) its correlation with tissue histamine levels in liver and muscle, after degranulation induced by a spectrum of agents, as a function of aging, and in diseases such as muscular dystrophy, (d) its persistence along with histamine in the muscle of mice after treatment with degranulating agents to which mice are generally resistant, and (e) the occurrence of an homologous activity in cells of rat peritoneal fluid which is released by treatment with degranulating agents in vivo and in vitro. It is entirely likely that the aforementioned liver "SH-type carboxypeptidase" of Haas & Heinrich (1979) is also of mast-cell origin since the behavior they report in studies on "subcellular" and "submitochondrial" localization is consistent with that expected of an enzyme located in the dense granules of mast cells. Parenthetically, the evidence that the liver carboxypeptidase is of a "novel SH-type"—inhibition by 100 μM Hg^{2+} and Cu^{2+} and inactivation by 3 mM *p*-(hydroxymercuri)benzoate—is not unequivocal. Inhibitions of CPA

by aromatic anions acting as substrate analogues are well-known, and high concentrations of heavy-metal ions can interact with proteins and substrates in many ways other than through sulfhydryl groups. Indeed, inhibition of CPA by various heavy-metal ions, including those utilized by Haas & Heinrich, has been known for many years and was thoroughly described by Coombs et al. (1962).

A major problem in purifying muscle CPA is its susceptibility to endogenous protease activity. Both trypsin-like and chymotrypsin-like (Figure 4) activities are present in muscle fractions and may be responsible for CPA lability. An endogenous serine protease plagued Haas & Heinrich and required the use of soybean trypsin inhibitor during the partial purification of liver carboxypeptidase. Similar precautions were taken by Everitt & Neurath (1980). The presence of high chymotrypsin-like activity in preparations containing CPA is not unexpected if the origin is, indeed, mast cells. A number of chymotrypsin-like enzymes have been isolated from a variety of rat tissues, including skin (Seppa & Jarvinen, 1978), liver (Jusic et al., 1976), muscle (Katunuma et al., 1975), and mast cells (Everitt & Neurath, 1979). It is reported that all of these enzymes are located in the granules of the mast-cell component of these tissues (Woodbury et al., 1978; Everitt & Neurath, 1979). The chymotrypsin-like activity (Figure 4) of rat muscle (and of peritoneal cells) is depleted coincidently with CPA when rats are treated with degranulating agents (Douglas, 1980), and it is solubilized by 2 M NaCl along with CPA from muscle residue [this report; compare the joint solubilization of liver "mitochondrial SH-type carboxypeptidase" and serine protease by 2 M NaCl (Haas & Heinrich, 1979)].

The many essential similarities in catalytic characteristics between muscle and pancreatic CPA leave no doubt that they are homologous. There are, however, some interesting differences in their behaviors as proteins. That the muscle enzyme is more labile is readily apparent during purification and during various manipulations such as in alteration of its metal ion complement. This is directly documented in Figure 6, where a difference of 20 °C is evident in the midpoint of the thermal stability curves for muscle and pancreatic CPA.

Another interesting comparison is in molecular weights. Highly purified muscle CPA preparations contain two proteins detected in gel electrophoretograms (Figure 2). Although we have not yet unequivocally demonstrated CPA activity associated with each band, that likelihood seems high. The molecular weights of these bands correlate very closely with two peaks of CPA activity repeatedly observed during molecular sieve chromatography of muscle CPA (Figure 3). The two chromatographic peaks are relatively enriched in one or the other of the electrophoretic bands in the predicted manner (Table II). Both electrophoretic bands are bound to two different types of a carboxypeptidase-specific affinity matrix, one containing potato inhibitor, the other containing the substrate analogue, benzylsuccinate. The molecular weights of the muscle CPA species, 39 300 and 35 700–37 800, are reproducibly larger than that of pancreatic CPA (34 500) on both gel electrophoresis and chromatography. The muscle species have sizes in the range reported for native subunit I of bovine pancreatic proCPA [37 900–40 500 (Petra, 1970)]. The liver carboxypeptidase of Haas & Heinrich (1979) has a molecular weight in this same range, 38 000 estimated by gel filtration, although a value of 34 500 was determined by gel electrophoresis. CPA from limpets, which may have diverged in evolution before the development of the zymogen function, has a molecular weight of 40 000 (Hass, 1979). It is tempting to speculate that a peptide in muscle mast cell CPA analogous to that cleaved from pancreatic proCPA does not have a zymogen-conferring property but may have evolved to function in binding CPA within the mast cell (see below).

