Structural and Functional Investigations on the Role of Zinc in Bifunctional Rat Peptidylglycine α-Amidating Enzyme†

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ABSTRACT: Bifunctional peptidylglycine α-amidating enzyme (α-AE) catalyzes the two-step conversion of C-terminal glycine-extended peptides to C-terminal α-amidated peptides and glyoxylate. The first step is the ascorbate-, O_2-, and copper-dependent hydroxylation of the α-carbon of the glycyl residue, producing an α-hydroxyglycine-extended peptide. The second step is the ascorbate-, O_2-, and copper-independent dealkylation of the carbinolamide intermediate. We show that α-AE requires 1.1 ± 0.2 mol of zinc/mol of enzyme for maximal (*S*)-*N*-dansyl-Tyr-Val-α-hydroxyglycine dealkylation activity. Treatment of the enzyme with EDTA abolishes both the peptide hydroxylation and the carbinolamide dealkylation activities. Addition of Zn(II), Co(II), Cd(II), and Mn(II) partially restores carbinolamide dealkylation activity to the EDTA-treated enzyme. Addition of Co(II) produces the greatest restoration of dealkylation activity, 32% relative to a control not treated with EDTA, while Mn(II) addition results in the smallest restoration of dealkylation activity, only 3% relative to an untreated control. The structure and coordination of the zinc center has been investigated by X-ray absorption spectroscopy. EXAFS data are best interpreted by an average coordination of 2−3 histidine ligands and 1−2 non-histidine O/N ligands. Since catalytic zinc centers in other zinc metalloenzymes generally exhibit only O/N ligands to the zinc atom, a zinc-bound water or hydroxide may serve as a general base for the abstraction of the hydroxyl proton from the carbinolamide intermediate. Alternatively, the zinc may function in a structural role.

Many biologically active peptide hormones have an α-amide moiety at their C-terminus (Eipper & Mains, 1988; Kopinska et al., 1992; Kreil, 1985), and in most cases, the α-amide is essential for biological activity (Merkler, 1994). The C-terminal α-amide arises by the post-translational, oxidative cleavage of a C-terminal glycine-extended prohormone to the active α-amidated peptide and glyoxylate (Bradbury et al., 1982; Eipper et al., 1983). This reaction is catalyzed by peptidylglycine α-amidating enzyme (α-AE, EC 1.14.17.3), a copper-dependent monooxygenase (Eipper et al., 1992b, 1993; Merkler et al., 1993) that has both structural and catalytic similarities to another copper-dependent monooxygenase, dopamine β-hydroxylase (DβH) (Boswell et al., 1996; Merkler et al., 1993; Southan & Kruse, 1989).

α-AE is a bifunctional enzyme that contains a monooxygenase domain (PHM, peptidyl α-hydroxylating monooxygenase) and a lyase domain (PAL, peptidylamidoglycolate lyase) which sequentially catalyze the conversion of peptidyl-Gly to peptidyl-NH_2 via a discrete peptidyl-α-hydroxyglycine intermediate (Eipper et al., 1991, 1992a; Katopodis et al., 1990; Ouafik et al., 1992; Young & Tamburini, 1989). The N-terminal domain, PHM, catalyzes the copper-, ascorbate-, and O_2-dependent hydroxylation of the glycine-extended substrate while the C-terminal domain, PAL, catalyzes the catalyzes the copper-, ascorbate-, and O_2-independent dealkylation of the carbinolamide intermediate (Scheme 1) (Eipper et al., 1991, 1992a; Katopodis et al., 1990; Merkler & Young, 1991; Ouafik et al., 1992; Young & Tamburini, 1989).

The enzyme is known to bind 2 copper atoms/PHM active site which redox cycle between Cu(II) and Cu(I) during catalysis (Boswell et al., 1996; Eipper et al., 1995; Freeman

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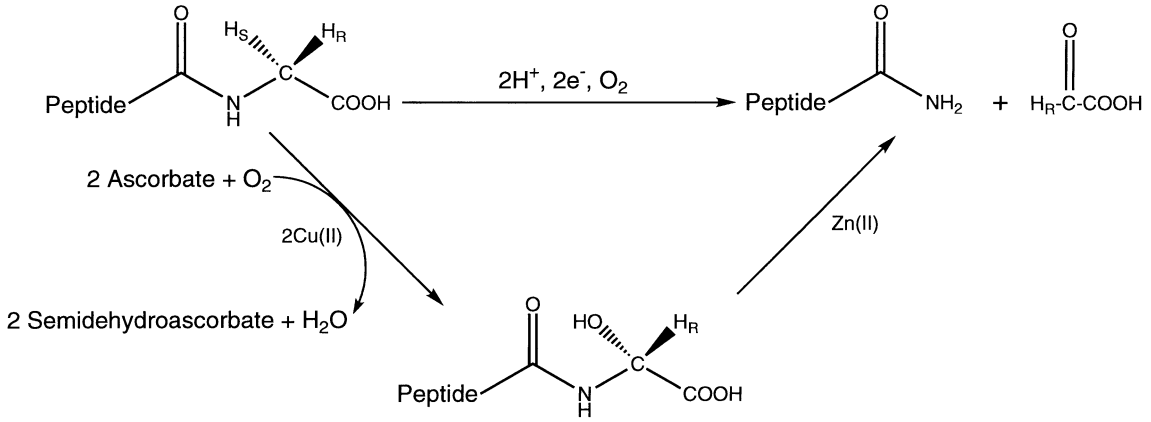
Scheme 1: Reaction Catalyzed by Bifunctional Peptidylglycine α-Amidating Enzyme



