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Differential Activation of Endopeptidase EC 3.4.24.15 toward Natural and Synthetic Substrates by Metal Ions

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The activity of endopeptidase EC 3.4.24.15 (thimet oligopeptidase, EP 24.15), as measured by cleavage of a quenched fluorescent substrate, 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-D-Lys (2,4-dinitrophenyl), was increased 2-3 fold by the addition of 1 mM Mn^{2+} or of 10 mM Ca^{2+}. The inhibitory capability of a specific EP. 24.15 inhibitor, N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate, was also increased at similar concentrations of these metal ions. However, the hydrolysis of naturally-occurring peptides, thought to be the physiological substrates for EP 24.15, was not affected by either Mn^{2+} or Ca^{2+}. These results suggest that the binding of synthetic analogs to the enzyme may differ significantly from the binding, and thus hydrolysis, of natural peptide substrates and caution against drawing conclusions about substrate interactions with the active site from data obtained with modified peptide ligands.

The metalloendopeptidase EC 3.4.24.15 (EP 24.15) is thought to participate in the metabolism of several neuropeptides (1, 2). Enzymatic activity of EP 24.15 can be measured by hplc analysis of degradation products of these peptides (3, 4), or by continuous spectrophotometric or fluorimetric assay with modified chromogenic or fluorogenic peptide-based substrates (1, 5).

This enzyme was initially characterized in rat brain (1), and was subsequently described in brain, muscle, testicular and embryonic tissue in a variety of species (2, 6-8). Zinc is absolutely required for enzymatic activity (1). The effect of other metal ions on activity on EP 24.15, particularly Mn^{2+} and Ca^{2+}, both in the presence and absence of Zn^{2+}, is somewhat contradictory (1, 8-11), possibly reflecting tissue differences and/or the presence of contaminating enzymes in even apparently-homogeneous samples. The availability of recombinant rat testes enzyme (12) has allowed us to characterize the effects of metal ions on highly-purified protein. We find that Mn^{2+} and Ca^{2+} at millimolar concentrations greatly enhance the activity of the enzyme toward artificial ligands, but not toward naturally-occurring substrates.

METHODS

Materials

Purified recombinant EP 24.15 (12) and double mutant EP 24.15 (C246S/C253S) (13) were the generous gifts of Prof. J. L. Roberts and Dr. M. J. Glucksman. The fluorescent substrate (QFS), 7-methoxycoumarin-4-acetyl-Pro-Leu-

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Abbreviations: EP 24.15, endopeptidase EC 3.4.24.15; GnRH, gonadotropin-releasing hormone; hplc, high performance liquid chromatography; TBS, Tris-buffered saline; TFA, trifluoroacetic acid; DTT, dithiothreitol; QFS, quenched fluorescent substrate; cFP, N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate; MCA, 7-methoxycoumarin-4-acetyl; Pz, 7-phenylazobenzyloxycarbonyl; Pz-peptide, Pz-Pro-Leu-Gly-Pro-D-Arg.

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Gly-Pro-D-Lys (2,4-dinitrophenyl), as well as bradykinin, were obtained from Auspep (Parkville, Australia). The product of QFS hydrolysis, 7-methoxycoumarin-4-acetyl-Pro-Leu-OH, and Pz-peptide, 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg, were from Novabiochem (La Jolla, CA). N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (cFP) was synthesized at the Baker Institute by Dr. J. H. Boublik and Mr. L. Lakat, and ^{125}I-cFP prepared by Ms. M. Fullerton. Hydroxyapatite was from Biorad, and dithiothreitol (DTT) from Calbiochem.

Enzyme Assays

QFS assay. The fluorescent assay (5) is similar to that described by Barrett *et al*. (14). Enzyme was added to Tris-buffered saline (TBS) (25 mM Tris, pH 7.4, containing 125 mM NaCl). DTT was included at 0.1 mM unless stated otherwise, and $MnCl_2$, $CaCl_2$ or other salts added as noted. All divalent cations were in the form of dichlorides. Total volume was 2.5 mL. The reaction was initiated by addition of the QFS substrate in 20 μL DMSO, final concentration 4.5 μM, and samples incubated for 30 min at 37° C. Reactions were stopped by addition of 25 μL 100 mM $ZnCl_2$, and the tubes allowed to reach room temperature before measuring fluorescence on a Perkin-Elmer LS-5 luminescence spectrometer (E_x = 314 nM; E_m = 418 nm). Enzymatic cleavage of the QFS was quantified by comparison with a standard curve (50-3200 pmol) of the fluorescent product, 7-methoxycoumarin-4-acetyl-Pro-Leu-OH. The quantity of enzyme in each incubation was adjusted so that the amount of product would lie within the linear portion of the standard curve. For the wild type enzyme, this was 0.25-0.5 μg protein/assay. Because of the much higher specific activity of the double mutant, approximately one-tenth as much enzyme was required.

