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Activity of Copper-Substituted Carboxypeptidase A toward Oligopeptides and Depsipeptides†

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ABSTRACT: Cu(II)-substituted carboxypeptidase A catalyzes the hydrolysis of oligopeptides and their depsipeptide (ester) analogues. Stopped-flow fluorescence assays demonstrate that relative to the zinc enzyme the Cu enzyme can have k_{cat}/K_m values up to 24% toward esters but only up to 2.5% toward the corresponding peptides. Adding Zn(II) to the copper enzyme reveals a slow exchange process that correlates with an increase in peptidase activity and with changes in the Cu(II) electron paramagnetic resonance spectra. Low concentrations of 1,10-phenanthroline (OP) (0.1–2.5 μM) markedly increase activity toward furanacryloyl-Phe-Phe (up to 8% of the zinc enzyme), but higher concentrations inhibit, resulting in complete inhibition at 0.8 mM OP. The non-metal-binding, hydrophobic analogues *m*- and *p*-phenanthroline are only activators of peptide hydrolysis, even at 1 mM. Activation is likely due to a modifier binding to a hydrophobic locus and either displacing an inhibitory peptide binding mode or inducing a conformational change in the active site.

The replacement of Zn(II) in several metalloenzymes by Cu(II) usually leads to nearly or totally inactive derivatives as is observed for carbonic anhydrase (Bertini et al., 1978), alkaline phosphatase (Lazdunski et al., 1970), liver alcohol dehydrogenase (Maret et al., 1983), and thermolysin (Holmquist & Vallee, 1974).

The activity of the mixed Cu–Zn derivative of *Aeromonas* aminopeptidase, on the other hand, is increased about 60-fold compared to that of the native 2Zn enzyme (Prescott et al., 1985), and Cu_2–Cu_2 superoxide dismutase is about as efficient a catalyst as the Zn_2–Cu_2 dimer (Fee, 1973; Fee & Briggs, 1975). Except for the reported hydrolysis of a thiol ester catalyzed by Cu-substituted carboxypeptidase A (Cu-carboxypeptidase A)[1] with a k_{cat} of 0.04 s^{-1} and a K_m of 94 μM (Schneider et al., 1976), no peptidase and esterase activity data are available for this metallo derivative. The reconstitution of apocarboxypeptidase A with all other VIIB–IIB (groups 7, 8, 9, 10, and 12 in 1985 notation) divalent metal ions of the first transition series (Mn, Fe, Co, Ni, Zn), however, restores the catalytic activity to different but significant extents (Coleman & Vallee, 1960, 1961; Auld & Vallee, 1970; Auld & Holmquist, 1974; King & Fife, 1983; Vallee et al., 1983; Dua & Gupta, 1984), esters generally being hydrolyzed more readily than peptides.

Since the electronic and paramagnetic spectral properties of Cu(II) (Bertini & Scozzafava, 1981) could render it a useful probe of carboxypeptidase for the investigation of its reaction mechanism, we reexamined the question of the activity of this metalloenzyme toward oligopeptides and their depsipeptide analogues under experimental conditions that we improved substantially compared to earlier studies (Coleman & Vallee, 1961).

Materials and Methods

Carboxypeptidase A was substituted with Cu(II) by suspending the affinity chromatography purified (Bicknell et al., 1985) enzyme crystals (Sigma Chemical Co., catalog no. C 0261) 4 times in 0.01 M 1,10-phenanthroline/0.01 M Mes, pH 7.0, buffer for 40 min and washing 5 times with metal-free buffer (Auld & Holmquist, 1974), followed by adding the desired amount of an aqueous solution of $CuSO_4$ (Puratronic, Johnson Matthey) to the dissolved enzyme. Solutions of the apoenzyme and of the metallo derivatives were prepared immediately before the experiments. Substrates were synthesized

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[1] Abbreviations: Cu(II)-carboxypeptidase A, Cu(II)-substituted carboxypeptidase A; RET, radiationless energy transfer; Dns or dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; FA, furanacryloyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; OP, 1,10-phenanthroline; EPR, electron paramagnetic resonance; Bz, benzoyl; OPhe, L-β-phenyllactate.

