Redox Cycling of Enzyme-Bound Copper during Peptide Amidation

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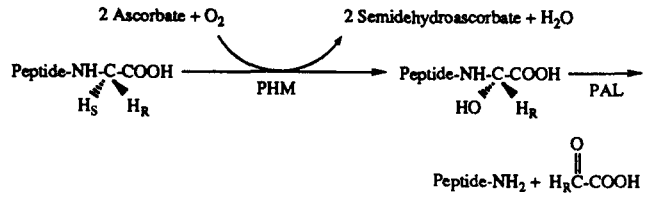
Approximately 50% of all known mammalian peptide hormones possess a C-terminal α-amide moiety,[1] and structure–activity studies demonstrate that the C-terminal α-amide is critical to the bioactivity of most amidated peptide hormones.[2] Peptidylglycine α-amidating enzyme (α-AE, EC 1.14.17.3) is a bifunctional, copper-dependent monooxygenase[3,4] which catalyzes the oxidative cleavage of glycine-extended peptides to C-terminal α-amidated peptides plus glyoxylate.[5] Peptides hydroxylated at the α-carbon of the C-terminal glycyl residue are intermediates in the amidation reaction[6] (Scheme I). The initial oxidative reaction is catalyzed by peptidyl α-hydroylating monooxygenase (PHM, EC 1.4.17.3), while the second step, dealkylation, is catalyzed by peptidyl amidoglycolate lyase (PAL, EC 4.3.2.5). Proteolytic separation of the monofunctional enzymes PHM and PAL is controlled by the alternate splicing of the α-AE mRNA.[4d] Ramer et al.[8] have shown that the *pro-S* proton is stereospecifically abstracted from the C-terminal glycyl α-carbon. Ping et al.[9] have shown that the product of the PHM reaction is *S*-peptidyl-α-hydroxyglycine, which is then dealkylated by PAL. Recent results from our laboratory[10] are consistent with the tandem stereochemical course of the PHM and the PAL reactions depicted in Scheme I.

The role played by enzyme-bound copper in the redox chemistry of α-AE has not been elucidated. In some models for the amidation reaction[3b,11] it has been proposed that the copper undergoes a reduction/oxidation cycle from Cu(II) to Cu(I) and back to Cu(II) with the concomitant oxidation of peptidyl-Gly to peptidyl-α-hydroxyglycine. Support for this hypothesis comes from work on dopamine β-hydroxylase (DβH) which demonstrated that redox cycling of DβH-bound copper correlated with tyramine hydroxylation to octopamine.[12] We report here the first evidence that (1) α-AE-bound Cu(II) is anaerobially reduced to Cu(I) by added reductant, (2) α-AE-bound Cu(I) is catalytically competent, and (3) α-AE-bound Cu(I) is quantitatively reoxidized to Cu(II) only in the presence of both O_2 and peptidyl-Gly substrates.

Scheme I. Reaction Catalyzed by Bifunctional α-AE[a]



[a] The two moles of semidehydroascorbate produced per α-AE turnover disproportionate to ascorbate and dehydroascorbate[7] unless a chemical or enzymatic trap for semidehydroascorbate is included in the assay solution.[3c]

The EPR spectrum of copper-free α-AE[13,14] reconstituted with 2.5 mol of $^{63}CuCl_2$/α-AE active sites is shown in Figure 1A. Excess ^{63}Cu(II) was added to ensure saturation of the two inferred copper binding sites of α-AE.[15,16] Spin counting of Figure 1A indicates that all the added copper is spin active, 0.9 ± 0.1 spins/Cu(II) added. The anaerobic addition of 0.5 mol of ascorbate/mol of $^{63}CuCl_2$ resulted in ~95% reduction of Cu(II) to the EPR-silent Cu(I) (Figure 1B). Oxygen was then admitted to the α-AE/Cu(I) solution and incubated at room temperature for 30 min. The resulting EPR spectrum (Figure 1C) indicated that 20 ± 2% of the Cu(I) was oxidized back to Cu(II). The recovery of only ~20% of the EPR signal in the presence of O_2, but not peptide substrate, suggested either that the Cu(I) centers were unavailable to interact with O_2 until peptidyl-Gly was bound or that the copper·O_2 complex was in a spin-coupled state with O_2 providing a bridging ligand for superexchange. Note that the concentration of Cu(I) oxidized back to Cu(II) after incubation in O_2 was approximately equal to the concentration of copper in excess of the assumed copper binding capacity of α-AE. Addition of 1.0 mol of *N*-dansyl-Tyr-Val-Gly/mol of α-AE active site to the sample shown in Figure 1C resulted in the quantitative oxidation of the remaining Cu(I) to Cu(II) in the time (≤5 min) taken to perform the experiment (Figure 1D). Subsequent HPLC