Hplc analysis. For hplc analysis of the products from the QFS assay, samples were incubated as described above, and the fluorescence recorded. The same samples were then subjected to separation on a Novapak C18 column (8.00 mm i.d. × 100 mm) contained within a radial compression module (Waters Associates). A 2-mL sample was injected, and constituents eluted with a linear (30 min) gradient from 3% to 100% solvent B (70% CH_3CN/0.08% TFA; solvent A = 0.08% TFA) at a flow rate of 1 mL/min. Bradykinin digestion was carried out in a total volume of 250 μL TBS, with additions as described above. The amount of peptide was 20 μg, and enzyme was 0.5 μg. Samples were incubated for intervals from 10-60 min, and the reaction terminated by addition of $ZnCl_2$. Samples of 100 μL were analyzed by hplc (3, 4), using a linear gradient of 3% to 70% solvent B over 30 min.

Pz-peptide hydrolysis. Hydrolysis of Pz-peptide was assayed according to Morales and Woessner (8). Peptide, 100 μg, was incubated with 1 μg of recombinant enzyme in a total volume of 250 μL for 30 min at 37° C. The buffer was TBS containing DTT, and metal ions as indicated. The reaction was terminated by addition of 400 μL 5% citric acid, and the product extracted with 2 mL ethyl acetate. After clarification of the extract with anhydrous Na_2SO_4, the absorbance of samples at 320 nm was determined.

Inhibitor binding assay. cFP binding was assessed by a hydroxyapatite binding method (15). Enzyme, ~130 nM, was incubated in 1 mM phosphate buffer, pH 7.4, containing 0.1 mM DTT, with unlabeled 10 nM cFP and 20,000 cpm of ^{125}I-cFP in a total volume of 200 μL, for 30 min at 37° C. Divalent cations were added as noted. Controls for non-specific binding contained an equivalent concentration of bovine serum albumin in place of EP 24.15, and controls for divalent cations included 20 mM Mn^{2+} or Ca^{2+} without enzyme. After incubation, 200 μL hydroxyapatite suspension was added to each sample (suspension prepared by suspension of 1 g of hydroxyapatite in 6 mL of phosphate buffer, followed by repeated settling, aspiration of supernatant and replacement of buffer). The samples containing hydroxyapatite were incubated on ice for 30 min, with frequent mixing, and were then microfuged for 3-5 min. The supernatant was removed and saved. The pellet was resuspended in 300 μL phosphate buffer, and washed a total of 3 times. Radioactivity was determined for all supernatants, including washes, and pellets, in a Riastar gamma counter (Packard). Percent binding was determined as the amount of radioactivity in the pellet compared to the total in all fractions. Non-specific binding was 2-3%.

RESULTS

In the QFS assay, activity of EP 24.15 was increased by the presence of $MnCl_2$ or $CaCl_2$, as shown in fig. 1. Mn^{2+} produced a maximum 2- to 3-fold increase between 1 and 10 mM, and Ca^{2+} between 5 and 20 mM. This effect was not produced with other divalent metal ions, which either had no effect (Mg^{2+}) or were inhibitory at these concentrations (Zn^{2+}, Cu^{2+}, Co^{2+}).

EP 24.15 is thiol-activated (4, 11), but the effect of metal ions did not appear to be a result of direct interaction with cysteine residues on the enzyme. The activation with manganese or calcium was observed in the presence or absence of dithiothreitol, as shown in fig. 2A, although the control level in the absence of DTT is much lower than in the presence of thiol reagents. In addition, a mutant of EP 24.15, with cysteines at positions 246 and 253 mutated to serines,