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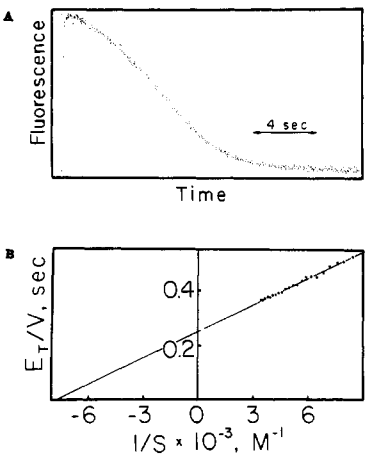

FIGURE 1: (A) Stopped-flow fluorescence observation of the formation and breakdown of the ES complex of the copper enzyme (19 μM) and Dns-Ala-Gly-Phe (0.3 mM). Excitation was at 285 nm, and the substrate dansyl emission above 430 nm was observed. Assay conditions in Table I. (B) Determination of the kinetic parameters k_{cat} and K_m from the oscilloscope trace in (A) as previously described (Lobb & Auld, 1980, 1984). Linear regression analysis yields values of 4 s^{-1} for k_{cat} and 130 μM for K_m.

and characterized as previously described (Galdes et al., 1983; Peterson et al., 1982).

All solvents were rendered metal free by extraction with dithizone in CCl_4 (0.001%), and precautions were taken to avoid contamination by adventitious metal ions (Thiers, 1957). Glassware and plastic ware were placed in 6 N HNO_3 for several hours and thoroughly rinsed with deionized water. The residual Zn content of the enzyme and substrate solutions was measured by atomic absorption spectroscopy using a Perkin-Elmer Model 2280 instrument.

The kinetic data were obtained by measuring RET between enzyme tryptophanyl residues and the fluorescent N-dansylated substrates (Lobb & Auld, 1980, 1984) or from initial rate measurements with a Durrum-Gibson stopped-flow instrument; details have been described elsewhere (Lobb & Auld, 1980). All internal parts of the instrument used for storage and mixing of the reaction solutions were washed several times with a solution of a metal chelating agent (0.01 M 1,10-phenanthroline/0.01 M Mes, pH 7.0) and thoroughly rinsed with deionized water prior to use. The effluent reaction mixtures were checked for their Zn concentration by atomic absorption spectroscopy.

For the EPR spectra, the samples were frozen in liquid nitrogen prior to measurement at 4.2 K on an X-band Varian E-9 spectrometer which was interfaced to an Apple IIe computer and equipped with an Air Products Heli-Tran transfer line.

RESULTS

Catalytic Parameters. The hydrolysis of N-dansylated oligopeptides and their ester analogues by Cu(II)-carboxypeptidase A was monitored by stopped-flow fluorescent assays. An example of the formation and breakdown of the copper enzyme–substrate complex is shown in Figure 1A; hydrolysis is complete in 20 s at 19 μM enzyme. Determination of the kinetic parameters for this particular substrate, Dns-Ala-Gly-Phe, yields values of k_{cat} and K_m of 4 s^{-1} and 130 μM, respectively (Figure 1B). Thin-layer chromatography demonstrates that bond cleavage occurs only at the C-terminal peptide bond.

Activity data for the copper enzyme toward a series of corresponding tripeptide and ester substrates (Table I) demonstrate that the latter are generally hydrolyzed more readily than the former. For esters, the k_{cat} values are 8–17% and the k_{cat}/K_m values are 1.8–24% of those for the zinc enzyme. The corresponding values for the peptides are much lower, generally in the range of 1–4%. The K_m values are increased up to 5-fold.

Table I: Kinetic Parameters of Copper-Substituted Carboxypeptidase A[a]

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\times 10^2$ s^{-1} μM^{-1})
Dns-Gly-Gly-Phe	2.1 (1.5)	600 (170)	0.35 (0.89)
Dns-Gly-Gly-OPhe	19 (15)	77 (130)	25 (11)
Dns-Ala-Gly-Phe	4.5 (2.6)	140 (95)	3.3 (2.5)
Dns-Ala-Gly-OPhe	14 (9.0)	32 (38)	44 (24)
Dns-Gly-Ala-Phe	7.9 (3.9)	170 (270)	4.5 (1.6)
Dns-Gly-Ala-OPhe	0.64 (8.1)	43 (430)	1.5 (1.8)
Dns-Ala-Ala-Phe	1.9 (1.7)	91 (340)	2.1 (0.49)
Dns-Ala-Ala-OPhe	0.66 (17)	18 (430)	3.7 (3.9)

[a] The kinetic analyses were performed by RET methods (Lobb & Auld, 1980, 1984). Average values of at least four measurements are given. Assay conditions: 1 M NaCl, 50 mM Mes, pH 7.0, 20 °C, with enzyme (19 μM) and substrate (0.1–0.3 mM). Relative values to the Zn(II) enzyme in percent are given in parentheses.

