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Carboxypeptidase of *Streptomyces griseus*. Implications of Its Characteristics†

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ABSTRACT: Carboxypeptidase from *Streptomyces griseus* has been isolated by use of a new, highly efficient affinity chromatographic procedure. The enzyme isolated in this manner consists of a single polypeptide chain with a molecular weight of 41 200. It is reversibly inhibited by *o*-phenanthroline and other metal chelators and contains 1 g-atom of zinc/mol. The absorption and magnetic circular dichroic spectra of the cobalt-substituted enzyme are virtually identical with those previously observed for bovine carboxypeptidase A. Moreover, chemical modification studies suggest the importance of tyrosyl, arginyl, and glutamyl residues for catalytic activity, all of which have been demonstrated to be essential for the activity of bovine carboxypeptidase A. Importantly, the *S. griseus* carboxypeptidase exhibits unique properties not previously observed in other zinc carboxypeptidases. It contains 2 g-atoms of tightly bound calcium which appears to function in protein stabilization in concert with two disulfide bridges. In marked contrast to any of the metallocarboxypeptidases known presently, the *S. griseus* enzyme hydrolyzes C-terminal basic peptide substrates and their exact ester analogues with kinetic parameters comparable to those of the corresponding neutral C-terminal substrates. These properties of this bacterial enzyme, combined with its close mechanistic similarity to bovine carboxypeptidase A, suggest that it may be the postulated but yet to be identified intermediate between endopeptidases and the carboxypeptidases.

Proteolytic enzymes are known to operate by at least four different mechanisms, each characterized by a distinctive constellation of functional amino acid residues (Hartley, 1960; Neurath & Bradshaw, 1970). However, the data presently available cannot resolve whether each of the four classes originated separately and independently of the others or whether some had a common ancestor. Moreover, in those functional classes which contain both exo- and endopeptidases, an evolutionary relationship between them has not yet been demonstrated.

A metalloendopeptidase has been postulated to have given rise to both the vertebrate carboxypeptidases A and B via a single intermediate carboxypeptidase possessing the combined specificities of the A and B enzymes (Neurath & Bradshaw, 1970). However, such an intermediate has not been isolated and hence the evolutionary link between the metalloendopeptidases and the metallocarboxypeptidases has not been established. Why or when in the course of evolution a single carboxypeptidase with A and B specificities was abandoned in favor of multiple enzymes with more limited specificities is not known. If on an evolutionary time scale this event occurred late, a single functionally homologous exopeptidase

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corresponding to the ancestor of both carboxypeptidases A and B, having the specificities of each, might be expected in lower organisms such as bacteria.

Here we describe the large-scale isolation, purification, and characterization of a metallocarboxypeptidase from *Streptomyces griseus*, using a CABS-Sepharose[1] affinity resin which is highly efficient for the carboxypeptidases (T. J. Bazzone, L. Cueni, and M. Sokolovsky, unpublished experiments). The molecular weight, amino acid composition, and response to an active site-specific chemical reagent (L-BAMP) of this CABS-Sepharose purified enzyme differ from those reported earlier (Seber et al., 1976; Gage-White et al., 1977; Narahashi et al., 1977). In addition to its catalytically essential zinc atom, the enzyme contains approximately 2 g-atoms of Ca^{2+} which appears to stabilize its structure. Since this enzyme also exhibits dual specificity toward both neutral and basic substrates, it may provide the first evidence for the proposed evolutionary development of exo- and endopeptidases.

Materials and Methods

Pronase, grade B, a commercially available extract from *S. griseus*, was purchased from Calbiochem; Sepharose-4B, Sephadex G-50, electrophoresis calibration standards, and PAA 4/30 acrylamide gradient gels were from Pharmacia Fine Chemicals; *m*-phenanthroline was from G. F. Smith Chemical Co.; cyanogen bromide, *o*-phenanthroline, 8-hydroxyquinoline-5-sulfonic acid, tetranitromethane, phenylglyoxal, and *m*-chloroperbenzoic acid were from Aldrich Chemical Co., Inc.; *N*-hydroxysuccinimide and 5,5′-dithiobis(2-nitrobenzoic acid) were from Pierce Chemical Company; DL-benzylsuccinic acid was from Burdick and Jackson Co.; 2-furanacrylic acid and dipicolinic acid were from Eastern Organic Chemicals; Mes, Tris, Hepes, L-leucine, ε-aminocaproic acid, glycine, Z-Gly-Gly-Phe, Z-Gly-Gly-Nle, and Bz-Gly-Lys were from Sigma Chemical Corp.; and spectroscopically pure metal sulfates were from Johnson-Matthey. Furanacryloyl substrates were synthesized according to the method of Blumberg & Vallee (1975). Bz-Gly-Gly-Leu was synthesized as previously described (Auld & Vallee, 1970). All other peptides were from Vega-Fox Chemicals.

FA-Gly-Leu. FA-Gly-ONSu (Blumberg & Vallee, 1975) (5 mmol) was dissolved in 30 mL of dioxane to which was added a solution of L-leucine (5.3 mmol) in 50 mL of water containing 1.5 g of $NaHCO_3$. After the mixture was stirred for 3 h, the mixture was concentrated to 40 mL, acidified to pH 1 with 1 N HCl, and extracted with ethyl acetate. The ethyl acetate was dried with $MgSO_4$ and evaporated to dryness. The residue was dissolved in hot water from which yellow crystals appeared on cooling The product had a melting point of 204–205 °C and exhibited a single spot on thin-layer chromatography on silica gel when developed with butanol–acetic acid–water (4:1:1). Anal. Calcd for $C_{15}H_{20}N_2O_5$ (308.33): C, 58.43; H, 6.54; N, 9.09. Found: C, 58.31; H, 6.51; N, 9.10.

