^{18}O Isotopic ^{13}C NMR Shift as Proof That Bifunctional Peptidylglycine α-Amidating Enzyme Is a Monooxygenase[†,‡]

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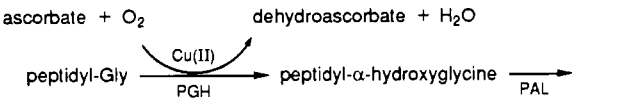
ABSTRACT: The biosynthesis of C-terminal α-amidated peptides from their corresponding C-terminal glycine-extended precursors is catalyzed by peptidylglycine α-amidating enzyme (α-AE) in a reaction that requires copper, ascorbate, and molecular oxygen. Using bifunctional type A rat α-AE, we have shown that O_2 is the source of the α-carbonyl oxygen of pyruvate produced during the amidation of dansyl-Tyr-Val-[α-^{13}C]-D-Ala, as demonstrated by the ^{18}O isotopic shift in the ^{13}C NMR spectrum of [α-^{13}C]lactate generated from [α-^{13}C]pyruvate in the presence of lactate dehydrogenase and NADH. In addition, one-to-one stoichiometries have been determined for glyoxylate formed/dansyl-Tyr-Val-Gly consumed, pyruvate formed/dansyl-Tyr-Val-D-Ala consumed, dansyl-Tyr-Val-NH_2 formed/ascorbate oxidized, and dansyl-Tyr-Val-NH_2 formed/O_2 consumed. Quantitative coupling of NADH oxidation to dansyl-Tyr-Val-NH_2 production using *Neurospora crassa* semidehydroascorbate reductase showed that two one-electron reductions by ascorbate occurred per α-AE turnover. The stoichiometry of ~1.0 dansyl-Tyr-Val-NH_2 produced/ascorbate oxidized observed in the absence of a semidehydroascorbate trap resulted from the disproportionation of two semidehydroascorbate molecules to ascorbate and dehydroascorbate.

Posttranslational, C-terminal amidation is required for the full biological activity of many peptide hormones (Ferrier & du Vigneaud, 1966; Burgus et al., 1970; McGuigan & Thomas, 1972; Couture et al., 1979; Guttman, 1980) and arises from the oxidative cleavage on the N-terminal side of the α-carbon of a C-terminal, glycine-extended precursor (Bradbury et al., 1982; Eipper et al., 1983). Ample evidence suggests that this oxidative cleavage is a two-step process (Young & Tamburini, 1989; Tajima et al., 1990; Kato et al., 1990; Katopodis et al., 1990). The first step, catalyzed by peptidylglycine hydroxylase (PGH),[1] is the ascorbate, O_2, and copper-dependent formation of peptidyl-α-hydroxyglycine, and the second step, catalyzed by peptidylaminoglycolate lyase (PAL), is the ascorbate, O_2, and copper-independent conversion of peptidyl-α-hydroxyglycine to the amidated peptide and glyoxylate (Young & Tamburini, 1989; Kato et al., 1990; Katopodis et al., 1990, 1991; Eipper et al., 1991; Merkler & Young, 1991). This reaction has been proposed to proceed according to eq 1.



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Although distinct proteins that catalyze the individual steps of the amidation reaction have been described (Kato et al., 1990; Katopodis et al., 1990, 1991; Suzuki et al., 1990; Eipper et al., 1991), we have previously shown that the rat type A 75-kDa protein, called peptidylglycine α-amidating enzyme (α-AE), contains both PGH and PAL activities on a single polypeptide chain (Merkler & Young, 1991). The selective inactivation of PGH, but not PAL, by ascorbate also strongly suggests that the rat type A enzyme is bifunctional (Merkler et al., 1992). The 75-kDa enzyme lacks an internal dibasic cleavage site (Bertelsen et al., 1990), which likely prevents the proteolytic separation of the two activities. The biological significance of these multiple enzyme forms is unclear; however, a novel regulatory scheme probably exists for the expression of PGH, PAL, and α-AE activities.