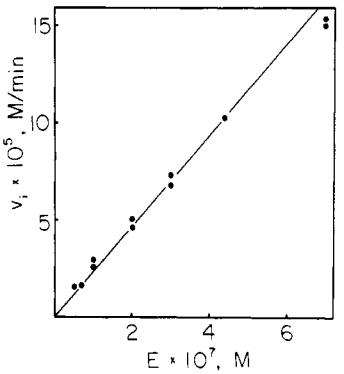

FIGURE 2: Dependence of the initial rate of hydrolysis of FA-Phe-Phe (0.4 mM) on the concentration of the Cu(II) enzyme; experimental aspects of the assay have been described (Peterson et al., 1982). Assay conditions: 1 M NaCl/50 mM Hepes, pH 7.5, 20 °C.

The linear dependence of the initial rate for hydrolysis of the furanacryloyl peptide FA-Phe-Phe on the Cu enzyme concentration is illustrated in Figure 2. The parameters k_{cat} = 8 s^{-1} (240 s^{-1}) and K_m = 1.9 × 10^{-4} M (6.3 × 10^{-5} M) for this substrate (values for the zinc enzyme are in parentheses) were obtained by initial rate analyses in stopped-flow experiments.

Effect of 1,10-Phenanthroline on Catalysis. To demonstrate that the activities observed for copper carboxypeptidase are dependent on the metal at the active site of the enzyme, the effect of metal removal by adding a chelating agent, 1,10-phenanthroline (OP), was examined. Surprisingly, it was found that binding of phenanthroline derivatives to the copper enzyme leads to a significant increase in activity. In the presence of 2.5 μM OP, the ratio of v_i/v_c for the hydrolysis of FA-Phe-Phe is elevated up to a 4-fold (Figure 3). Under these conditions, the activity of the Cu enzyme toward this peptide is increased to 8% of that of the zinc enzyme. A similar result was obtained by assaying the substrate FA-Gly-Phe. The inhibitor solution was checked for its Zn concentration by atomic absorption spectroscopy and found to contain less than 0.01% relative to the enzyme concentration. This clearly excludes any explanation based on contamination for the native enzyme (Figure 3). The meta and para derivatives of phenanthroline show a similar activating effect on Cu(II)-carboxypeptidase A but at higher concentrations (v_i/v_c ~ 4 at 10^{-3} M inhibitor).

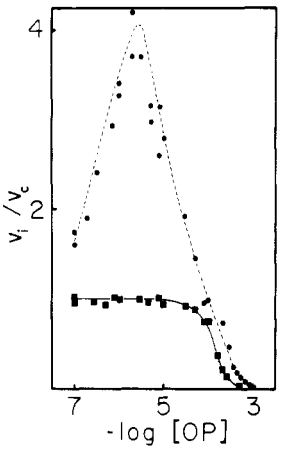

FIGURE 3: Effect of 1,10-phenanthroline concentration on the hydrolysis of 0.4 mM FA-Phe-Phe by 2 nM zinc enzyme (■) and 0.2 mM FA-Phe-Phe by 0.4 μM copper enzyme (●). v_i and v_c are the velocities in the presence and absence of the inhibitor, respectively.

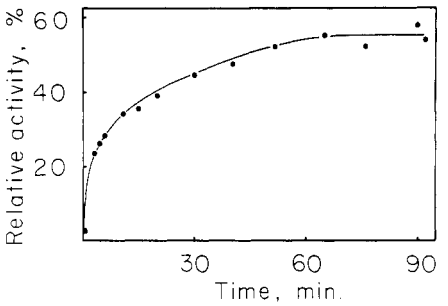

FIGURE 4: Examination of the rate of exchange of zinc for copper in Cu(II)-carboxypeptidase A (8.6 μM) upon addition of 1 equiv of zinc. Assay conditions: Dns-Ala-Ala-Phe, 0.25 mM, in 1 M NaCl/50 mM Hepes, pH 7.0, 20 °C.