et al., 1993; Kulathila et al., 1994). The redox chemistry at the PHM-bound copper atoms is important for the reductive activation of molecular oxygen (Karlin & Tyeklár, 1993). Spectroscopic studies indicate that the Cu(II) coordination of the oxidized enzyme is typical of a type 2 copper protein with an average coordination per copper atom of 2−3 histidines and 1−2 O/N atoms as equatorial ligands (Boswell et al. 1996; Eipper et al., 1995). Upon reduction, the non-histidine oxygen/nitrogen ligands are displaced and the average coordination per Cu(I) is 2.5 histidines and 0.5 sulfur ligand (most likely from a methionyl residue). Note that the mutation of Met^{314} to Ile in bovine PHM produces a catalytically inactive protein (Eipper et al., 1995) and that the coordination of a sulfur atom to one of the Cu(I) in reduced DβH has previously been observed (Blackburn et al., 1991; Reedy & Blackburn, 1994).

In contrast to the role played by copper in PHM catalysis, there has been a long-standing debate concerning the role, if any, of metal ions in PAL catalysis. Eipper et al. (1991) first reported that EGTA and EDTA inhibited bovine PAL, a result subsequently verified when Merkler et al. (1993) showed that EDTA inhibited the PAL activity of bifunctional rat α-AE. However, other chelators such as *o*-phenanthroline and diethyl dithiocarbamate had little or no effect on either bovine or rat PAL (Eipper et al., 1991; Young & Tamburini, 1989) and studies with copper-free bifunctional α-AE showed that copper was required only for PHM activity (Kulathila et al., 1994). On the basis of these data, Kulathila et al. (1994) argued that EDTA inhibition might not result from metal chelation but from the binding of EDTA to PAL. There is precedence for this suggestion because Colombo et al. (1984) found that DβH bound 0.5−0.75 mol of EDTA/mol of subunit and that the stoichiometry of EDTA binding was only reduced to ∼0.25 mol of EDTA/mol of subunit upon dialysis against $MgCl_2$.

The data presented here demonstrate that rat bifunctional α-AE contains tightly bound Zn(II). Treatment of denatured enzyme with the metallochromic reagent 4-(2-pyridylazo)-resorcinol (PAR) shows that α-AE contains 1.1 ± 0.2 mol of zinc/mol of enzyme. X-ray absorption (XAS) spectra of α-AE also show the presence of zinc, ∼1.0 mol of zinc/mole of enzyme, with a coordination of 5 ± 1 O/N donor atoms as ligands. Incubation of the bifunctional enzyme with EDTA results in complete loss of both the PHM and PAL activities. PAL activity is partially restored upon the addition of Cd(II), Co(II), Mn(II), and Zn(II) while PHM activity is only restored upon the addition of Cu(II). The finding of a Zn(II) requirement for the PAL-catalyzed dealkylation of carbinolamides is surprising, because model studies indicate that a metal is not required for this chemistry (Bundgaard & Kahns, 1991; Mounier et al., 1997).

EXPERIMENTAL SECTION

General. Rabbit muscle lactate dehydrogenase, bovine catalase, *N*-dansyl-Tyr-Val-Gly, (*R*,*S*)-α-hydroxyhippuric acid, 4-(2-pyridylazo)resorcinol disodium salt, and NADH were purchased from Sigma, and Chelex-100 resin (200−400 mesh) was obtained from Bio-Rad. (*S*)-N-Dansyl-Tyr-Val-α-hydroxyglycine was synthesized as described (Young & Tamburini, 1989). Discrimination between (*R*)- and (*S*)-*N*-dansyl-Tyr-Val-α-hydroxyglycine is based on the α-AE-catalyzed conversion of only the (*S*)-isomer to *N*-dansyl-Tyr-Val-NH_2 (Kawahara et al., 1992; Ping et al., 1992). All other reagents were of the highest quality available from commercial sources.

Enzyme. Chinese hamster ovary cells which secrete recombinant type A rat medullary thyroid carcinoma α-AE into the culture media were grown in a Wheaton stirred tank bioreactor (Matthews et al., 1994). The bifunctional enzyme was purified as described by Miller et al. (1992) except that the final gel filtration step (Sephacryl S-300) was carried out using 20 mM HEPES/NaOH pH 7.8, 50 mM NaCl, 0.001% (v/v) Triton X-100. The purified α-AE was ≥95% pure as judged by SDS-PAGE and, unless otherwise noted, had a PHM specific activity >7.0 units/mg. One unit of PHM activity is defined as the amount of enzyme necessary to convert 1.0 μmol of *N*-dansyl-Tyr-Val-Gly to *N*-dansyl-Tyr-Val-NH_2 in 1.0 min at 37 °C in 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 100 μg/mL catalase, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10 mM sodium ascorbate, and 20 μM *N*-dansyl-Tyr-Val-Gly.[1]

Enzyme Activity. Amidation activity was routinely measured using HPLC separation of *N*-dansyl-Tyr-Val-NH_2 from *N*-dansyl-Tyr-Val-Gly as described by Jones et al. (1988). A modification of this HPLC method (Consalvo et al., 1992)