[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; OP, *o*-phenanthroline; FA, 2-furanacryloyl; Bz, benzoyl; CABS-Sepharose, ε-aminocaproyl-*p*-aminobenzylsuccinyl-Sepharose-4B; –ONSu, *N*-hydroxysuccinimide; MCD, magnetic circular dichroism; CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Z, *N*-carbobenzoxy; –OArg, arginic acid; –OPhe, β-phenyllactic acid; –OLeu, α-hydroxyisocaproic acid; HQSA, 8-hydroxyquinoline-5-sulfonic acid; Nle, norleucine; $NaDodSO_4$, sodium dodecyl sulfate; CPD, *S. griseus* carboxypeptidase; CPD-A, bovine carboxypeptidase A; CPD-B, bovine carboxypeptidase B; L-BAMP, *N*-bromoacetyl-*N*-methyl-L-phenylalanine.

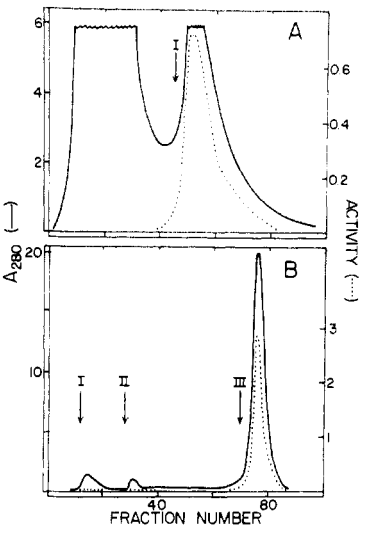

FIGURE 1: Purification of CPD by affinity chromatography on CABS–Sepharose. Commercial crude Pronase, desalted on Sephadex G-50 and equilibrated with 10 mM Mes and 5 mM $CaCl_2$, pH 6.0, was applied to the affinity column (2.6 × 8 cm; panel A). The column was washed with 10 mM Mes and 5 mM $CaCl_2$, pH 6.0, and the bound enzyme was eluted with 1 M NaCl at the same pH (I). The fractions containing activity were pooled and, after concentration by pressure dialysis, reapplied to the CABS-Sepharose column (panel B). The column was washed with 10 mM Mes, 5 mM $CaCl_2$, and 0.25 M NaCl, pH 6.0 (I), and with 10 mM Mes, 5 mM $CaCl_2$, and 0.50 M NaCl, pH 6.0 (II). Elution of the bound enzyme was accomplished with 0.1 M Tris and 0.2 M NaCl, pH 8 (III). Fraction size: 6.8 mL. Activity was measured by addition of 5 μL of eluate to 700 μL of 5 mM FA-Gly-Leu, and 0.1 M Tris, pH 8.0. Activity is expressed as micromoles hydrolyzed per minute.

FA-Gly-OLeu. Z-Gly was coupled to α-hydroxyisocaproic acid benzyl ester by the carbonyl bis(imidazole) method of Gisin et al. (1969). The oily product was hydrogenated catalytically in methanol at atmospheric pressure, 10% Pd/C, for 4 h, followed by evaporation of the methanol to yield a white powder. The resultant product, Gly-OLeu, exhibited a single yellow spot on thin-layer chromatography by use of ninhydrin for detection and was used without further purification. The product, Gly-OLeu (10 mmol), was dissolved in 30 mL of 50% dioxane–water to which 2.7 g of $NaHCO_3$ was added. Immediately, 8 mmol of FA-ONSu dissolved in 30 mL of dioxane was added, and the mixture was allowed to stir for 20 min. The reaction was then acidified to pH 1 with HCl, evaporated in vacuo to 20 mL, and extracted with ethyl acetate. The pooled ethyl acetate fractions were dried over $MgSO_4$ and evaporated to a brown oil. All attempts to crystallize FA-Gly-OLeu failed, and consequently the product was converted to the sodium salt by careful neutralization in 50% methanol–water to pH 7, followed by lyophilization to a tan powder. This product was a substrate for the enzyme, giving the spectral changes expected for cleavage of the ester bond (Holmquist & Vallee, 1976). It exhibited a single spot on thin-layer chromatography, indicating a minimum of 90% purity.

CABS-Sepharose was prepared by the method of T. J. Bazzone, L. Cueni, and M. Sokolovosky, (unpublished experiments).

Enzyme Isolation. Pronase (27.4 g) was dissolved in 10 mM Mes and 5 mM $CaCl_2$, pH 6.0, and the solution was adjusted to pH 6.0 with solid Mes. This material was passed through a Sephadex G-50 column (5.0 × 50 cm) equilibrated with 10 mM Mes and 5 mM $CaCl_2$, pH 6.0. Fractions with activity toward FA-Gly-Leu were pooled and applied to a CABS-Sepharose column (2.6 × 18 cm) equilibrated with 10 mM

Mes and 5 mM $CaCl_2$, as shown in Figure 1. The column was washed with the same buffer, and the bound enzyme was eluted with 10 mM Mes, 5 mM $CaCl_2$, and 1 M NaCl, pH 6.0. The eluate was concentrated by ultrafiltration in an Amicon cell equipped with a PM-10 membrane, then diluted 20-fold with 10 mM Mes and 5 mM $CaCl_2$, pH 6.0, and concentrated again to be reapplied to the affinity column equilibrated with 10 mM Mes and 5 mM $CaCl_2$, pH 6.0. The column was washed with two intermediate ionic strength buffers, 10 mM Mes, pH 6.0, 0.25 M NaCl and 0.50 M NaCl, and then the enzyme was eluted with 0.1 M Tris and 0.2 NaCl, pH 8.0.

Composition. An $A_{280}^{1\%}$ of 17.1 was obtained at two protein concentrations (2.7 and 2.0 mg/mL in 50 mM Tris, pH 7.5) based upon the refractive index by use of a synthetic boundary forming cell in a Spinco Model E ultracentrifuge.

Disc gel electrophoresis at pH 8.9 by use of 10% acrylamide (Davis, 1964) was performed by means of a Bio-Rad Laboratories Model 150A gel electrophoresis cell.