Because of the similarities between the reactions catalyzed by PGH and dopamine β-hydroxylase (Villafranca, 1981; Stewart & Klinman, 1988), it has been assumed that the production of 1 mol of amidated peptide required the input of two electrons from the oxidation of ascorbate to dehydroascorbate and that the origin of the α–carbonyl oxygen of glyoxylate was O_2 (Glembotski et al., 1984). Although chemically reasonable, these assumptions have not been experimentally verified.

We report in this article the elucidation of the stoichiometric relationships between substrates and products, show that 2 mol of ascorbate undergoes oxidation during amidation to produce 2 mol of semidehydroascorbate, and demonstrate by an ^{18}O–^{13}C NMR isotopic shift that O_2 is the source of the α-carbonyl oxygen of pyruvate generated during the amidation of dansyl-Tyr-Val-D-Ala.

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[1] Abbreviations: α-AE, peptidylglycine α-amidating enzyme; CHO, chinese hamster ovary; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; DβH, dopamine β-hydroxylase; DCC, dicyclohexylcarbodiimide; DHA, dehydroascorbic acid; DMAP, 4-(dimethylamino)pyridine; EDTA, ethylenediaminetetraacetic acid; FMOC, 9-fluorenylmethoxycarbonyl; LDH, lactate dehydrogenase; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAL, peptidylamidoglycolate lyase; PGH, peptidylglycine hydroxylase; ppb, parts per billion; SDR, semidehydroascorbate reductase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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MATERIALS AND METHODS

Materials. $H_2{}^{18}O$ (98.1 atom %) was from Isotec, Inc., $^{18}O_2$ (97–98 atom %) was from Cambridge Isotope Labs, and sodium [α-^{13}C]lactate and DL-[α-^{13}C]alanine were from MSD Isotopes. NADH was from US Biochemicals, and dehydroascorbic acid was from Aldrich. Lactate dehydrogenase, catalase, and all other chemicals were of the highest quality available from Sigma.

Synthesis of Dansyl-Tyr-Val-[α-^{13}C]-DL-Ala. 9-Fluorenylmethyl chloroformate (316 mg, 1.2 mmol) in 2 mL of dioxane was added dropwise to a stirred solution of [α-^{13}C]-DL-alanine (100 mg, 1.1 mmol) in 3.5 mL of 10% sodium carbonate at 0 °C (Bodanszky & Bodanszky, 1984). After being stirred at 0 °C for 1 h and at room temperature overnight, the reaction mixture was poured into 60 mL of water and washed with two 30-mL volumes of diethyl ether. The aqueous phase was cooled in an ice bath and acidified to pH 3 with aqueous HCl (12 N). The suspension of precipitated amino acid was kept cold for several hours, then collected by suction filtration, washed with ice/water, and dried in vacuo to yield 330 mg (95%) of FMOC-[α-^{13}C]-DL-alanine.

A symmetrical anhydride of FMOC-[α-^{13}C]-DL-Ala was prepared using DCC in *N*-methylpyrrolidone/dichloromethane and coupled to a *p*-alkoxybenzyl alcohol (Wang) resin in the presence of a catalytic amount of DMAP. Synthesis of Tyr-Val-[α-^{13}C]-DL-Ala was performed on an ABI 431A peptide synthesizer starting from 0.25 mmol of FMOC-[α-^{13}C]-DL-Ala resin. Following removal of the final FMOC group, the peptide was dansylated on the resin as described (Consalvo et al., 1989). The peptide was deprotected and cleaved from the resin with 95% TFA for 2 h at room temperature. The peptide diastereomers were separated and purified by HPLC on a Vydac C_{18} column using an acetonitrile gradient in 0.1% (v/v) TFA(aq). Landymore-Lim et al. (1983) showed that D-alanine-extended peptides are substrates for α-AE while L-alanine-extended peptides are not. On the basis of these results, dansyl-Tyr-Val-[α-^{13}C]-D-Ala was identified as the purified diastereomer that was amidated by α-AE. In parallel experiments, the other purified diastereomer, dansyl-Tyr-Val-[α-^{13}C]-L-Ala, showed no conversion to dansyl-Tyr-Val-NH_2. Aliquots of each peptide were characterized by amino acid analysis using phenylisothiocyanate precolumn derivatization and reverse-phase HPLC (Jones et al., 1987). FAB-MS: $(M-H)^+$ for Tyr-Val-[α-^{13}C]-DL-Ala calcd 313, found 313. FAB-MS: $(M-H)^+$ for dansyl-Tyr-Val-[α-^{13}C]-L-Ala calcd 586, found 586.