Bovine pancreatic CPA is known to occur in two allotypic forms (Petra, 1970) due to the occurrence of linked replacements of amino acid residues at three sites within the molecule. Such an explantion is unlikely to account for the two forms observed for muscle CPA; they differ sufficiently in molecular weight to differ by around 15 amino acid residues in total length. Furthermore, two forms in the same approximate proportion have been found in every one of many preparations from different rats; this would be unlikely if the forms were allelic variants. No evidence for zymogen precursors of muscle CPA has been observed. Despite innumerable experiments on the physiology, purification, and characterization of muscle CPA, we have not by design or otherwise detected any increase in measurable CPA activity. No evidence for a zymogen form of the mast cell chymotrypsin-like enzyme has been found either (Everitt & Neurath, 1979). Although it is conceivable that the apparently smaller muscle CPA protein is derived from the larger by some cleavage reaction, indication of such a conversion has not materialized. Both are always present, and their proportion does not seem to vary whether or not protease inhibitors are included during purification or when preparations are purposefully incubated under conditions leading to progressive loss of activity. When proteolysis is encouraged, although peaks I and II CPA remain in evidence, albeit diminished, apparent breakdown products do appear in electrophoretograms in the form of several peptides in the 10 000–20 000-dalton range (Figure 2). These smaller peptides are observed upon denaturing gel electrophoresis of CPA samples from the 30 000–40 000-dalton range of molecular sieve chromatography experiments and CPA samples from potato inhibitor–affinity or substrate analogue–affinity chromatography experiments. These results suggest that the peptides are fragments of CPA which remain associated under nondenaturing conditions and retain the binding properties of CPA. Whether they are enzymatically active is unknown. The most likely accounting remaining then for forms I and II of muscle CPA is that they are homologous products of genes evolved from an original precursor gene or that they are forms derived from a single gene product by some mechanism not commonly observed or perturbed during experimental manipulations.

Some interesting similarities and differences exist between the recent report on rat periotoneal mast cell CPA (Everitt & Neurath, 1980) and this report on rat muscle CPA. The pH–activity profiles with hippuryl-L-phenylalanine are nearly identical. Although the K_m and k_{cat} values with this substrate which they report for mast cell and pancreatic enzyme are 2–6-fold lower than those presented in Table IV, the most significant comparison is probably the agreement of both reports that under their respective conditions of assay pancreatic CPA is 3–4-fold more active than mast cell or muscle enzyme as a peptidase. Both reports are also in excellent agreement on the relative values for kinetic parameters for mast cell or muscle and pancreatic enzyme acting on hippuryl-L-phenyllactate. Again, the 2-fold higher k_{cat} values we report might be attributable to some effect of our preparation procedure as mentioned under Results and Discussion. The most puzzling difference is in the molecular weights. Everitt & Neurath find a molecular weight for peritoneal mast cell CPA similar to that of pancreatic CPA, whereas by two different techniques directly comparing muscle and pancreatic enzymes we find a difference of about 4000 daltons. Everitt & Neurath (1980)

used urea-containing gels to determine molecular weight, whereas we did not. Presumably, urea was used to minimize the possibility of errors due to protein aggregation (Everitt & Neurath, 1979). It is doubtful that the absence of urea accounts for the higher molecular weights we observed. In early experiments, we saw no effect of urea on the relative mobilities of carboxypeptidases and marker proteins. The general use of urea in $NaDodSO_4$–polyacrylamide gel electrophoresis has been characterized as unsound (Nielson & Reynolds, 1978), and counteracting effects on detergent binding and protein denaturation have been documented (Takagi & Kubo, 1979). The net result can be erroneous estimates of molecular weight. The presence of urea has been shown to lower molecular weights of some proteins (Abraham & Cooper, 1976) by about the amount the estimates of this report and those of Everitt & Neurath (1979) differ. Abraham & Cooper (1976) also noted conversion of a single band pattern in the absence of urea to a multiple banding pattern in the presence of urea. In this report, two bands of CPA are demonstrated in the presence of urea; Everitt & Neurath (1980) only describe one CPA species in its presence. Until more detailed studies are performed, it must be left conceivable that muscle and peritoneal mast cells contain different forms of CPA.