Polyacrylamide slab gel electrophoresis in the presence of 1% sodium dodecyl sulfate and 0.1% β-mercaptoethanol was performed according to the method of Weber et al. (1972) by use of a Bio-Rad Laboratories Model 220 apparatus. The gels contained 10% acrylamide and were stained with Coomassie blue.

Gradient gel electrophoresis was performed by use of Pharmacia PAA 4/30 polyacrylamide gradient gels with β-alanine–acetate buffer, pH 4.5, with a Pharmacia GE-4 gel electrophoresis instrument (Margolis & Kendrick, 1968).

Amino acid analyses were carried out with a Durrum Model D-500 amino acid analyzer. Tryptophan was determined by magnetic circular dichroism (Holmquist & Vallee, 1973). Cysteine was determined as cysteic acid after performic acid oxidation (Bailey, 1962). Free cysteine was estimated after reaction with DTNB (Ellman, 1959). Neutral sugar determinations were carried out by use of the phenol–sulfuric acid procedure (Dubois et al., 1956). The molecular weight of the enzyme was determined by sedimentation equilibrium (Yphantis, 1964) by use of a Spinco Model E analytical ultracentrifuge. The protein (0.35 mg/mL) was dialyzed against 10 mM Tris and 0.1 mM NaCl, pH 8.0, and the centrifugation was performed at 29 500 and 23 150 rpm for 16 h. The partial specific volume, 0.72, was determined from the amino acid composition (Cohn & Edsall, 1943).

Inhibition of the enzyme was examined after incubation with OP, HQSA, or dipicolinic acid for 20 h, 4 °C, followed by dilution into an assay mixture containing the same inhibitor concentration. These studies were performed in the absence of added calcium.

The zinc and calcium content of the enzyme was determined by flame (Fuwa & Vallee, 1963) and graphite furnace (Perkin-Elmer Model 603 manual) atomic absorption methods, respectively. The enzyme was dialyzed extensively against 50 mM Tris, pH 7.5, prior to metal measurements. Zinc-free enzyme (11 mg/mL) was prepared by dialysis against 20 mM Hepes and 10 mM OP, pH 7.5, followed by extensive dialysis against 20 mM Hepes, pH 7.5. Calcium-free enzyme was prepared by dialysis against 50 mM EDTA and 20 mM Hepes, pH 7.5, followed by extensive dialysis against buffer without EDTA and addition of a stoichiometric amount of zinc. The apoenzymes were reconstituted with spectroscopically pure metal salts. Calcium chloride solutions were extracted with dithizone to remove contaminating trace metals.

Kinetics. Activities were measured spectrophotometrically at 340 nm for the furanacryloyl substrates (Blumberg & Vallee, 1975) and at 256 nm for Bz-Gly-Arg, Bz-Gly-OArg, Bz-Gly-Lys, and Bz-Gly-OPhe (Sokolovsky, 1972) in 0.1 M Tris, pH 8.0, 25 °C. Peptidase activities toward all other N-blocked di- and tripeptides were determined by use of the automated ninhydrin assay of Auld & Vallee (1970). The purification of CPD was followed by measurement of activities against FA-Gly-Leu at 353 nm rather than at 340 nm because of the high initial absorbance at the substrate concentration used (5 mM).

The effect of Ca^{2+} on the thermal stability of CPD was determined by incubation of 2.2×10^{-6} M enzyme at 64 °C in 0.1 M Hepes, pH 7.5, containing various amounts of Ca^{2+}. Aliquots were assayed for peptidase activity toward 1 mM Bz-Gly-Lys, 0.1 M Tris, pH 8.0.

Throughout, precautions were taken to avoid contamination with adventitious metals (Thiers, 1957). Deionized distilled water was used to prepare all buffers which were extracted subsequently with 0.01% dithizone in CCl_4.

Spectra of the Cobalt Enzyme. Absorption spectra of the Co^{2+}-substituted enzyme were obtained with a Cary 219 spectrophotometer. MCD and CD measurements of the cobalt enzyme were performed by use of a Cary 61 circular dichroic spectropolarimeter equipped with a Varian Model V-4145 superconducting magnet energized by a Varian V-4106 superconducting magnet power supply. MCD measurements were made at a magnetic field strength of 40 kG. Absorption and MCD measurements were performed at an enzyme concentration of 0.26 mM.

Chemical Modifications. All chemical modifications were carried out at an enzyme concentration of 2.3×10^{-5} M according to procedures established for bovine carboxypeptidase A. Modification with tetranitromethane (Sokolovsky et al., 1966) was carried out at 0.1 mM TNM and 0.1 M Tris, pH 8.0. *N*-(Bromoacetyl)-*N*-methyl-L-phenylalanine (Hass et al., 1972) was used at 10 mM in the same buffer, and phenylglyoxal modification (Riordan, 1973) was carried out in 50 mM sodium bicarbonate, pH 7.5, at 20 mM reagent. Activities were measured against FA-Gly-Leu and FA-Gly-OLeu, 0.1 M Tris, pH 8.0, after 3 h of reaction. In all cases the unmodified enzyme was found to retain full activity when incubated without reagent.

Results

Enzyme Purification. Solutions of Pronase were passed through Sephadex G-50 before application to the CABS-Sepharose column to equilibrate the protein solution with the starting buffer for the affinity column and to remove a brown pigment which discolored the crude material. Carboxypeptidase activity was present only in the fractions eluting in the void volume of the Sephadex G-50 column. The pooled fractions were applied to CABS-Sepharose, and, after washing with the starting buffer to remove most of the inactive protein, the enzyme was eluted by addition of 1 M NaCl to the buffer (Figure 1A). The pooled activity peak was then concentrated, dialyzed, and reapplied to the CABS-Sepharose. A change of pH from 6.0 to 8.0 was used to elute the enzyme (Figure 1B). A one-step affinity chromatographic procedure was found to be insufficient, since elution of the enzyme at pH 8.0 resulted in the release of a small amount of brown pigment.