At OP concentrations higher than 3 μM, the chelating binding mode of the inhibitor becomes more important. Applying the relationship $\log \phi = pK_I + \bar{n} \log [I]$ with $\phi = v_c/v_i - 1$, $pK_I = -\log K_I$, and I = 1,10-phenanthroline, a value for $\bar{n} \sim 2$ was determined for both the copper and the zinc enzyme. This suggests that under these conditions the metal is removed from the enzyme by OP rather than forming a stable 1:1 complex of OP and the enzyme ($\bar{n} = 1$) (Pollack et al., 1983). Inactivation by extracting copper may thus prevent a more pronounced activation. A 50% inhibition of the Cu(II) enzyme is achieved with 0.3 mM OP (0.1 mM for the zinc enzyme), and concentrations higher than 0.8 mM abolish the activity entirely. With the 2,9-dimethyl derivative of OP ("neocupreine"), no activation at lower inhibitor concentrations is observed, and the 50% inactivation concentration is shifted to a value of 1.8 mM.

Metal Exchange Studies. Metal exchange experiments were performed to determine the stability of Cu(II) binding to the enzyme active site. The time course for the replacement of Cu(II) in copper carboxypeptidase A by Zn(II) was followed by measuring enzyme activity toward Dns-Ala-Ala-Phe periodically subsequent to the addition of an equimolar amount of Zn(II) to the copper enzyme (Figure 4). The zinc was added directly to Cu(II)-carboxypeptidase A in the stopped-flow syringe. Maximal activity corresponding to only 60% of that of the fully reconstituted zinc enzyme occurs after about 1 h.

The substitution process correlates with changes in the EPR spectrum of Cu(II)-carboxypeptidase A whose structural parameters are shown in Table II and compared with the values for a solution of $CuSO_4$. After 3-min incubation time with zinc, the features of the EPR spectrum are mainly those of copper carboxypeptidase A. After 70 min, a progressive change in the band positions leads to a form which represents the spectrum of solvated Cu(II) in buffer and partially of Cu(II)-carboxypeptidase A, indicating that the metal exchange under these conditions is not complete, in agreement with the activity measurements (Figure 4).

Table II: EPR Parameters of Copper-Substituted Carboxypeptidase A

	$g_\parallel$	$g_\perp$	$A_\parallel$ ($\times 10^{-4}$ cm^{-1})
Cu(II)-carboxypeptidase A[a]	2.336	2.049	142.5
$CuSO_4$	2.260	2.035	170.9
Cu(II)-carboxypeptidase A[b]	2.327	2.057	134.7

[a] Conditions: apocarboxypeptidase (0.3 mM) plus $CuSO_4$ (0.3 mM) in 1 M NaCl/50 mM Hepes, pH 7.5. [b] Rosenberg et al. (1975).

Discussion

The present study shows that divalent copper reconstitutes metal-depleted carboxypeptidase A to an active esterase and peptidase. The activity of the copper enzyme was confirmed under improved experimental conditions compared to earlier studies which indicated this enzyme to be inactive (Coleman & Vallee, 1961). The conventional initial rate experiments used in those studies required low enzyme concentrations. Assays of apocarboxypeptidase A and metal derivatives with low activity are particularly difficult under such conditions because the background contamination of the solutions with zinc often is much greater than the enzyme concentrations used in the assay. Accordingly, the combination of carefully designed experimental conditions in order to avoid metal contamination (see Materials and Methods) and the use of high enzyme concentrations with stopped-flow assays as in the present study provides an essential advantage: The activities of the apoenzyme solutions were found to be essentially those predicted on the basis of residual Zn contents of the reaction solutions measured by atomic absorption spectroscopy (~1% of enzyme concentration).