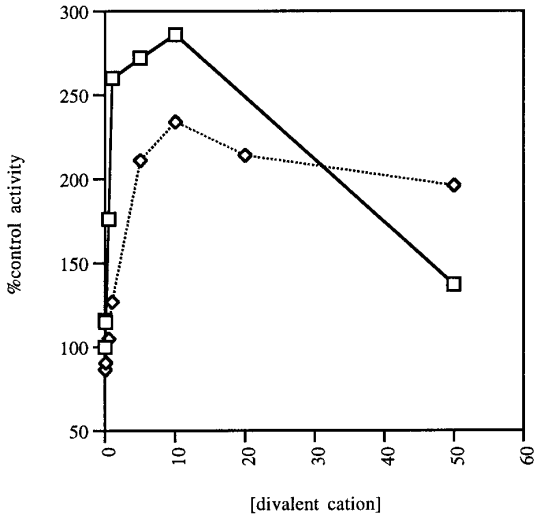

FIG. 1. Activation of EP 24.15 by Mn^{2+} and Ca^{2+}. Activity was determined by QFS assay, as described in the text. TBS buffer included 0.1 mM DTT and $MnCl_2$ (open squares) or $CaCl_2$ (open diamonds) to the final concentration given on the graph. All data points were obtained at least in duplicate, and the relative activities are the mean of 2-5 experiments.

not subject to thiol activation (13), is activated by both manganese and calcium (fig. 2B). The enzyme in extracts (soluble and nuclear) from rat testes is similarly activated (data not shown).

Since enzymatic activity in the QFS assay is deduced from the increase in fluorescence attributed to the product, the possibility that the ions were enhancing fluorescence of the substrate and/or the product was examined. Separation and quantification of the QFS substrate and product by hplc after incubation with enzyme (Table I) showed that the increase in fluorescence exactly paralleled that obtained for the area of the product peak, thus excluding a direct effect of Mn^{2+} or Ca^{2+} on substrate/product fluorescence.

It was further verified that the activation with metal ions observed was not an artifact of the fluorescent assay by demonstration that the increase in product was linear with time and with enzyme concentration, in the presence or absence of the metal ions (data not shown). Additionally, standard curves for the product of the QFS assay, 7-methoxycoumarin-4-acetyl-Pro-Leu-OH, were identical in the presence or absence of ions.

To test the possibility that the enzyme was depleted of zinc, and thus the observed effects reflected Mn^{2+} or Ca^{2+} substitution in the active site (1, 8, 11), low concentrations of Zn^{2+} (25 μM) were included. Mn^{2+} (1 mM) or Ca^{2+} (10 mM) continued to increase product formation 2-3-fold over levels obtained with the zinc-treated enzyme.

Hydrolysis of the Pz-peptide, on which the QFS substrate is based, as determined spectrophotometrically, was also enhanced by manganese or calcium ions, although the range of activating concentrations was narrower than for the QFS substrate, and the maximum degree of activation was only $\sim$1.5-fold. Results for Mn^{2+} activation are presented in Table II.

When the effective and specific inhibitor for EP 24.15, N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (cFP) (16), was incubated at less than saturating levels with the enzyme and substrate, the inhibitory effect was increased in the presence of 10 mM Mn^{2+} (figure 3). A similar enhancement of inhibition was observed when the substrate was bradykinin: in this case, the area of the product peak (bradykinin$^{1-5}$), separated by hplc, was 63% of control in the presence of 1.6 μM cFP alone, and 43% in the presence of the same concentration of cFP plus