Table I summarizes the purification. From 27.4 g of Pronase, a total of 316 mg of purified enzyme was obtained with a recovery of 91% of the total activity toward FA-Gly-Leu.

In previous purifications of CPD, Z-Gly-Leu has been used as substrate. We have found the chromogenic substrate FA-Gly-Leu to be superior for this purpose since its hydrolysis

Table I: Purification of CPD

purification step	total protein[a] (g)	sp act.[b] [(μmol/min)/mg]	act. recovered (%)
starting material	15.0	1.16	100
Sephadex G-50	9.1	2.00	104
CABS-Sepharose I	0.564	33.0	106
CABS-Sepharose II	0.316	50.8	91

[a] Based on $A_{280}^{1\%} = 17.1$. [b] Measured from initial velocity of FA-Gly-Leu hydrolysis at 5 mM substrate.

Table II: Amino Acid Compositions

residue	mol/mol of enzyme: CPD	bovine CPD-A[e]	bovine CPD-B[f]
Asp	43	29	28
Thr	19[a]	26	27
Ser	33[a]	32	27
Glu	39	25	25
Pro	18	10	12
Gly	32	23	22
Ala	42	20	22
Val	20[b]	16	14
Met	9	3	6
Ile	18[b]	20	16
Leu	16	23	21
Tyr	22	19	22
Phe	11	16	12
His	6	8	7
Lys	11	15	17
Arg	23	11	13
Trp	9[c]	7	8
half-cysteine	4[d]	2	7

[a] Extrapolated to zero time. [b] Value after 72 h of hydrolysis. [c] Determined by MCD. [d] Determined as cysteic acid after performic acid oxidation. [e] From Bradshaw et al. (1969). [f] From Titani et al. (1975).

can be followed spectrophotometrically at a wavelength well removed from that where the background absorption of protein interferes.

Composition. CPD obtained in the above manner migrates as a single band on acrylamide disc gel electrophoresis at pH 8.9 in both the absence and presence of $NaDodSO_4$. A single band is also observed in gradient gel electrophoresis where its migration indicates a molecular weight of nearly 40 000. Yphantis high-speed sedimentation equilibrium centrifugation resulted in linear plots of ln c vs. r^2 at both 29 500 and 23 150 rpm, indicating homogeneity and yielding molecular weights of 41 300 and 41 200, respectively. A molecular weight of 41 200 was used for all calculations.

Table II shows the amino acid composition of CPD and also shows that it differs distinctly from that of CPD-A and CPD-B. The enzyme does not contain free -SH groups, as determined with DTNB; thus, the four cysteines are organized as two S–S bridges. The molecule does not contain neutral sugars and exhibits a ratio of A_{280}/A_{260} of 2.11, consistent with the absence of nucleic acid moieties. CPD isolated in this manner contains 1.1 ± 0.05 g-atoms of zinc and 1.9 ± 0.2 g-atoms of calcium per mol of enzyme, based on a molar absorptivity at 280 nm of 7.04×10^4 M^{-1} cm^{-1} calculated from the molecular weight of 41 200 and $A_{280}^{1\%}$ of 17.1 determined by refractive index in the synthetic boundary cell of the ultracentrifuge.

The three chelating agents OP, HQSA, and dipicolinic acid inhibit CPD in both a time-dependent and a reversible manner. The degree of inhibition depends upon the chelator concentration (Figure 2) with the following pK_I and $\bar{n}$ values (Coombs et al., 1962): OP, $pK_I = 3.6$, $\bar{n} = 1.6$; HQSA, $pK_I = 3.8$, $\bar{n} = 1.9$; and dipicolinic acid, $pK_I = 3.0$, $\bar{n} = 1.6$. Addition of excess Zn^{2+} to the inhibited enzyme restores activity instantaneously and completely in all three cases. Under identical conditions, the nonchelating isomer *m*-phenanthroline does not inhibit the enzyme.

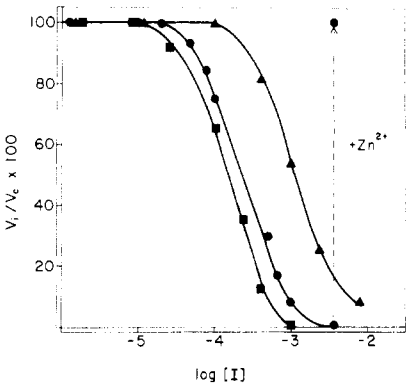

FIGURE 2: Inhibition of activity toward FA-Gly-Leu by OP (●), HQSA (■), and dipicolinic acid (▲). Enzyme (1.1×10^{-5} M) was incubated overnight in 0.05 M Tris, pH 7.9, with the indicated concentrations of chelator. The assay mixture contained the same concentration of chelator. Activity is completely restored in each case on addition of Zn^{2+}. It is indicated here by an arrow for the reversal of OP inhibition.

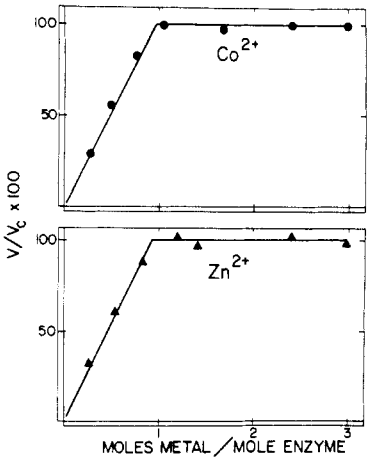

FIGURE 3: Titration of apoenzyme with Zn^{2+} or Co^{2+} in 0.1 M Tris, pH 7.5. Addition of Zn^{2+} or Co^{2+} to the apoenzyme, prepared by dialysis against OP (see Materials and Methods) restores activity instantaneously. Activity is monitored with FA-Gly-Leu. Full restoration of activity correlates exactly with 1 g-atom of metal/mol of enzyme.