Enzyme Purification. Neurospora crassa (ATCC 24740) was cultured for 5 days at 30 °C in 5 L of Fries minimal medium (Beadle & Tatum, 1945). The mycelia were collected by filtration and were disrupted using a Biospec bead beater (Bartlesville, OK) according to the manufacturer's instructions for *Candida*. Semidehydroascorbate reductase (SDR) was partially purified through the ammonium sulfate precipitation step (Schulze et al., 1972) and was stored at −70 °C. SDR was assayed spectrophotometrically by measuring the decrease in A_{340} following the addition of enzyme to 100 mM Tris-HCl, pH 7.3, 10 mM ascorbate, 7.5 mM dehydroascorbate, and 100 μM NADH. One unit of SDR activity was the amount of enzyme necessary to oxidize 1 μmol of NADH in 1 min at 37 °C under these conditions. The final SDR had a specific activity of 0.24 unit/mg and had been purified 2.5-fold from the disrupted mycelia.

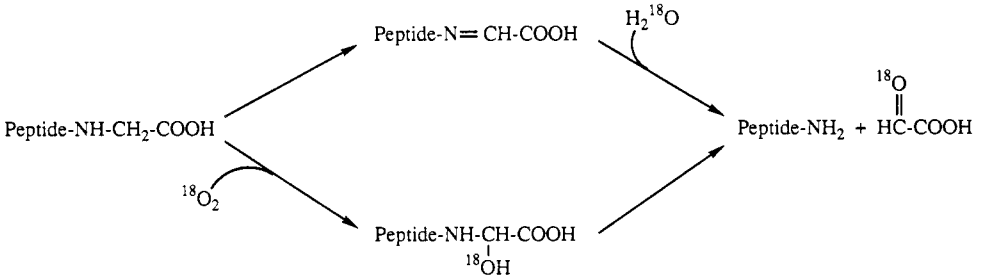
Recombinant type A rat medullary thyroid carcinoma α-AE was expressed in cultured mouse C127 cells (Beaudry et al., 1990) or CHO cells (Miller et al., 1992) transfected with an α-AE expression vector specifically constructed for each cell type. The expression vectors each contained α-AE cDNA modified to terminate translation at the dibasic cleavage site preceding the transmembrane and cytoplasmic domains, thereby permitting the secretion of a soluble 75-kDa enzyme into the culture medium (Beaudry et al., 1990). The C127 α-AE was purified using a rapid, two-step procedure (Merkler & Young, 1991). The resulting C127 α-AE preparation had a specific activity of ~0.6 μmol min^{-1} mg^{-1} and was ~20% pure as judged by SDS–PAGE. The enzyme expressed in CHO cells was purified using a protocol we have developed (Miller et al., 1992). The purified CHO α-AE was 70–90% pure and showed a specific activity of >2.0 μmol min^{-1} mg^{-1}. As previously discussed (Merkler et al., 1992), it was difficult to correlate the specific activity of α-AE with its purity.