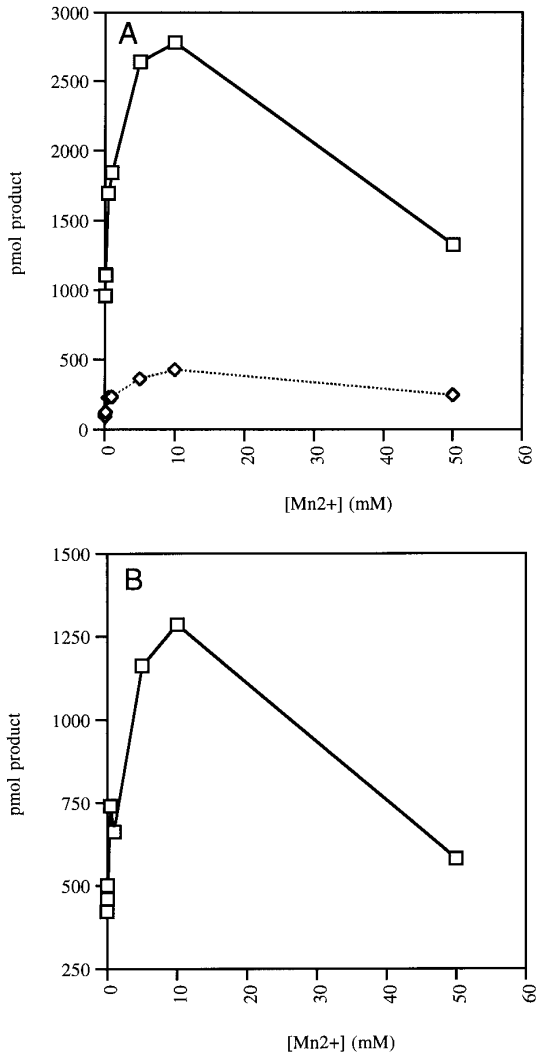

FIG. 2. Activation by Mn^{2+} in the presence or absence of thiol reagents. (A) Effect of addition of DTT: Wild-type enzyme was assayed using the QFS method in the presence (open squares) or absence (open diamonds) of 0.1 mM DTT. $MnCl_2$ was added to the noted concentrations. Pmol of product produced were determined from a standard curve of MCA-Pro-Leu-OH. Similar results were obtained using $CaCl_2$ (data not shown). All data points were obtained at least in duplicate. (B) Activation of the double mutant by Mn^{2+}: EP 24.15 C246S/C253S was assayed by the QFS method in the presence of DTT, and Mn^{2+}, as noted. Similar results were obtained using $CaCl_2$ in the absence of DTT (data not shown). All data points were obtained at least in duplicate.

10 mM Mn^{2+}. The conclusion that divalent cations are increasing the affinity with which ligands bind to the enzyme is further supported by data for binding of radioactively-labeled cFP to EP 24.15 (figure 4). The percentage of inhibitor remaining bound to the enzyme in the presence of calcium or manganese was almost double that observed in the absence of these ions.

In spite of the marked enhancement of activity with the QFS substrate, we found no evidence that hydrolysis of the natural substrates bradykinin or GnRH (free acid) was affected by Mn^{2+} or Ca^{2+}.

TABLE I
Comparison of MCA-Pro-Leu-OH Production by Fluorescent and HPLC Assays

	pmol product	
	By fluorescence (% control)	By peak area (% control)
Enzyme alone	686	681
+1 mM Mn^{2+}	1675 (244)	1750 (257)
+10 mM Ca^{2+}	1969 (287)	1910 (280)

Note. QFS assay was carried out as described in the text. The amount of product was determined by interpolation from a standard curve of MCA-Pro-Leu-OH (50-3200 pmol). These results are noted under ''pmol product: by fluorescence.'' The same samples were then subjected to hplc analysis, followed by peak integration. Two-milliliter samples of MCA-Pro-Leu-OH standards (800–3200 pmol) were also injected in the same system, and a standard curve was constructed. These results are given as ''pmol product: by peak area.''

DISCUSSION

Previous studies of the effects of metal ions on EP 24.15 have generally focussed on the active-site Zn^{2+}. When this ion is removed from the enzyme by dialysis against EDTA, the enzyme is inactivated, and several ions at low concentrations, including Mn^{2+} and Ca^{2+}, can replace Zn^{2+} (1, 8, 11). Under these conditions, (*i.e.*, when zinc is absent), higher concentrations of metal ions were found to be inhibitory.

In several cases, using fluorogenic or chromogenic substrates, activation by Mn^{2+} or Ca^{2+} has been reported. Using a quenched fluorescent substrate for Pz-peptidase, thought to be

TABLE II
Effect of Manganese on Hydrolysis of the Pz-Peptide

$[Mn^{2+}]$	% of control activity
0	100
0.1	112
1	127
5	145
10	132
20	110

Note. Assay was carried out according to Morales and Woessner (8), as described in the text. Incubations were carried out at least in duplicate, and the results presented are the average of 2 separate experiments. Activity levels were corrected by subtraction of blanks, containing substrate and buffer (with or without Mn^{2+}).

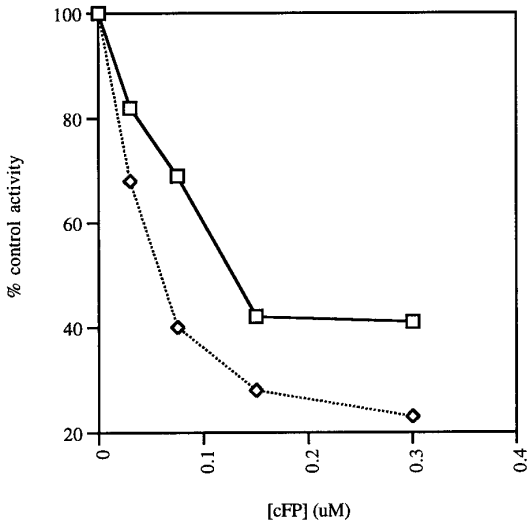

FIG. 3. Enhancement of cFP inhibition in presence of Mn^{2+}. EP 24.15 was incubated with increasing amounts of cFP, as noted, and assayed using the QFS substrate in the presence (open squares) or absence (open diamonds) of 10 mM Mn^{2+}. ''% control activity'' refers to the activity obtained in the absence of cFP for each series. The control level in the presence of Mn^{2+} was ~3-fold that of the level in the absence of Mn^{2+}. All data points were obtained at least in duplicate.