Table III: Activities of Metal-Substituted CPD

	$V/V_{Zn} \times 100$: peptidase[a]	esterase[b]		$V/V_{Zn} \times 100$: peptidase[a]	esterase[b]
apoenzyme	0	0	Mn^{2+}	60	120
Zn^{2+}	100	100	Cd^{2+}	0	80
Co^{2+}	1100	110	Cu^{2+}	0	0
Ni^{2+}	80	10	Co^{3+c}	0	0

[a] 1 mM Bz-Gly-Arg and 0.1 M Tris, pH 8.0. [b] 1 mM Bz-Gly-O-Arg and 0.1 M Tris, pH 8.0. [c] The exchange inert Co^{3+} derivative was generated in situ by oxidation of Co^{2+}-CPD (2.3×10^{-5} M; 0.1 M Tris, pH 8.0) with 10^{-4} M *m*-chloroperbenzoic acid. Unreacted enzyme (10%) was removed by passage over the CABS-Sepharose column (Van Wart & Vallee, 1977). The Co^{3+}-CPD appeared relatively stable ($t_{1/2} > 24$ h) compared to CPD-A ($t_{1/2} < 2$ h).

Zinc is essential for both peptidase and esterase activities. Dialysis of CPD against OP reduces both the zinc content and the activity to less than 1% of the control. Restoration of

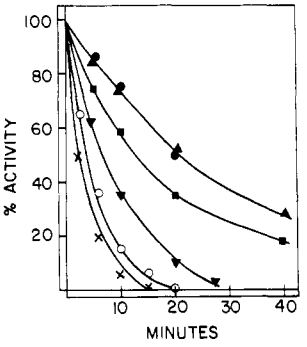

FIGURE 4: Effect of added Ca^{2+} on the stability of CPD (2.2×10^{-6} M) at 64 °C in 0.1 M Hepes, pH 7.5. Activity against 1 mM Bz-Gly-Lys, 0.1 M Tris, pH 8.0, was measured at 256 nm and expressed as percent of starting activity. (×) No added Ca^{2+}; (▼) 0.1 mM Ca^{2+}; (■) 1 mM Ca^{2+}; (▲) 5 mM Ca^{2+}; (●) 30 mM Ca^{2+}; (○) 30 mM Ca^{2+}, 50 mM dithiothreitol.

Table IV: Kinetic Parameters for CPD-Catalyzed Hydrolysis of Peptides

substrate	[substrate] (mM)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1} × 10^{-3})
FA-Gly-Leu	1–5	13	6300	480
Z-Gly-Leu	0.5–45	7.7	1600	210
Z-Phe-Leu	0.05–2	<0.05	2100	>42000
Z-Gly-Gly-Leu	0.2–23	0.57	2600	4600
Bz-Gly-Gly-Leu	0.4–20	0.57	810	1400
Z-Gly-Gly-Phe	0.5–22	0.96	87	91
Z-Gly-Gly-Nle	0.2–43	0.36	4100	11000

activity to the apoenzyme correlates exactly with the binding of 1 g-atom of Zn^{2+} or Co^{2+} per mol of enzyme (Figure 3). Table III shows the activities obtained on adding other metals, in 20% excess over enzyme, to the apoenzyme.

The 2 g-atoms of calcium in native CPD is not involved in enzymatic activity. Both the native enzyme and the enzyme containing only 1 g-atom of zinc but less than 0.1 g-atom of Ca^{2+} exhibit identical specific esterase and peptidase activities.

Calcium, however, significantly enhances the heat stability of CPD. In the absence of added Ca^{2+}, incubation at 64 °C abolishes peptidase activity completely within 15 min (Figure 4). Increasing concentrations of Ca^{2+}, up to 5 mM, progressively retard the rate of inactivation. Increasing the Ca^{2+} concentration from 5 to 30 mM has no further effect. Calcium stabilizes the zinc-free enzyme to the same degree as the native enzyme. However, in the presence of 50 mM dithiothreitol the stabilizing effect of added calcium is abolished (Figure 4).

Specificity. The substrate specificity of pure CPD was explored by investigation of the effects of variation of substrate structure on the hydrolytic kinetic parameters.

Comparison of the kinetic parameters for the dipeptides Z-Gly-Leu and FA-Gly-Leu indicates effects of the blocking groups on both K_m and k_{cat} (Table IV). Replacement of Gly in Z-Gly-Leu by Phe greatly reduces K_m while only slightly affecting k_{cat}. An increase in the length of the substrate by one Gly residue from Z-Gly-Leu to Z-Gly-Gly-Leu or Bz-Gly-Gly-Leu significantly improves kinetic efficiency (k_{cat}/K_m). The tripeptides with Leu or Nle in the C-terminal position are cleaved more efficiently than that with Phe. Only the C-terminal peptide bond of these tripeptides is cleaved; Z-Gly-Gly is not hydrolyzed. The enzyme will not hydrolyze substrates bearing a C-terminal Pro as in Z-Gly-Pro and Z-Ala-Pro nor will it hydrolyze those with acidic C-terminal amino acids such as Z-Gly-Asp. Since Z-Gly-D-Phe is not cleaved, the enzyme requires an L-amino acid in the C-terminal position.

Table V: Kinetic Parameters for the Hydrolysis of Matched Ester–Peptide Pairs by CPD

substrate	[substrate] (mM)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1} × 10^{-3})
FA-Gly-Leu	1–5	~13	6300	480
FA-Gly-OLeu	0.05–1	0.42	8400	20000
Bz-Gly-Arg	0.1–2	1.5	220	150
Bz-Gly-OArg	0.05–1	0.15	23000	150000
Bz-Gly-Phe	1–10	2.8	140	50
Bz-Gly-OPhe	0.2–1	0.61	15000	25000
Bz-Gly-Lys[a]	0.25–3	0.78	2000	2600

[a] The exact ester analogue of this substrate has not been synthesized yet.