Amidation Assay. The amidation of dansyl-Tyr-Val-Gly or dansyl-Tyr-Val-D-Ala was monitored by isocratic elution from a C_{18} reverse-phase HPLC column (4.6 × 100 mm, 5 μm, 120 Å, Keystone Scientific Hypersil ODS) as described (Jones et al., 1988). Oxygen consumption during amidation was measured using a Yellow Springs oxygen electrode. Standard assays to determine specific activity were carried out at 37 °C by the addition of α-AE into 100 mM MES, pH 6.0, 30 mM KCl, 30 mM KI, 1 μM $CuSO_4$, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 100 μg/mL catalase, 20 μM dansyl-Tyr-Val-Gly, and 10 mM ascorbate. At 1 atm and 37 °C, the concentration of dissolved O_2 was assumed to be 200 μM (Morrison & Billet, 1952). At regularly timed intervals, aliquots (30–50 μL) were removed and added to one-fifth volume (6–10 μL) of 6% (v/v) TFA(aq) to stop the reaction. Under these conditions, one unit is the amount of α-AE necessary to amidate 1 μmol of dansyl-Tyr-Val-Gly in 1 min.

^{18}O Incorporation into [α-^{13}C]Lactate. [α-^{13}C]Lactate was generated from dansyl-Tyr-Val-[α-^{13}C]-D-Ala at 37 °C by the addition of 0.77 unit of α-AE to 2.0 mL of 100 mM MES, pH 6.0, 30 mM KCl, 30 mM KI, 1 μM $CuSO_4$, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 100 μg/mL catalase, 1.4 mM dansyl-Tyr-Val-[α-^{13}C]-D-Ala, 3 mM ascorbate, 2 mM NADH, and 2 units/mL LDH. In some reactions, the solvent was 100% $H_2{}^{16}O$ and in others, $H_2{}^{18}O/H_2{}^{16}O$ (50:50). Reactions in 100% $^{18}O_2$ were carried out in sealed vials which had first been exhaustively flushed with O_2-free N_2 (Fieser, 1924) and then filled with $^{18}O_2$. Amidation was allowed to proceed until 60–70% conversion to dansyl-Tyr-Val-NH_2 was achieved, accumulating ~2 μmol of [α-^{13}C]-lactate, and was terminated by the addition of 0.4 mL of 6% (v/v) TFA(aq). Following termination of the reaction, the pH of the solution was adjusted to 6–7 by the addition of KOH. The samples were lyophilized to dryness and stored at −20 °C for subsequent analysis by ^{13}C NMR spectroscopy. For the NMR experiments, the lyophilized samples were dissolved in 1.5 mL of a solution containing 20 mM Na_2-EDTA and 20% (v/v) D_2O. ^{1}H-Decoupled ^{13}C NMR spectra (75.47 MHz) were recorded at 20 °C on a Bruker AM300 spectrometer with the following acquisition and processing parameters: 3000-Hz sweep width, 8-μs pulse width (45° pulse), 5.5-s acquisition time, 0.5-s relaxation delay, and 0.1-Hz line broadening. The samples were contained in 8-mm NMR tubes and 1400–2200 scans were accumulated for each sample. Assignment of the resonance to the α-carbon of lactate was made by the addition of authentic lactate to a sample lacking dansyl-Tyr-Val-[α-^{13}C]-D-Ala.

Quantitation of Glyoxylate or Pyruvate Produced during the Amidation of Dansyl-Tyr-Val-Gly or Dansyl-Tyr-Val-D-Ala. Since glyoxylate and pyruvate are substrates for LDH (Holbrook et al., 1975), the net decrease in A_{340} ($\Delta\epsilon_{340} = 6.22 \times 10^3$ M^{-1} cm^{-1}) caused by complete reduction of glyoxylate

Scheme I: Proposed Mechanisms for α-AE



or pyruvate in the presence of excess NADH was used to quantify these compounds. Following termination of the amidation reactions by heating at 100 °C for 5 min, 330–440 μM NADH and 0.06–0.07 unit/mL LDH were added, and the net decrease in A_{340} was measured. Corrections for nonspecific NADH oxidation were made by running blanks that lacked α-AE.