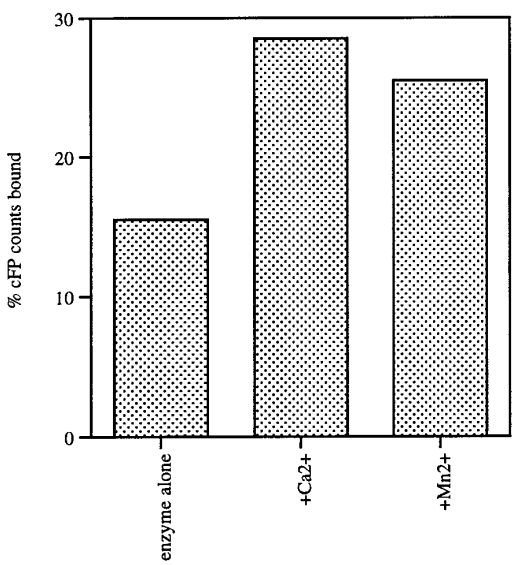

FIG. 4. Effect of Ca^{2+} and Mn^{2+} on the binding of ^{125}I-cFP to recombinant EP 24.15 EP 24.15 (2 μg) was incubated with ^{125}I-cFP and non-radioactive cFP, in phosphate buffer containing DTT, with or without 20 mM $CaCl_2$ or $MnCl_2$, as indicated. Separation of bound from free ligand was carried out by addition of hydroxyapattite, followed by centrifugation and wash. See text for details. All determinations were carried out in duplicate, with a range of $\leqslant$ 2%.

identical to EP 24.15, Tisljar and Barrett (10) described activation by 10 mM $CaCl_2$. Similarly, using the substrate carbobenzoxy-Gln-Lys-Leu-*p*-nitroanilide, McDermott and Gibson (9) identified a Mn^{2+}-activated enzyme in brain, which was characterized as EP 24.15. These results, along with the current data, suggest the inclusion of 1-10 mM $MnCl_2$ or 10-50 mM $CaCl_2$ in assay buffers greatly enhances the sensitivity of the fluorogenic substrate assay.

A similar effect has been reported by Lloyd *et al.* for aminopeptidase P (17). These authors found that the hydrolysis by aminopeptidase P of some, but not all, substrates is enhanced by Mn^{2+}, and that some inhibitors of the enzyme exert their effects only in the presence of the metal ion. Our finding that the hydrolysis of the Pz-peptide is also enhanced by metal ions suggests that a bulky group in the N-terminal region of the substrate or inhibitor interacts with Mn^{2+} or Ca^{2+}, thereby increasing affinity of the enzyme for the ligand. Because the effect on Pz-peptide hydrolysis is less than that for fluorogenic or chromogenic substrates, the presence of a second bulky group at the C-terminus may also be necessary for maximal activation.

The observation that the recombinant enzyme is activated by Mn^{2+} and Ca^{2+} verifies earlier findings, though does not clarify the physiological role, if any, of these ions in the enhanced activity of EP 24.15 *in vivo.* EP 24.15 is both an intra-cellular and extra-cellular enzyme. Extra-cellularly, there is the possibility that the enzyme would be exposed to levels of Ca^{2+} approaching those utilized in this study (18); however, it is unlikely that an intracellular enzyme would encounter high levels of Ca^{2+} or that Mn^{2+} levels inside or outside the cell would reach millimolar concentrations. The sequence of EP 24.15 has no clear homology to any catalogued calcium-binding proteins. The ions may be mimicking a natural activator, not yet identified. Furthermore, it is possible that the peptide substrates postulated to be the natural ones (*e.g.*, bradykinin and GnRH (free acid)) may not be the endogenous substrates, and that hydrolysis of the ''true'' substrates is enhanced by metal ions or other activators. Alternatively, the substrates employed in these assay systems may bind to the enzyme in a manner quite dissimilar to that of natural substrates, and only this artificial binding is enhanced by metal ions. Our data for binding of the inhibitor cFP certainly suggest that there is a direct effect on binding of ligands in the presence of calcium and manganese.

In addition to providing both insight into the way synthetic substrates and inhibitors fit into the active site of EP 24.15 and increased assay sensitivity, these results raise a cautionary note in interpretation of data from assays and binding studies carried out with artificial substrates, especially given the rising popularity of assays with quenched fluorescent substrates (19).

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