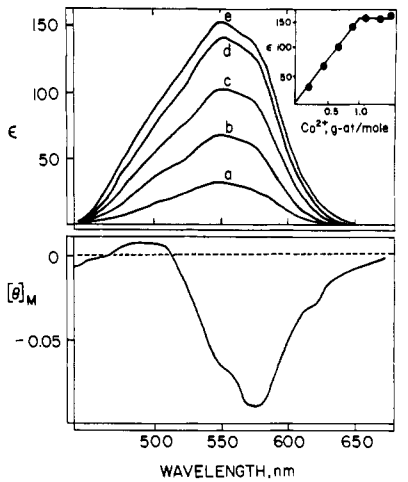

FIGURE 5: Absorption and magnetic circular dichroic spectra of cobalt CPD (2.61×10^{-4} M) in 20 mM Hepes, pH 7.5. Panel A. Spectral titration of apo CPD (20 mM Hepes, pH 7.5) with Co^{2+}. Visible absorption spectra were recorded after addition of 0.21 (a), 0.42 (b), 0.64 (c), 0.85 (d), and 1.05, 1.3, and 1.5 (e) g-atoms of Co^{2+} per mol of enzyme. The ϵ_{550} increases linearly up to 1 g-atom of Co^{2+} (inset). Panel B. MCD spectrum of the fully reconstituted cobalt enzyme was measured at 40 kG. The units of $[\theta]_M$ are deg cm^2 $dmol^{-1}$ G^{-1}.

CPD hydrolyzes the exact ester analogues of typical peptide substrates for mammalian carboxypeptidases A and B (Table V). The peptide analogues of these esters are hydrolyzed as well but with values of k_{cat}/K_m 2–3 orders of magnitude lower than their corresponding ester analogues. The Lineweaver-Burk plots from which the kinetic parameters for the substrates of Tables IV and V have been extrapolated are linear over the entire substrate concentration ranges examined.

Spectral Properties of the Cobalt Enzyme. The Co^{2+}-substituted enzyme exhibits a visible absorption spectrum with a maximum at 550 nm (ϵ = 154) with shoulders near 580 and 510 nm (Figure 5A). This spectrum correlates with the binding of 1 g-atom of cobalt (inset). Cl^-, a relatively weak ligand (Phillips & Williams, 1966), does not perturb the visible spectrum up to 0.7 M. On the other hand, N_3^- and CN^-, both strong ligands, shift the maximum to 580 nm (ϵ = 210) (not shown) when added in excess (0.05 M NaN_3 or 0.005 M NaCN). The MCD spectrum is characterized by a pronounced negative band at 575 nm and a smaller positive band near 495 nm (Figure 5B).

Chemical Modifications. The reagents *N*-bromoacetyl-*N*-methyl-L-phenylalanine, tetranitromethane, and phenylglyoxal were used under conditions previously employed for site-specific modification in CPD-A. All three reagents inhibit both the esterase and peptidase activities of CPD, but only the

enzyme modified with *N*-bromoacetyl-*N*-methyl-L-phenylalanine is completely devoid of both esterase and peptidase activities. Modification with tetranitromethane results in an enzyme with only 5% peptidase but 25% esterase activity. Similarly, phenylglyoxal inhibits both activities: 5% peptidase and 15% esterase activity remained after 3 h.

Discussion

The CPD purified by Seber et al. (1976) was reported to have a molecular weight of 30 300 as determined by ultracentrifugation. The molecular weight of 41 200 determined here, obtained by use of the same method and buffer systems employed previously (Seber et al., 1976), differs significantly. This may be due to limited proteolysis occurring in the course of the lengthy isolation procedure employed earlier, a conclusion supported by significant differences in the amino acid composition of the respective products. The enzymes prepared by the two different methods are similar only in regard to the presence of two S–S bridges.

The CPD from *S. griseus* described here is a metallopeptidase containing 1 g-atom of zinc and 2 g-atoms of calcium per mol of protein. The zinc is removed by OP, HQSA, and dipicolinic acid. The inhibition by these chelating agents is time dependent and is reversible by addition of zinc. Compared with most other zinc metalloenzymes, the pK_I of 3.6 for the inhibition by OP is unusually low (Auld et al., 1975). The number of inhibitor molecules bound to the metal, $\bar{n}$, exceeds 1 for all three inhibitors, consistent with metal ion removal (Coombs et al., 1962).

The zinc atom of CPD is required for activity since the zinc-free apoenzyme is devoid of both peptidase and esterase activities. However, other divalent metal atoms can substitute for zinc and restore esterase and peptidase activities, albeit to differing degrees (Table III). The cobalt(II), nickel, and manganese derivatives are all active esterases and peptidases while the cadmium derivative exhibits only esterase activity. The copper and cobalt(III) derivatives are inactive. Except for the rather high peptidase activity of the cobalt(II) derivative, this behavior resembles that of bovine carboxypeptidases A and B (Riordan, 1974; Van Wart & Vallee, 1977) and, in conjunction with the markedly different kinetic parameters for ester and peptide hydrolysis (vide infra), is consistent with differences in the mechanisms of ester and peptide hydrolysis, as reported for bovine carboxypeptidase A (Auld & Holmquist, 1974).

Like all other metallocarboxypeptidases, CPD requires a free C-terminal carboxyl group and the L configuration in the C-terminal position; CPD will not cleave C-terminal proline, glycine, or aspartic acid. CPD hydrolyzes N-blocked peptide substrates containing either a neutral or basic side chain in the C-terminal residue (Tables IV and V). Changes in blocking group from FA to Bz for dipeptides and from Z to Bz for the tripeptides predominantly affect k_{cat} in both instances (Table IV). Likewise, changing of the penultimate residue in Z-Gly-Leu to Phe affects primarily K_m and results in an enormous increase in k_{cat}. These effects are quite similar to those observed previously with CPD-A (Auld & Vallee, 1970; Klyosov & Vallee, 1977). However, a change in the C-terminal residue from Leu to Phe in the N-blocked tripeptides results in a large decrease in k_{cat} ($\sim$50-fold). This contrasts with CPD-A where k_{cat} is nearly identical for the two substrates but K_m for Z-Gly-Gly-Phe is more than 20-fold lower than for Z-Gly-Gly-Nle (Klyosov & Vallee, 1977). These differences likely reflect dissimilarities in the topographies of the C-terminal side-chain binding site of the two enzymes which may be related to the dual specificity of CPD for both neutral and basic substrates (vide infra).