Quantitation of Ascorbate Oxidation during Amidation. Ascorbate oxidation was monitored by the decrease in A_{250} [$\Delta\epsilon_{250}$ = 8.55 × 10^3 M^{-1} cm^{-1}; Daglish (1951)] at 37 °C resulting from the addition of α-AE to 950 μL of 100 mM MES, pH 6.0, 30 mM KCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, 12.5 μM dansyl-Tyr-Val-Gly, and 48 μM ascorbate. Rates were corrected for nonspecific ascorbate oxidation by running controls containing heat-denatured (5 min at 100 °C) α-AE. Copper and catalase were omitted to minimize nonenzymatic ascorbate oxidation,[2] and KI was omitted to minimize background absorbance.

Quantitation of Semidehydroascorbate Formed during Amidation. Dansyl-Tyr-Val-Gly amidation was coupled to semidehydroascorbate reductase at 37 °C by the addition of α-AE to 1.0 mL of 100 mM Tris-HCl, pH 7.3, 10 mM ascorbate, 30 μM dansyl-Tyr-Val-Gly, 100 μM NADH, 0.018–0.066 unit/mL SDR, and 50 μM $CuSO_4$/unit of SDR. The SDR preparation apparently chelated copper and inhibited α-AE. The addition of 50 μM $CuSO_4$/unit of SDR was required to give acceptable amidation rates. Rates were corrected for nonspecific NADH oxidation by running controls that lacked α-AE.

RESULTS

^{18}O Incorporation into [α-^{13}C]Pyruvate Generated by the Amidation of Dansyl-Tyr-Val-[α-^{13}C]-D-Ala. Two different mechanisms have been proposed for peptidyl-Gly amidation (Scheme I). Bradbury et al. (1982) proposed a dehydrogenation reaction to form a C-terminal *N*-acylimine which would be hydrolyzed to peptidyl-NH_2 and glyoxylate.[3] A later proposal involved the direct α-hydroxylation of the C-terminal glycine at the expense of O_2 and the subsequent conversion of the peptidyl-α-hydroxyglycine to peptidyl-NH_2 and glyoxylate (Bateman et al., 1985; Bradbury & Smyth, 1987). Recent evidence strongly favors formation of the peptidyl-α-hydroxyglycine (Young & Tamburini, 1989; Tajima et al., 1990; Bongers et al., 1992). Further evidence in support of the direct hydroxylation mechanism would be provided by the incorporation of ^{18}O from $^{18}O_2$ into the α-carbonyl oxygen of glyoxylate. Unfortunately, glyoxylate readily hydrates to the hemiacetal [K_{eq} = [glyoxylate hydrate]/[glyoxylate] = 260; Rendina et al. (1984)] and would exchange incorporated ^{18}O for ^{16}O from the aqueous solvent. Landymore-Lim et al. (1983) showed that *D*-alanine-extended peptides are slow, alternate substrates for α-AE.[4] Since pyruvate hydrates only slightly at pH ≥3 [K_{eq} = [pyruvate hydrate]/[pyruvate] = 0.06; Pocker et al. (1969)], the LDH-catalyzed reduction of pyruvate, generated by the amidation of dansyl-Tyr-Val-D-Ala, would serve to fix the incorporated ^{18}O label.