[1] The time-dependent conversion of *N*-dansyl-Tyr-Val-Gly to *N*-dansyl-Tyr-Val-NH_2 was routinely used throughout these studies to determine α-AE activity. This is a measure of PHM activity. The rate of O_2 consumption equals the rate of *N*-dansyl-Tyr-Val-NH_2 production (Merkler et al., 1992). The data of Consalvo et al. (1992) show a lag in the production of *N*-dansyl-Tyr-Val-NH_2 and the formation of a steady-state concentration of (*S*)-*N*-dansyl-Tyr-Val-α-hydroxyglycine. Both of these results are consistent with PAL serving as a coupling enzyme for PHM (Rudolph et al., 1979).

was used to both measure the accumulation of (*S*)-*N*-dansyl-Tyr-Val-α-hydroxyglycine during the amidation of *N*-dansyl-Tyr-Val-Gly and to measure PAL activity by monitoring the dealkylation of (*S*)-dansyl-Tyr-Val-α-hydroxyglycine to *N*-dansyl-Tyr-Val-NH_2.

A spectrophotometric assay employing lactate dehydrogenase (LDH) as a coupling enzyme was also used to measure PAL activity. The basis of this assay is the LDH-catalyzed reduction of glyoxylate to glycolate with the concomitant oxidation of NADH to NAD^+. The standard solution for the spectrophotometric assay was 100 mM MES/KOH pH 6.0, 100 mM KCl, 1% (v/v) ethanol, 500 μM (*R,S*)-α-hydroxyhippuric acid, 170 μM NADH, 19 μg/mL α-AE, and 61 U/mL LDH.

Zinc Determination Using 4-(2-Pyridylazo)resorcinol (PAR). All protein dialysis and reagent dilutions were performed with 50 mM HEPES/KOH pH 7.5 which had been treated with Chelex-100 in a batch mode to remove adventitious metals. Zinc(II) standards from 0 to 30 μM were prepared by dilution from a 10 mM $ZnCl_2$ stock prepared in 50 mM HEPES/KOH pH 7.5. All Zn(II) standards contained 3% (v/v) perchloric acid (PCA). Enzyme samples were exhaustively dialyzed against 50 mM HEPES/KOH pH 7.5 and then denatured by the addition of one-tenth volume of 30% (v/v) PCA. An aliquot of each dialyzed enzyme sample was removed prior to PCA addition for subsequent determination of the enzyme concentration. Absorbance at 500 nm was measured after the addition of 950 μL of 100 μM PAR (prepared in 500 mM HEPES/KOH pH 7.5) to 50 μL of Zn(II) standards or 50 μL of the denatured enzyme samples. The Zn(II) content of the denatured enzyme samples was determined from a standard curve of A_{500} vs [Zn(II)]. The protocol described for the use of PAR to determine [Zn(II)] was adapted from literature procedures (Hunt et al., 1985; Jefferson et al., 1990). The protein concentration was determined either by amino acid analysis or by the Bradford dye-binding method using bovine serum albumin as a standard (Bradford, 1976). Previous results have shown that both methods yield the same protein concentration (Kulathila et al., 1994).

Preparation of Apo-Enzyme and the Restoration of PAL Activity upon Metal Addition. Apo-enzyme was prepared by incubating α-AE (27 μg/mL, specific activity = 4.1 units of PHM activity/mg) for 10 h at 4 °C (or 1 h at 37 °C) in 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, and 10 mM EDTA. A control was prepared by incubating the enzyme under similar conditions without EDTA. In order to examine the restoration of dealkylation activity upon metal addition, an aliquot of apo-α-AE (5 μL) was added to 545 μL of 110 mM MES/NaOH pH 6.0, 33 mM NaCl, 1.1% (v/v) ethanol, 0.0011% (v/v) Triton X-100, and 0 or 182 μM desired metal, incubated at 37 °C for 5 min, and the dealkylation reaction was initiated by the addition of 50 μL of 192 μM (*S*)-*N*-dansyl-Tyr-Val-α-hydroxyglycine. At regularly timed intervals, a 30 μL aliquot was removed and added to 6 μL of 6% (v/v) trifluoroacetic acid, and the percent conversion of (*S*)-*N*-dansyl-Tyr-Val-α-hydroxyglycine to *N*-dansyl-Tyr-Val-NH_2 was determined by HPLC (Consalvo et al., 1992). The ratio of [added metal]/[residual EDTA] was 2.2 in the solution used to measure the regain of PAL activity. The protocol described here produced the maximum regain in PAL activity upon metal addition. Preliminary experiments surveying different incubation conditions, different EDTA concentrations, and different ratios of [added metal]/[residual EDTA] in the final dealkylation assay solution provided the same general results but resulted in lower levels of restored PAL activity.

Restoration of Only PHM Activity upon Copper Addition. Enzyme (4.6 mg/mL, specific activity = 13.1 units of PHM activity/mg) was incubated in 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, and 0 or 10 mM EDTA. After 1 h at 37 °C, an aliquot (10 μL) was added to the assay solution (2990 μL) such that the final solution contained 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10 μg/mL catalase, 63 μM *N*-dansyl-Tyr-Val-Gly, 6.0 mM sodium ascorbate, and either 3.0 μM $Cu(NO_3)_2$ or 36 μM $Cu(NO_3)_2$. The α-AE-dependent consumption of O_2 was measured in one sample using a Yellow Springs Instrument Model 53 oxygen monitor, and in a second, matched sample, the concentrations of *N*-dansyl-Tyr-Val-Gly, (*S*)-*N*-dansyl-Tyr-Val-α-hydroxyglycine, and *N*-dansyl-Tyr-Val-NH_2 were determined as a function of time after enzyme addition using the HPLC procedure of Consalvo et al. (1992).