Previous studies of the carboxypeptidase from *S. griseus* have suggested that this enzyme possesses a specificity much like a mammalian carboxypeptidase B as a result of its ability to cleave both neutral and basic carboxyl terminal amino acids (Gage-White et al., 1977; Narahashi et al., 1977). The more extensive kinetic studies here, while consistent with this previously observed behavior, indicate a unique aspect of this specificity, contrasting with any of the presently known carboxypeptidases: CPD exhibits kinetic parameters toward basic substrates which are comparable to those for neutral substrates (Table V). Thus, the K_m values for the three Bz-blocked peptides in Table V are closely similar. This behavior is analogous for the corresponding esters. On the other hand, for bovine carboxypeptidase B, which also cleaves both neutral and basic C-terminal residues, the binding of neutral substrates is orders of magnitude weaker than that for basic substrates [Alter et al. (1977) and references cited therein]. Thus, for human carboxypeptidase B the K_m value for the hydrolysis of Bz-Gly-Phe (70 mM) is 600-fold higher than for Bz-Gly-Arg (unpublished experiments). This property of CPD would seem consistent with the apparent absence in bacteria of a separate enzyme for basic substrates as elaborated by higher organisms; a single enzyme exhibiting dual specificity suffices.

When we allow for certain kinetic differences, CPD appears very similar to bovine carboxypeptidase A with respect to the geometry of the metal binding site, if spectra can serve as the bases for such a judgment; the visible absorption and MCD spectra of the two cobalt(II) enzymes are nearly identical. In CPD-A, the imidazole nitrogens of two histidyl residues and the carboxyl oxygen of a glutamic acid residue plus a water molecule constitute the zinc ligands (Quiocho & Lipscomb, 1971). They are arranged in a distorted tetrahedral (or pentacoordinate) coordination geometry (Latt & Vallee, 1971; Holmquist et al., 1975). Anions like N_3^- and CN^- perturb the spectrum of the cobalt(II)-CPD, indicating that the enzyme readily accommodates at least one ligand in addition to those contributed by the protein. Such spectral effects have not been observed in CPD-A, probably due to the high concentration of NaCl required for solubility (C. A. Spilburg, unpublished experiments). Anion effects have been seen in carbonic anhydrase in a more pronounced form and have similarly been attributed to binding to the active site metal (Lindskog, 1963, 1966).

The similarity in active site metal coordination in CPD-A and CPD indicated by the spectral data suggests that specific chemical modifications of active site residues might reveal mechanistic similarities among these enzymes. Indeed, the chemical modification data are consistent with the involvement of tyrosine, arginine, and glutamic acid in the catalytic activities of CPD and hence suggest mechanistic similarities to CPD-A (Sokolovsky et al., 1966; Riordan, 1973; Hass et al., 1972). These results, taken in conjunction with the spectra and kinetic evidence, suggest that, functionally, the CPD of *S. griseus* is very similar to the vertebrate carboxypeptidases. Thus, it seems that, as the species evolved, the mechanism of action of metallocarboxypeptidases remained similar. Specificity appears to be the primary functional feature to have changed, possibly due to specialized demands as organisms became more complex.

The 2 g-atoms of calcium in the enzyme apparently does not serve a catalytic role, since the native enzyme containing two bound calcium ions has the same specific activity as the calcium-free enzyme. In the absence of added calcium (Figure

5), incubation at 64 °C quickly inactivates the enzyme. However, addition of calcium markedly stabilizes the enzyme and suggests that calcium has a role in maintaining its tertiary structure. Until now, tightly bound calcium has not been detected in a carboxypeptidase from any organism.

From a structural point of view, CPD is unique in another way. The complete absence of disulfides and the presence of calcium in many bacterial extracellular enzymes have been noted (Pollock & Richmond, 1962; Matsubara & Feder, 1971) and have led to the suggestion that intramolecular cross-links involving calcium were the predecessors of disulfide bridges (Hsiu et al., 1964). The weaker calcium salt bridge probably emerged very early due to ready availability of this ion in the environment and the instability of disulfides in the reducing atmosphere likely prevalent during the very early stages of development of living organisms (Fahey et al., 1977). The transition from a reducing to an oxidizing environment allowed the development of the more stable disulfide linkages to replace the weaker calcium salt bridges. Thus, at present, higher organisms utilize disulfide bridges almost exclusively for stabilization of extracellular proteases. The *S. griseus* carboxypeptidase, however, contains *both* tightly bound calcium and disulfide bridges (two of each). Both serve to stabilize the enzyme, since high stability at 64 °C is only obtained if the disulfide bridges are intact (Figure 4) and excess calcium is present in the buffer. The bacterium *S. griseus* might represent one of the earlier aerobes to produce extracellular proteolytic enzymes equipped with disulfide bridges while at the same time retaining some additional benefits from its inherited calcium salt bridges.

Attempts to provide evidence that exopeptidases arose subsequent to and directly from endopeptidases have thus far not been possible due to the lack of an available endopeptidase–exopeptidase pair from a single organism. Metalloendopeptidases have not been found in the vertebrate pancreas and, conversely, metallocarboxypeptidases have not been widely detected in microorganisms. The presence of both a metalloendopeptidase (Morihara et al., 1968) and a metallocarboxypeptidase in *S. griseus* provides a system through which the postulated development of metallocarboxypeptidases from a metalloendopeptidase (Neurath & Bradshaw, 1970) can be examined critically. If both enzymes contain calcium as an integral part of their tertiary structure and, further, if overall structure homology is found, convincing evidence for or against a genetic relationship of these two enzymes would be in hand.[2] It would be of special interest to identify the alterations in the active site constellation responsible for the change in functionality from endo- to exopeptidases.