Kinetic parameters for the hydrolysis of N-dansylated tripeptides and depsipeptides by copper carboxypeptidase A are shown in Table I. Although the activity is generally much less compared to the enzyme derivatives of the Cu-flanking 3d metals, it is clear that this enzyme is active, catalyzing esters more efficiently than peptides. With the ester substrate Dns-Ala-Ala-OPhe, a k_{cat} value of 17% relative to that with the native enzyme was determined, and k_{cat}/K_m ratios for esters were found to be as high as 24% (Table I). The K_m values for all substrates with a penultimate alanine residue are 3–5-fold higher than those for the zinc enzyme while those with a glycine residue are 0.5–1.7 that of the zinc enzyme. Such changes in the specificity of the zinc and copper enzyme may reflect slight structural changes in the active site of the enzymes.

The discrepancy between the preference of d^9 Cu(II) to planar or strongly Jahn Teller distorted tetrahedral or octahedral complexes, respectively, and the pseudotetrahedral array of active-site ligands in carboxypeptidase A has often been reported to be one reason for the inactivity of this enzyme (Bertini & Scozzafava, 1981; Jameson, 1981). An explanation for the relatively small catalytic efficiency based solely on differences in metal coordination geometries, however, does not seem to be sufficient. In both the native enzyme and cobalt carboxypeptidase A, the metal ions are throught to be bound by the two off-axis oxygens of Glu-72, two His nitrogens, and a water molecule in a quasi-tetrahedral coordination geometry (Rees et al., 1983). The metal ligand environment in nickel

carboxypeptidase A, which can be as active a peptidase as the zinc enzyme (Auld & Vallee, 1970), on the other hand, according to recent crystallographic studies, is best described as octahedral-like, where the sixth position is vacant (Hardmann & Lipscomb, 1984). These metals are, therefore, at the opposite ends of a series of increasingly square-pyramidal coordination geometry in carboxypeptidase A derivatives in the order Zn, Co, Hg, Mn, Cd, and Ni (Rees, 1985). Since the metal coordination in catalytically active carboxypeptidases apparently can show some change, while the structural changes of the protein in the crystalline state are considered minimal (Hardman & Lipscomb, 1984), the restoration of activity with divalent copper may not be surprising.

Possible structural differences of apo- and metallocarboxypeptidases in solution were recently suggested from proteolytic susceptibility studies of these enzymes toward serine proteases (Bicknell et al., 1985). Because of the apparent structural flexibility of the dissolved enzyme, the lower activity of Cu(II)-carboxypeptidase A could also be a result of local conformational changes in the protein with improperly oriented functional groups. Support for this hypothesis is gained from the results of studies of the effect of 1,10-phenanthroline (OP) on activity. At low inhibitor concentrations, the activity of the copper enzyme toward the hydrolysis of FA-Phe-Phe was increased significantly with a maximum v_i/v_c of 4 at a concentration of 2.5 μM OP (Figure 3). The binding of OP to a hydrophobic region of the enzyme and subsequent changes in the protein conformation resulting in increased activity could serve as an explanation for this activation. Additional evidence for this possible binding mode arises from the fact that the nonchelating, hydrophobic meta and para derivatives of phenanthroline at concentrations up to 1 mM are also activators of the copper enzyme.

Activation of the zinc enzyme catalyzed hydrolysis of dipeptides (e.g., Bz-Gly-Phe) has been reported (Davies et al., 1968). Such activation was suggested to occur by a modifier competing with the peptide for a nonproductive peptide-binding locus, but not for a productive binding locus. Viewed in this manner, activation of peptide hydrolysis can be regarded as release of self-competitive peptide inhibition (Davies et al., 1968). Further kinetic studies will be needed to determine if such a mechanism pertains for the activation of the copper enzyme.

The substitution of Zn(II) in metalloenzymes by paramagnetic metal ions and the spectroscopic characterization of the catalytically active metal derivatives have proved to be a powerful tool for the investigation of enzyme reaction mechanisms (Vallee et al., 1983). Cryospectrokinetic studies of cobalt-substituted carboxypeptidase A have recently led to the identification of two intermediates in its catalysis (Galdes et al., 1983; Geoghegan et al., 1983; Auld et al., 1984). Examination of the spectral and kinetic properties of the intermediates in the copper enzyme catalysis could provide insight into the reasons for its lower catalytic potency. Changes in the pre-steady-state parameters in the reaction of Cu(II)-carboxypeptidase A in comparison to other metallo derivatives (Galdes et al., 1983; Auld et al., 1984) may reflect the observed decrease in catalysis by the copper enzyme. Experiments concerning this aspect are under way.

Acknowledgments

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