Perhaps the most interesting feature of muscle CPA is its apparently rather specific binding to a substance in crude preparations which is separated effectively by the coupled organic mercurial–affinity column technique (Table VII). Pancreatic CPA has little, if any, affinity for this substance. Perhaps the peptide analogous to that which confers zymogen status to pancreatic proCPA is involved in muscle CPA association with the binding substance. Experiments with the pancreatic zymogen could reveal whether the zymogen-conferring peptide confers affinity for the binding substance. Study of this presumed binding material holds substantial interest in the context of enzyme packaging in mast cell granules and granules in general, in relation to binding interactions of the histamine–serotonin–heparin type in mast cells, and in terms of the physiology of release of enzymes from mast cells.

The catabolic potential of the phenylalanine-releasing enzyme system—of which CPA and the chymotrypsin-like activity are presumably the key elements—in muscle is impressive. In homogenates, the endogenous system releases free phenylalanine residues and continues the process until at least 50% of these residues (Reed, 1975) and 25% of all amino acid residues are released as free amino acids (Meyer & Reed, 1975). Clearly, the system has a high capacity to contribute to tissue remodelling, to inflammation-related invasion of tissue, to extensive injury repair, and to pathological processes such as those in muscle-wasting diseases. What role it might play in the controlled catabolism of muscle during starvation or in the day-to-day turnover of muscle protein remains to be elucidated, although it is likely that it does not play a major role. Certainly, the activity of this proteolytic system, presumably mast-cell associated, is so overwhelmingly high that any study of proteolytic activity with whole muscle preparations is subject to great interpretative problems and must account for the muscle chymotrypsin-like activity and for the muscle CPA described in this report. It is likely that similar considerations apply to most solid tissues of higher organisms.

References

Abraham, G., & Cooper, P. D. (1976) *Anal. Biochem. 73*, 439–446.

Ager, S. P., & Hass, G. M. (1977) *Anal. Biochem. 83*, 285–295.

Andrews, P. (1965) *Biochem. J. 96*, 595–606.

Anson, M. L. (1937) *J. Gen. Physiol. 20*, 663–669.

Auld, D. S., & Vallee, B. L. (1970) *Biochemistry 9*, 602–609.

Bender, M. L., Whitaker, J. R., & Menger, F. (1965) *Proc. Natl. Acad. Sci. U.S.A. 53*, 711–716.

Bergmeyer, H. U., Gawehn, K., & Grassl, M. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) 2nd Engl. ed., Vol. I, pp 425–522, Academic Press, New York.

Bodwell, J. E. (1980) *Diss. Abstr. Int. B 41*, 160.

Bodwell, J. E., & Meyer, W. L. (1979) *11th International Congress of Biochemistry*, Toronto, Canada, July 1979, Abstr., p 234.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Coleman, J. E., & Vallee, B. L. (1961) *J. Biol. Chem. 236*, 2244–2249.

Coombs, T. L., Felber, J. P., & Vallee, B. L. (1962) *Biochemistry 1*, 899–905.

Davies, R. C., Riordan, J. F., Auld, D. S., & Vallee, B. L. (1968) *Biochemistry 7*, 1090–1099.

Douglas, M. L. (1980) M.S. Thesis, University of Vermont.

Drechsler, E. R., Boyer, P. D., & Kowalsky, A. G. (1959) *J. Biol. Chem. 234*, 2627–2634.

Driedger, P. E., & Blumberg, P. M. (1978) *Anal. Biochem. 87*, 177–186.

Everitt, M. T., & Neurath, H. (1979) *Biochimie 61*, 653–662.