Since CPD has both dual specificity and virtually the same active site characteristics as the vertebrate carboxypeptidases, it may be functionally analogous to the proposed direct ancestor of CPD-A and CPD-B. However, because of the enormous phylogenetic gulf between bacteria and vertebrates, structural homology of CPD and the vertebrate enzymes need not exist. In fact, the great differences in the amino acid composition (Table II) and the absence of calcium in the vertebrate enzymes support this possibility. Where in the course of evolution the dually specific carboxypeptidase was abandoned to be replaced by two specialized enzymes and whether this happened before or after calcium was lost from the enzyme are not known. Isolation and characterization of carboxypeptidases from species intermediate between bacteria and vertebrates might answer these questions.

[2] Addition of terbium to the *S. griseus* carboxypeptidase results in enhancement of terbium fluorescence upon irradiation at 280 nm, characteristic of radiationless energy transfer from aromatic amino acid side chains. The fluorescence is quenched partly in the cobalt-substituted enzyme. This method has already been used to measure the intramolecular distances between structural and active site metals (Horrocks et al., 1975; Holmquist, unpublished experiments).

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Dissociation of Guanosine Nucleotide·Elongation Factor G·Ribosome Complexes†

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ABSTRACT: The spontaneous dissociation of complexes containing elongation factor G (EF-G), the ribosome, and either GDP plus fusidic acid, guanyl-5′-yl imidodiphosphate, or guanyl-5′-yl methylene diphosphonate has been measured and it follows biphasic kinetics that can be resolved into two first-order decay rates. This suggests the existence of two classes of complexes with apparent dissociation rate constants (k) differing 5–20-fold. The values of k and the distribution of complexes between the fast and the slowly decaying class depend on the conditions in which the dissociation occurs but not on the conditions in which the complexes are formed. Rapid transitions of complexes from one to the other class occur only when the chemical environment in which the dissociation takes place is modified. Thus, increasing the concentration of NH_4Cl or adding the antibiotic thiostrepton accelerates the decay and converts slowly dissociating into fast dissociating complexes. In contrast, addition of misreading-inducing aminoglycoside antibiotics of the neomycin, kanamycin, streptomycin, and gentamicin (but not hygromycin) groups slows down the decay. For neomycin B at 10 μM, this effect is due to the conversion of fast into slowly decaying complexes. A model to explain the results involving conformational transitions of the complexes is proposed.

To study the function of elongation factor (EF) G,[1] an *Escherichia coli* protein involved in ribosomal translocation, the ability of the factor to interact with guanosine nucleotides and the ribosome in the absence of protein synthesis has been very valuable (for reviews, see Lucas-Lenard & Lipmann, 1971; Modolell & Vázquez, 1975). With GDP and with the nonhydrolyzable GTP analogues, guanyl-5′-yl methylene diphosphonate (Gpp(CH_2)p) and guanyl-5′-yl imidodiphosphate (Gpp(NH)p), the interaction leads to formation of ternary complexes containing 1 mol of each of guanosine nucleotide, EF-G, and ribosomes (Bodley et al., 1970; Lin & Bodley, 1976; Eckstein et al., 1971; San-Millán et al., 1977). Recent observations are consistent with an ordered mechanism for the formation and dissociation of ternary complexes: EF-G binds guanosine nucleotide first, and then the ribosome; the ternary complex spontaneously dissociates into a guanosine nucleotide·EF-G complex, which then dissociates into its components (Rohrbach & Bodley, 1976; Girbés et al., 1977a). Moreover, the ternary complex containing GDP is stabilized by fusidic acid and it is usually studied in the form of a quaternary complex containing one molecule of this antibiotic (Bodley et al., 1970; Willie et al., 1975).

To gain further insight into the mechanism of breakdown and the properties of the ternary and quaternary complexes, we have now studied their spontaneous dissociation under conditions in which reactions leading to complex re-formation and interactions between complexes and free constituents have been halted by high dilution. The experimental decay curves obtained are consistent with the existence of at least two classes of interconvertible complexes with different dissociation rates. Moreover, the influence of ionic environment, temperature, and antibiotics on the rates of decay and on the interconversion between complexes has been examined.

Experimental Procedure

Materials. Preparation of 1 M NH_4Cl washed *E. coli* MRE600 ribosomes and EF-G has been described elsewhere (Modolell & Vázquez, 1973; Parmeggiani et al., 1971). One A_{260} unit of ribosomes was assumed to be equivalent to 27.7 pmol (Koppel, 1974). Labeled guanosine nucleotides were from The Radiochemical Centre, Amersham. Their specific activities, determined by isotopic dilution (Modolell et al., 1973), were: [^{3}H]GDP, 7230 and 6820 cpm/pmol; [^{3}H]-Gpp(NH)p, 7860 and 4630 cpm/pmol; [^{3}H]Gpp(CH_2)p, 1040 cpm/pmol; they were adjusted to convenient, lower values by addition of known amounts of unlabeled nucleotides. Counting efficiency was 30%. Antibiotics were gifts from the following sources: thiostrepton, Squibb Institute for Medical Research (Dr. B. Stearns); sisomicin, verdamicin, gentamicin C_1, and gentamicin C_{1a}, Schering Corp. (Dr. Waitz); kanamycins A and B, Bristol Laboratories (Dr. F. Leitues); tobramycin and hygromycin B, Lilly Laboratories; neamine (= neomycin A), neomycins B and C, and bluensomycin, Upjohn Co.; ribostamycin, Maiji Seika Kaisha, Ltd., Japan (Dr. I. Matsuda); paromomycin, Parke-Davis.

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[1] Abbreviations used: EF-G, elongation factor G; Gpp(CH_2)p, guanyl-5′-yl methylene diphosphonate; Gpp(NH)p, guanyl-5′-yl imidodiphosphate.

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