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(13) Copper-free α-AE was prepared by exhaustive dialysis against 100 mM MES pH 6.0, 100 mM KCl, and 30 mM KI. Copper analysis was done by atomic absorption spectroscopy, and the protein concentration was determined using the bicinchoninic acid (BCA) assay. The protein concentration obtained using the BCA assay is equivalent within experimental error to that obtained by amino acid analysis (Young, S. D.; Freeman, J. C; Snyder, L. M., unpublished results). The reference for the BCA protein assay is Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. *Anal. Biochem.* **1985**, *150*, 76–85.
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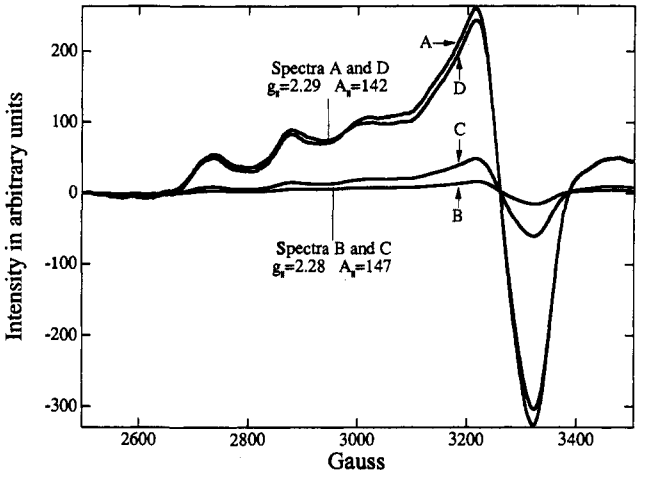

Figure 1. Electron paramagnetic spectra of α-AE under the following conditions: (A) anaerobic copper-free 63 μM α-AE (4.7 mg/mL) + 158 μM $^{63}CuCl_2$; (B) anaerobic 61 μM α-AE + 154 μM $^{63}CuCl_2$ + 76 μM ascorbate; (C) aerobic 61 μM α-AE + 154 μM $^{63}CuCl_2$ + 76 μM ascorbate; (D) aerobic 60 μM α-AE + 150 μM $^{63}CuCl_2$ + 74 μM ascorbate + 63 μM *N*-dansyl-Tyr-Val-Gly. Spectrum B was collected after a 10-min incubation at room temperature. Spectrum C was obtained after a 30-min exposure of the sample to the atmosphere. Air was gently bubbled up from the bottom of the solution with a syringe at 5-min intervals over the 30-min period to ensure complete dissolution of O_2 into the solution contained in the long, narrow EPR tube. Following the 30-min incubation in the presence of air, *N*-dansyl-Tyr-Val-Gly was added and spectrum D immediately collected. All spectra were collected at 113 K after the α-AE samples had been flash frozen by immersion in an isopentane/dry ice slush. The irradiation conditions for all the spectra were 63 mW of power at 9.44 GHz. The specific activity of the α-AE was 4.6 μmol of *N*-dansyl-Tyr-Val-Gly amidated/min/mg at 37 °C using the standard conditions of Miller et al.[13]

analysis[17] revealed complete amidation of *N*-dansyl-Tyr-Val-Gly to *N*-dansyl-Tyr-Val-NH_2 (data not shown). In summary, we have shown that copper in the presence of α-AE undergoes a reduction/oxidation cycle during the enzymatic-catalyzed amidation of *N*-dansyl-Tyr-Val-Gly.[18] The spectrum in Figure 1A is consistent with α-AE-bound type 2 copper, and the reoxidation of Cu(I) upon O_2 and *N*-dansyl-Tyr-Val-Gly addition (Figure 1C) strongly suggests that Cu(I) is also enzyme-bound. The data in Figure 1 also provide some insights into the kinetic mechanism for α-AE. The anaerobic reduction of α-AE-Cu(II) in the absence of peptide substrate is evidence that ascorbate binds first, in accordance with the kinetic mechanism of the catalytically similar enzyme, DβH.[19] The quantitative oxidation of α-AE–Cu(I) only in the presence of O_2 and *N*-dansyl-Tyr-Val-Gly, coupled with production of *N*-dansyl-Tyr-Val-NH_2, indicates than an EPR-silent α-AE–Cu(I)·O_2·peptidyl-Gly ternary complex gives rise to products, again consistent with the kinetic mechanism proposed for DβH.[19,20] Experiments to determine the copper stoichiometry per α-AE active site and to further define the role of copper in enzymatic peptide amidation are currently underway.

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(18) A reviewer noted that the results in Figure 1 imply that copper-free α-AE is inactive. Repeated attempts to verify this experimentally have been unsuccessful. Assays of 10–100 nM copper-free α-AE invariably showed 20–30% of the initial velocity found in assays containing 1 μM added $CuSO_4$ which was subsequently attributed to adventitious copper, from 0.2–0.5 mol of Cu(II)/active site. Treatment of the rat α-AE with metal chelators produced inactive enzyme which could be restored to full initial activity by the addition of Cu(II).[15] Dialysis of porcine α-AE vs EDTA also produced inactive enzyme which could be restored to ≥90% of the original activity only by the addition of Cu(II). The addition of other metal ions restored activity only marginally, ≤4% of original activity, see: Bradbury, A. F.; Smyth, D. G. In *Biogenetics of Neurohormonal Peptides*; Thorell, J., Håkanson, R., Eds.; Academic Press: New York, 1985; pp 171–186.

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