Everitt, M. T., & Neurath, H. (1980) *FEBS Lett. 110*, 292–296.

Folk, J. E., & Schirmer, E. W. (1963) *J. Biol. Chem. 238*, 3884–3894.

Gates, B. J., & Travis, J. (1973) *Biochemistry 12*, 1867–1874.

Haas, R., & Heinrich, P. C. (1979) *Eur. J. Biochem. 96*, 9–15.

Hass, G. M. (1979) *Arch. Biochem. Biophys. 198*, 247–254.

International Union of Biochemistry (1973) *Enzyme Nomenclature*, Elsevier, Amsterdam.

International Union of Biochemistry (1979) *Enzyme Nomenclature*, Academic Press, New York.

Jusic, M., Seifert, S., Weiss, E., Haas, R., & Heinrich, P. C. (1976) *Arch. Biochem. Biophys. 177*, 355–363.

Katunuma, N., Kominami, E., Kobayashi, K., Banno, Y., Suzuki, K., Chichibu, K., Hamaguchi, Y., & Katsunuma, T. (1975) *Eur. J. Biochem. 52*, 37–50.

Lacko, A. G., & Neurath, H. (1970) *Biochemistry 9*, 4680–4690.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

March, S. C., Parikh, I., & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149–152.

Marinkovic, D. V., & Marinkovic, J. N. (1976) *Biochem. Med. 14*, 125–134.

McCaman, M. W., & Robins, E. (1962) *J. Lab. Clin. Med. 59*, 885–890.

Meyer, D. H., Meyer, W. L., & Kakulas, B. A. (1974) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33*, 1412.

Meyer, W. L., & Reed, J. P. (1975) *Fed. Prod., Fed. Am. Soc. Exp. Biol. 34*, 511.

Meyer, W. L., Little, B. W., Feussner, J. R., & Meyer, D. H. (1972) in *Research in Muscle Development and the Muscle Spindle* (Banker, B. Q., Przybylski, R. J., Van Der Meulen, J. P., & Victor, M., Eds.) pp 195–217, Excerpta Medica, Amsterdam.

Meyer, W. L., Smith, D. B., Stearns, K., & Little, B. W. (1973) in *Basic Research in Myology* (Kakulas, B. A., Ed.) pp 242–247, Excerpta Medica, Amsterdam.

Nielsen, T. B., & Reynolds, J. A. (1978) *Methods Enzymol. 48*, 3–10.

Nwizu, C. S., Meyer, D. H., & Meyer, W. L. (1974) *Int. Congr. Ser.-Excerpta Med. No. 334*, 7.
Peterson, L. M., Sokolvsky, M., & Vallee, B. L. (1976) *Biochemistry 15*, 2501–2508.
Petra, P. H. (1970) *Methods Enzymol. 19*, 460–503.
Reed, J. P. (1975) M.S. Thesis, University of Vermont.
Reed, J. P., & Meyer, W. L. (1974) *Int. Congr. Ser.-Excerpta Med. No. 334*, 46.
Riordan, J. F., & Vallee, B. L. (1963) *Biochemistry 2*, 1460–1468.
Ryan, C. A., Hass, G. M., & Kuhn, R. W. (1974) *J. Biol. Chem. 249*, 5495–5499.
Segel, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.
Seppa, H. E. J., & Jarvinen, M. (1978) *J. Invest. Dermatol. 70*, 84–89.
Takagi, T., & Kubo, K. (1979) *Biochim. Biophys. Acta 578*, 68–75.
Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., & Weigele, M. (1972) *Science (Washington, D.C.) 178*, 871–872.
Vallee, B. L., & Riordan, J. F. (1968) *Brookhaven Symp. Biol. No. 21*, 91–119.
Woodbury, R. G., Gruzenski, G. M., & Lagunoff, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2785–2789.