X-ray Absorption (XAS) Data Collection and Analysis. XAS data were collected at the Stanford Synchroton Radiation Laboratory (SSRL) on beam line 7.3, with a beam energy of 3 GeV and maximum stored beam currents between 100 and 50 mA. The Si(220) monochromator was detuned 50% to reject harmonics. The protein samples were measured as frozen glasses in 20% glycerol at 11−14 K in fluorescence mode using a 13-element Ge detector. To avoid detector saturation, the count rate of each detector channel was kept below 35 KHz, by adjusting the hutch entrance slits or by moving the detector in or out from the cryostat windows. Under these conditions, no dead-time correction was necessary. The summed data for each detector were then inspected, and only those channels that gave high-quality backgrounds free from glitches, drop outs, or scatter peaks were included in the final average. Sixteen scans of the raw data were averaged, the background was subtracted, and then the value was normalized to the smoothly-varying background atomic absorption using the EXAFS data reduction package EXAFSPAK (George, 1990). The experimental energy threshold ($k = 0$) was chosen as 9665 eV. Energy calibration was achieved by reference to the first inflection point of a zinc foil (9660.7 eV) placed between the second and third ion chambers. In any series of scans, the measured energy of the first inflection of the zinc foil spectrum varied by less than 1 eV. Averaged EXAFS data were referenced to the zinc calibration of the first scan of a series, since the energy drift in any series of scans was too small to perturb the EXAFS oscillations. For edge analysis, nine scans with absolute energy calibrations which varied by less than ±0.3 eV were averaged.

Data analysis was carried out by least-squares curve fitting utilizing curved-wave calculations as formulated by the SRS library program EXCURV (Binsted et al., 1988; Gurman, 1989; Gurman et al., 1984, 1986), using the methodology described in detail in previous publications from the Blackburn laboratory (Blackburn et al., 1991; Sanyal et al., 1993; Strange, et al., 1987). The parameters refined in the fit were as follows: E_0, the photoelectron energy threshold; R_i, the distance from Zn to atom *i*; and $2\sigma^2_i$, the Debye−Waller term for atom *i*. The coordination numbers were constrained (i)

Table 1: Zinc Stoichiometry for Bifunctional Peptidylglycine α-Amidating Enzyme

sample	[Zn(II)] (μM)[a]	[α-AE] (μM)	[Zn(II)]/[α-AE]
A	2.1	1.7[b]	1.2
B	2.4	1.8[b]	1.3
C	5.4	6.2[b]	0.9
D	2.1	2.3[c]	0.9
E	8.7	6.4[c]	1.4
average (±SD)			1.1 ± 0.2

[a] The zinc concentration was determined using 4-(2-pyridylazo)resorcinol as described in the Experimental Section. [b] The protein concentration was determined using the Bradford dye-binding assay (Bradford, 1976). [c] The protein concentration was determined by amino acid analysis.

to integer values and (ii) so as to produce Debye−Waller factors within reasonable limits (first shell, $0 < 2\sigma^2 < 0.012$; second shell, $0.02 < 2\sigma^2$). Multiple scattering contributions from outer shell (C_2, C_3, N_4, and C_5) atoms of coordinated histidine rings were simulated using well-documented methodology described in previous publications (Blackburn et al., 1991, Sanyal et al., 1993, Strange, et al., 1987). The quality of the fits was determined using a least-squares fitting parameter, *F*, defined as

$$F^2 = (1/N)\sum k^6(\chi_i^{\text{theor}} - \chi_i^{\text{exp}})^2$$

referred to as the fit index, where *N* is the number of data points.

RESULTS AND DISCUSSION

Presence of an Enzyme-Bound Zinc. Previous data have established that two Cu(II) atoms were required per mole of enzyme for maximum PHM activity; however, a possible role for other metals in carbinolamide dealkylation has been suggested (Eipper et al., 1991). To address this issue, our initial experiments were designed to determine if bifunctional rat α-AE contained zinc.

PAR analysis of the supernatant obtained after perchloric acid denaturation of a sample of α-AE that had been exhaustively dialyzed against metal-free buffer indicated that Zn(II) was present in the enzyme. The Zn(II) stoichiometry determined from analysis of five samples from three different enzyme preparations was 1.1 ± 0.2 mol of Zn(II)/mol of α-AE (Table 1). The Zn(II) atom must bind more tightly to α-AE than the two Cu(II) atoms because the enzyme, as purified, contains 1.0 mol of Zn(II)/mol of enzyme (Table 1) and only 0.1−0.2 mol of Cu(II)/mol of enzyme (Boswell et al., 1996; Freeman et al., 1993; Kulathila et al., 1994). Zinc binds tightly to most zinc metalloenzymes, as evidenced by the difficulty frequently encountered in removing the protein-bound Zn(II) atom(s) (Coleman, 1983; Valle & Galdes, 1984). For example, a K_d of 1.0 pM has been determined for the binding of Zn(II) to apo-carbonic anhydrase at pH 7.0 (Lindskog & Nyman, 1964).

Zinc Dependence of the PAL Activity. The data in Table 1 show that bifunctional α-AE binds 1.0 mol of Zn(II)/mol of enzyme. Treatment of α-AE with EDTA produced an enzyme species unable to catalyze either peptide hydroxylation or carbinolamide dealkylation. The addition of Mn(II), Zn(II), Cd(II), or Co(II) to the EDTA-treated enzyme partially restored PAL activity (Figure 1). No restoration of PAL activity was detected upon the addition of Ca(II), Cu(II), Mg(II), or Fe(III). A comparison of the first-order

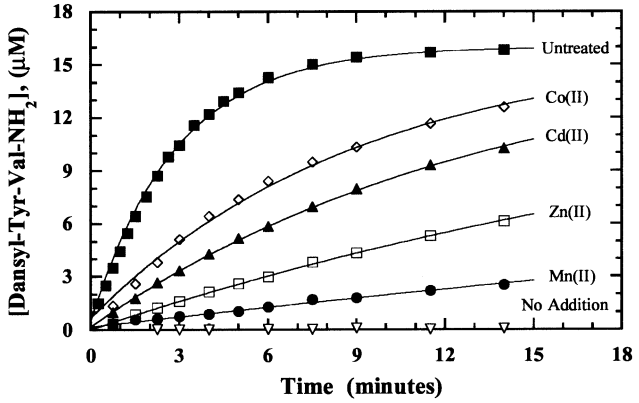


FIGURE 1: Restoration of PAL activity after EDTA treatment. Enzyme (27 μg/mL) was incubated for 10 h at 4 °C with 10 mM EDTA. Following EDTA treatment, an aliquot (5 μL) was removed, added to PAL assay solution containing 16 μM (*S*)-*N*-dansyl-Tyr-Val-α-hydroxyglycine and 0 or 167 μM metal, the subsequent time-dependent formation of *N*-dansyl-Tyr-Val-NH_2 was measured (see Experimental Section for complete details). EDTA was omitted from the untreated control, and this sample was assayed without the addition of metal to the assay solution. The symbols represent the experimentally determined values for [*N*-dansyl-Tyr-Val-NH_2], and the lines were drawn using the first-order rate constants in Table 2.

Table 2: First-Order Rate Constants for the Conversion of (*S*)-*N*-Dansyl-Tyr-Val-α-hydroxyglycine to *N*-Dansyl-Tyr-Val-NH_2 as a Function of the Metal Added to Apo-α-AE[a]

metal[b]	k (s^{-1})	$k_{metal}/k_{control}$
untreated control[c]	$(57.6 \pm 1.0) \times 10^{-4}$	1.0
no addition[d]	$\sim 0.06 \times 10^{-4}$	0.001
Mn(II)[e]	$(1.9 \pm 0.1) \times 10^{-4}$	0.03
Zn(II)[e]	$(5.8 \pm 0.1) \times 10^{-4}$	0.10
Cd(II)[e]	$(12.3 \pm 0.1) \times 10^{-4}$	0.21
Co(II)[e]	$(18.4 \pm 0.4) \times 10^{-4}$	0.32

[a] The first-order rate constants were obtained by linear regression of the data in Figure 1 to $\ln([S]_0/([S]_0 - [P]_t)) = kt_1$ with $[S]_0 = 16$ μM and $[P]_t$ = the concentration of *N*-dansyl-Tyr-Val-NH_2 at time = *t*. The reported errors are the standard error. [b] Metal added to carbinolamide dealkylation assay solution. [c] EDTA was not included in the incubation solution for the untreated control, see Experimental Section. [d] Calculated from the formation of ~80 nM *N*-dansyl-Tyr-Val-NH_2 at 14 min. [e] For each different added metal, a control was run for which EDTA was omitted from the incubation solution, but the relevant metal was included in the carbinolamide dealkylation assay solution. In each case, the first-order rate constant obtained was within experimental error of $k_{control}$, 57.6×10^{-4} s^{-1}.

rate constants for *N*-dansyl-Tyr-Val-NH_2 formation from the data in Figure 1 indicates that Co(II) is most effective in restoring PAL activity, with a $k_{Co}/k_{control} = 0.32$, and Mn(II) is least effective, $k_{Mn}/k_{control} = 0.03$ (Table 2). Addition of Zn(II) to EDTA-treated α-AE restored only 10% of the carbinolamide dealkylation activity.

The incomplete restoration of dealkylation activity upon metal addition suggests that apo-α-AE is unstable. The instability of the apo forms of zinc metalloenzymes, such as carboxypeptidase A (Auld & Holmquist, 1974), alcohol dehydrogenase (Sytkowski & Vallee, 1978, 1979), RNA polymerase (Speckhard et al., 1977; Wu et al., 1977), and Met-tRNA synthetase (Lando & Schimmel, 1993), has been reported. For *E. coli* RNA polymerase, only 30−50% of the original specific activity could be restored after reconstitution of the apo-enzyme with Zn(II) (Wu et al., 1977). Cobalt(II) substitution for Zn(II) in a zinc metalloenzyme usually results in an enzymatically active species (Vallee &