Molecular Characterization of Human Clathrin†

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ABSTRACT: Clathrin extracted from coated vesicles at pH 8.0 sediments as a single boundary with 8.1S sedimentation constant ($s^0_{20,w}$) of 8.1 ± 0.1 S. Sedimentation equilibrium gave a molecular weight (M_r) of 610 000 ± 30 000. The clathrin frictional ratio (pH 7.5) computed from $s^0_{20,w}$ and M_r is very large, i.e., 3.06 ± 0.18. Analysis of the circular dichroic spectrum in the far-ultraviolet showed that about half of the peptide residues are in a α-helical conformation. The molecular weight of a preparation of clathrin purified to homogeneity on a Sepharose CL-4B column in 6 M guanidine hydrochloride was 170 000 ± 26 000 by sedimentation equilibrium, which is in agreement with the values we and others obtained by sodium dodecyl sulfate gel electrophoresis. The 8.1S clathrin species may be regarded as the "native" promoter since (1) it is extracted from coated vesicles by an extremely mild procedure, (2) it is stable over considerable ranges of pH, temperature, and ionic strength, and (3) it readily polymerizes into characteristic closed lattice structures resembling those observed in coated vesicles in the electron microscope. The 8.1S clathrin molecule self-associates at pH 6.3 to form two very high molecular weight species with average sedimentation coefficients of 150 and 300 S. The sedimenting boundaries of both of these species have been analyzed to reveal their molecular heterogeneity. The two species observed by sedimentation velocity may correspond to the two sizes of coated vesicles previously reported to be present in some cells when observed by electron microscopy. Analysis of the sedimentation pattern in the ultracentrifuge also gives the amount of unreacted 8.1S clathrin from which the yield of polymerizable clathrin is obtainable. This methodology can therefore be employed to estimate the quality of the 8.1S preparation of clathrin and thereby affords an assay of its activity.

Coated pits are specialized regions of the plasma membrane that are characterized by an invagination of the cytoplasmic surface. Such regions have been recognized in many eukaryotic cells (Roth & Porter, 1964; Fawcett, 1965; Friend & Farquhar, 1967; Palade & Burns, 1968; Heuser & Reese, 1973; Pearse, 1975, 1976; Ockleford & Whyte, 1977; Goldstein et al., 1979) and have been referred to also as bristle coat areas. The bristle coat is believed to be the same coat observed in intracellular coated vesicles (Franke et al., 1976) which are thought to form by endocytosis of coated pits. Current evidence suggests numerous functions for coated pits and coated vesicles, including receptor-mediated endocytosis (Pearse, 1976; Ockleford & Whyte, 1977; Goldstein et al., 1979; Anderson et al., 1977; Gorden et al., 1978), specific exocytosis of newly synthesized protein (Ericsson, 1965; Dumont, 1969; Franke et al., 1976), and transfer of proteins (Maxfield et al., 1978; Roth et al., 1976) and hormones (Rodewald, 1973; Bradshaw, 1978; Hemmaplardh & Morgan, 1976; Anderson et al., 1978) among certain cellular organelles.

Evidence of the dynamic cellular physiology occurring at the coated pit and/or bristle coat areas has understandably led to much interest in these regions of membranes. Immunochemical and electron microscopic methods suggest the presence of clathrin in the coat of coated pits (Goldstein et al., 1979; Hemmaplardh & Morgan, 1976). Coated vesicles purified from tissue homogenates by differential centrifugation and sedimentation in sucrose gradients contain one major protein which is clathrin (Pearse, 1976) and several minor protein components (Goldstein et al., 1979; Blitz et al., 1977; Woods et al., 1978; Woodward & Roth, 1978; Keen et al., 1979).

The major protein present in the coated vesicle, and the one responsible for its coat structure, has been identified as clathrin by Pearse (1975, 1976) and verified by other investigators (Ockleford & Whyte, 1977; Blitz et al., 1977; Woods et al., 1978). Although the preparations of clathrin have been analyzed extensively by sodium dodecyl sulfate ($NaDodSO_4$)[1] gel electrophoresis, almost no description is available con-

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[1] Abbreviations used: Gdn·HCl, guanidine hydrochloride; $NaDodSO_4$, sodium dodecyl sulfate; NaMES, sodium 2-(*N*-morpholino)ethanesulfonic acid; EDTA, disodium ethylenediaminetetraacetate; Ag–Ab, antigen–antibody.

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