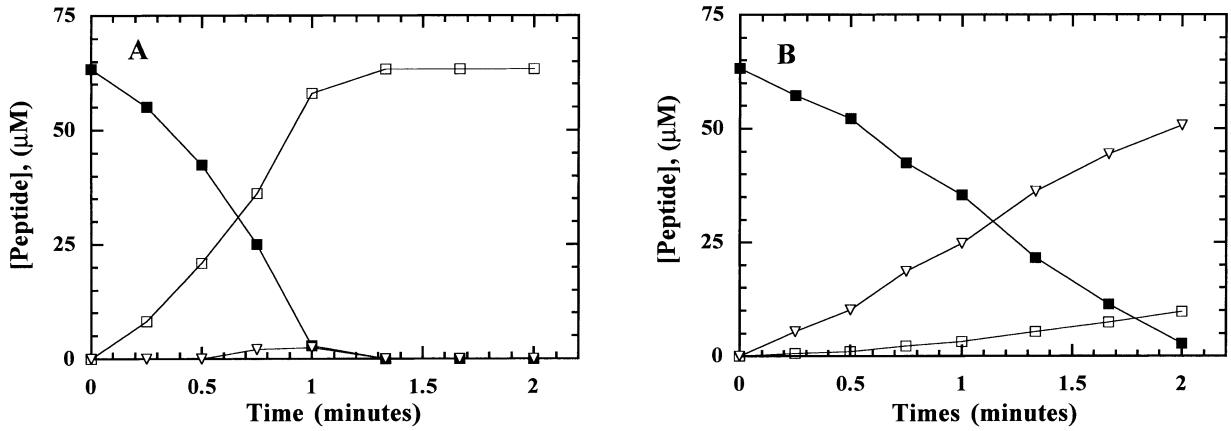


FIGURE 2: Restoration of only PHM activity upon copper addition to EDTA-treated bifunctional α-AE. Enzyme (4.6 mg/mL) was incubated with either 0 (A) or 10 mM EDTA (B). After 1 h at 37 °C, an aliquot (10 μL) was added to the assay solution such that the final solution contained 100 mM MES/NaOH pH 6.0, 30 mM NaCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 10 μg/mL catalase, 63 μM *N*-dansyl-Tyr-Val-Gly, 6.0 mM sodium ascorbate, and either 3.0 μM $Cu(NO_3)_2$ (A) or 36 μM $Cu(NO_3)_2$ (B). The concentrations of *N*-dansyl-Tyr-Val-Gly (■), (*S*)-*N*-dansyl-Tyr-Val-α-hydroxyglycine (▽), and *N*-dansyl-Tyr-Val-NH_2 (□) were determined as described (Consalvo et al., 1992).

Galdes, 1984), and in a few cases, the Co(II)-enzyme has a higher V/K than the Zn(II)-enzyme (Ben-Meir et al., 1993; Brown & Collins, 1991; Omburo et al.,1993; Pettigrew et al., 1985). The results in Figure 1 suggest, but do not prove, that Co(II)-substituted PAL is more active than Zn(II)-PAL.

Addition of Copper to Zinc-Depleted Bifunctional α*-AE.* Eipper et al. (1983) first described the copper dependence of peptide amidation, which was subsequently verified in other laboratories (Emeson, 1984; Kulathila et al., 1994). The addition of metals other than copper to copper-depleted PHM does not restore peptide hydroxylation activity (Eipper et al., 1983; Emeson, 1984; Kizer et al., 1986; Kulathila et al., 1994). Since the addition of Cu(II) to EDTA-treated, bifunctional α-AE does not restore PAL activity, copper addition is expected to only restore PHM activity. Experimentally, this would be manifested by the production of only (*S*)-*N*-dansyl-Tyr-Val-α-hydroxyglycine from *N*-dansyl-Tyr-Val-Gly when assaying EDTA-treated enzyme in the presence of Cu(II). As shown in Figure 2A, ≤2.5 μM (*S*)-*N*-dansyl-Tyr-Val-α-hydroxyglycine accumulated when 63 μM *N*-dansyl-Tyr-Val-Gly was amidated by untreated, bifunctional α-AE. In contrast, significant concentrations of (*S*)-*N*-dansyl-Tyr-Val-α-hydroxyglycine accumulated, to a maximum of 51 μM at 2.0 min, upon reaction of *N*-dansyl-Tyr-Val-Gly with EDTA-treated enzyme in a solution containing Cu(II) (Figure 2).[2] The data in Figure 2 provide additional evidence that Cu(II) is required only for peptide hydroxylation and Zn(II) is required only for carbinolamide dealkylation.

X-ray Absorption Spectroscopy. The XAS spectrum of bifunctional α-AE indicates the presence of both Cu and Zn K-absorption edges, with the Zn edge jump approximately half that of the Cu edge jump. This result confirms the metal stoichiometry of 1 Zn per 2 Cu. Figure 3 (A and B) shows

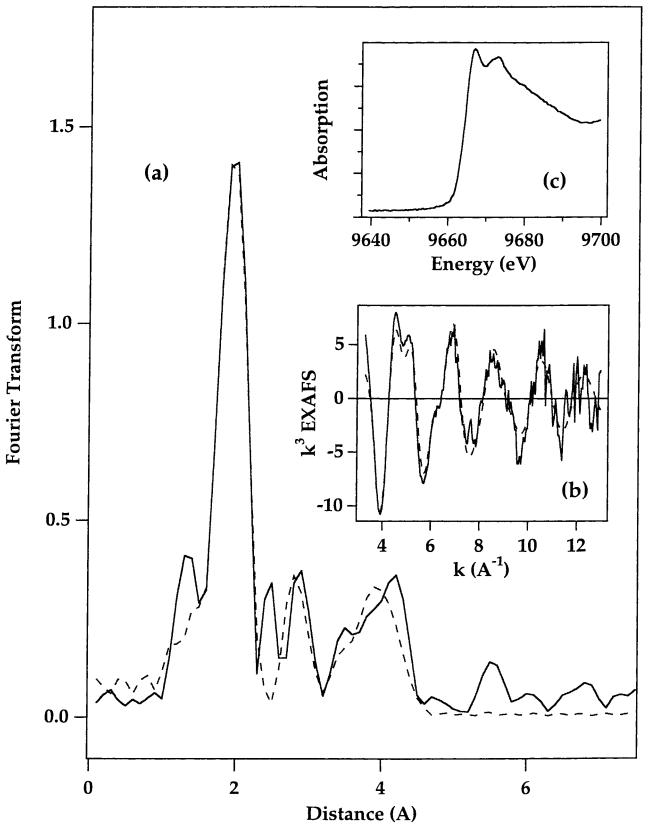


FIGURE 3: Simulation of the EXAFS Spectra of the Zinc Center in Bifunctional α-AE. The fits correspond to five N/O ligands at 1.98 Å, Debye−Waller ($2\sigma^2$) = 0.006 Å^2, multiple scattering contributions from three imidazole groups with outer shell Zn−C/N distances (Å) and Debye−Waller factors ($2\sigma^2$, Å^2) of 2.79 (0.012), 2.86 (0.012), 4.05 (0.020), and 4.13 (0.020), $\Delta E_0 = -26.5$ eV, FI = 4.71. (A) Experimental versus simulated Fourier transform; (B) experimental versus simulated background-substrated Zn K-EXAFS; (C) Zn K-edge. Errors are estimated as ±0.002 Å in the first shell distances and ±25% in the coordination numbers.

the Fourier transform Zn K-EXAFS. The presence of outer shells in the FT and characteristic beat patterns in the EXAFS at $k \approx 5$ and 7 indicate the presence of imidazole coordination from histidine amino acid side chains. Detailed curve fitting was performed to determine the number of histidine side chains and whether additional non-histidine coordinated

[2] Ideally, the addition of Cu(II) and Co(II) (or Zn(II)) to EDTA-treated α-AE would restore both the PHM and PAL activities. In such a situation, (*S*)-*N*-dansyl-Tyr-Val-α-hydroxyglycine would accumulate to concentrations significantly less than that observed in Figure 2B. This result was not obtained despite extensive variation in the ratios of Co(II)/Cu(II)/EDTA. Control experiments show that the specific activity of untreated α-AE for (*S*)-*N*-dansyl-Tyr-Val-α-hydroxyglycine dealkylation decreases 80%, from 20.3 to 4.3 s^{-1}, upon the the addition of 167 μM Cu(II), suggesting that Cu(II) inhibits PAL.

ligands were present. The results of the analysis suggested the presence of only O and N donor atoms and a first-shell coordination number greater than 4 or higher. Initial fitting to first-shell Fourier filtered data gave the best fit ($F = 0.229$) with 5 O/N ligands at 1.98 ± 0.01 Å. Alternative fits using 4 O/N or 6 O/N first-shell scatterers gave slightly poorer fits with F values of 0.305 and 0.241, respectively. Splitting the first shell gave no improvement in the fit. Outer shells from C_2/C_5 (C_β) and C_3/N_4 (C_γ) atoms of the imidazole rings were then added to the fit and refined against the raw data. Both single and multiple scattering pathways were included in this analysis. During the least-squares refinement, imidazole ring bond lengths and bond angles were allowed to vary by about 10% from idealized positions found in crystallographic zinc−imidazole complexes. Based on our considerable experience in simulation of the EXAFS of copper−imidazole coordination in proteins, Debye−Waller terms for the C_β shell were fixed at ~2 times the first shell value and for C_γ at ~3 times the first shell value. The results gave best fits for between 2 and 3 histidine ligands with Zn−N bond lengths of 1.98 Å. In agreement with the first-shell Fourier filter analysis, it was necessary to include two additional non-histidine O/N ligands at the same distance (1.98 Å) to obtain a satisfactory fit to the EXAFS and Fourier transform. Representative fits to 3 histidine and 3 non-histidine O/N ligands are shown as dashed lines in Figure 3 (A and B).

The EXAFS fitting results, thus, support 5 ± 1 O/N ligands at 1.98 ± 0.01 Å, with evidence that at least two of these are derived from histidine ligation. As is typical of EXAFS fitting methods, bond lengths are derived with much greater precision than coordination numbers. Bond lengths are themselves related to coordination number since as the coordination number increases, the bond length must also increase in order to maintain the oxidation state of the central metal. Therefore, it is possible to use the EXAFS-derived bond length to provide an independent estimate of the coordination number. This relationship has been quantified by Thorp in the procedure known as bond valence sum analysis (Thorp, 1992; Liu & Thorp, 1993). The bond valence (BV) of a metal−ligand bond with length r is calculated for the expression (Brown & Altermatt, 1985)

$$\mathrm{BV} = \exp[(r_0 - r)/B]$$

where $B = 0.37$ Å and r_0 is obtained from tabulated data given by Thorp (1992) and Liu & Thorp (1993). The bond valence sum (BVS), which should equal the oxidation state of the metal, is simply the sum of bond valences for all of the bonds around the metal atom. Using our EXAFS-derived bond length of 1.98 Å, we have calculated the BVS for Zn−N/O coordination numbers between 4 and 6. Since the EXAFS analysis cannot distinguish between O and N scatterers, we have used an average $r_0 = 1.740$. The resulting BVS values are 2.09, 2.61, and 3.12 for coordination numbers 4, 5, and 6, respectively. Since the oxidation state of the zinc must be 2.0, this analysis indicates that while curve-fitting results produce a slightly lower value of F for the 5-coordinate model, 4-coordination is more consistent with the observed Zn−N/O bond length.

Role of Zinc in the α-AE Catalyzed Dealkylation of Carbinolamides. Zinc is generally found to be 4- or 5-coordinate in structurally characterized zinc-containing metalloproteins (Vallee & Auld, 1990). These zinc sites can be divided into those which have a structural role and those with a catalytic role. Proteins with structural zinc generally (but not universally) contain thiolate ligation and include the structural site of alcohol dehydrogenase, the family of zinc-finger DNA-binding proteins ($Zn(Cys)_x(His)_{4-x}$, $x = 1-4$), and the zinc clusters of metallothioneins. Catalytic zinc sites, on the other hand, contain primarily oxygen and nitrogen donor groups as exemplified by the carbonic anhydrases ($[Zn(His)_3OH_2]$) and the metalloproteases ($[Zn(His)_2(Glu)OH_2]$ as found in carboxypeptidase A, carboxypeptidase B, and thermolysin or $[Zn(His)_3OH_2]$ as found in carboxypeptidase D and β-lactamase). Comparison of high-resolution X-ray crystallographic and XAS studies on carboxypeptidase A has shown that the four primary ligands to zinc are provided by His-69, His-196, the ϵ_1-oxygen of Glu-72, and a coordinated water molecule, while the ϵ_2-oxygen of the coordinated Glu-72 provides a weak fifth ligand to zinc at around 2.3 Å (Zhang et al., 1992). Five-coordinate zinc is also observed in the structurally distinct astacin family of metalloendoproteases which contain zinc coordinated by three histidines, water, and a more weakly bound tyrosine residue (Bode et al., 1992; Morante et al., 1996; Stöcker et al., 1993). Human neutrophil collagenase cocrystallized with the substrate analog Pro-Leu-Gly-hydroxylamine contains pentacoordinate zinc with three histidines and both oxygen atoms of the terminal hydroxamate group of the inhibitor as ligands.

In the present study, the elimination of PAL activity on removal of zinc by EDTA certainly suggests that Zn may be involved in carbinolamide dealkylation activity, and the 4−5 N/O coordination environment suggested from EXAFS analysis is consistent with such a role. The carbinolamide dealkylation reaction catalyzed by PAL proceeds nonenzymatically above pH 8 (Bundgaard & Kahns, 1991; Mounier et al., 1997) and, thus would appear to involve attack by OH^-. In this respect, the carbinolamide dealkylation reaction resembles the reactions catalyzed by other zinc-dependent hydrolases (Christianson & Lipscomb, 1989; Matthews, 1988; Merkler & Schramm, 1993; Silverman & Lindskog, 1988; Wilson et al., 1991). A zinc-coordinated water (or hydroxide) could serve as a general base to abstract the hydroxyl proton of the carbinolamide. On the other hand, attempts to reconstitute > 10% PAL activity by adding back zinc have proven difficult. This raises the possibility that Zn plays some essential structural role, such that its removal by EDTA causes a structural change that leads to the loss of activity. A third possibility is that binding of EDTA to α-AE is itself inhibitory and the restoration of activity by zinc is merely the result of the removal of inhibitory EDTA. We believe this latter explanation is unlikely since EDTA should have been removed more efficiently by both iron and copper, neither of which were able to restore any PAL activity.

A recent crystal structure of the monooxygenase catalytic core of α-AE (referred to as PHMcc) has revealed a binding site for divalent metal ions, Ni(II) and Cu(II), at the interface of two PHMcc molecules, with two histidines and four solvent molecules as ligands in an approximately octahedral configuration. In the crystal structure, this site functions as a crystal packing contact and is occupied by copper.[3] Concentrated samples of PHMcc prepared for XAS studies

[3] Prigge, S. T., and Amzel, L. M., personal communication.

on the catalytic copper centers of the monooxygenase domain also appear to contain 1 Zn per 2 Cu as estimated for X-ray fluorescence spectra (Blackburn, N. J., unpublished data). It is likely that the zinc observed in these PHMcc samples is coordinated at the same locus as the crystal packing site found in the crystal structure and arises because of aggregation of the PHMcc molecules in the concentrated solutions used for XAS. It is unclear whether the Zn that we find in the bifunctional enzyme could also bind in this site or indeed whether the site exists or is accessible to metal ions in the bifunctional enzyme, where the PAL domain presumably has extensive contact with the PHM domain. However, if the Zn binding site of α-AE is coincident with the divalent metal site of PHMcc, then the loss of PAL activity on zinc removal may suggest a role in the transport of the PHM product to the PAL active site. Further studies are underway to clarify the specific role of zinc in the peptide amidation reaction.

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