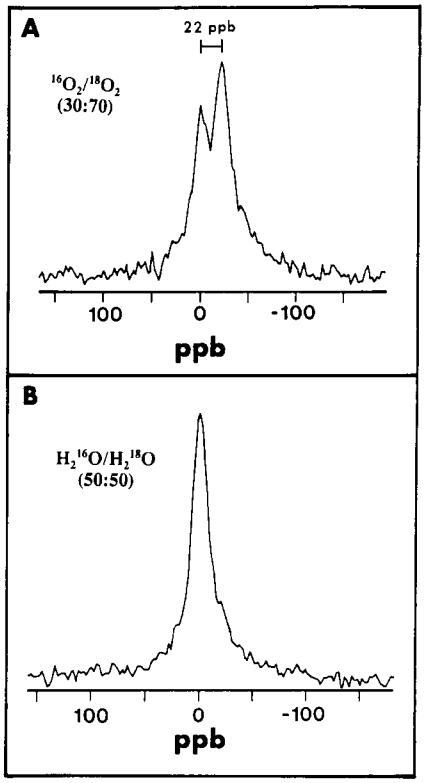


FIGURE 1: ^{13}C NMR spectrum of [α-^{13}C]lactate generated by the amidation of dansyl-Tyr-Val-D-Ala in $^{16}O_2/^{18}O_2$ (30:70) (A) or $H_2{}^{16}O/H_2{}^{18}O$ (50:50) (B). Chemical shifts are relative to authentic [α-^{13}C,^{16}O]lactate added to a sample lacking α-AE.

Amidations of dansyl-Tyr-Val-[α-^{13}C]-D-Ala were carried out separately in $^{16}O_2$ or $^{18}O_2$. These enzymatic reactions were terminated and subsequently mixed in a 30:70 proportion.[5] The resulting ^{13}C NMR signal of [α-^{13}C,^{16}O]lactate/[α-^{13}C,^{18}O]lactate is shown in Figure 1A. The spectrum shows two resonances, the largest of which is shifted upfield by 22 ppb. In contrast, the ^{13}C NMR signal of [α-^{13}C,^{16}O]lactate generated from amidation of dansyl-Tyr-Val-[α-^{13}C]-D-Ala in $H_2{}^{16}O/H_2{}^{18}O$ (50:50) is a single resonance (Figure 1B), indicating no incorporation of ^{18}O into lactate from $H_2{}^{18}O$.

Substrate/Product Stoichiometries. Amidation of dansyl-Tyr-Val-Gly should produce dansyl-Tyr-Val-NH_2 and gly-

[2] There is sufficient trace copper in the buffers (0.5–1.0 μM by atomic absorption) to support amidation.

[3] Ramer et al. (1988) pointed out that N-hydroxylation of the C-terminal glyine provides another route to the *N*-acylimine intermediate.

[4] At saturating ascorbate (3 mM), the ratio of the $(V/K)_{\text{dansyl-Tyr-Val-Gly}}$ to the $(V/K)_{\text{dansyl-Tyr-Val-D-Ala}}$ is 30 (Merkler, D. J., unpublished results).

[5] The valving necessary to mix $^{16}O_2/^{18}O_2$ in situ was unavailable. Since the ^{18}O-induced shift is small [18–29 ppb; Risley and Van Etten (1990)], the only reliable method to observe the ^{18}O–^{13}C NMR shift would be to mix the separate reactions carried out under $^{16}O_2$ or $^{18}O_2$.

Table I: Comparison of Peptide Consumption to Glyoxylate or Pyruvate Formation[a]

substrate (μM)	enzyme source	% conversn[b]	product (μM)	product generated/ peptide consumed
dansyl-Tyr-Val-Gly[c,d] (73)	C127	38 ± 7	glyoxylate (27 ± 9)	0.9 ± 0.2 (2)
dansyl-Tyr-Val-Gly[c,d] (145)	C127	27 ± 3	glyoxylate (43 ± 1)	1.1 ± 0.1 (2)
dansyl-Tyr-Val-Gly[c,d] (218)	C127	16 ± 3	glyoxylate (34 ± 3)	1.0 ± 0.3 (2)
dansyl-Tyr-Val-Gly[c,e] (145)	CHO	85 ± 2	glyoxylate (107 ± 10)	0.9 ± 0.1 (2)
dansyl-Tyr-Val-(D)Ala[c,f] (202)	CHO	66 ± 1	pyruvate (124 ± 6)	0.9 ± 0.03 (2)

[a] Pyruvate and glyoxylate were quantitated as described in Materials and Methods. The number of determinations is shown in the parentheses, and the errors are the standard deviation. Errors were propagated as described (Morrison & Uhr, 1966). [b] Percent conversion of the indicated substrate. Determined as described in Materials and Methods. [c] The assay conditions were 100 mM MES, pH 6.0, 30 mM KCl, 30 mM KI, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 100 μg/mL catalase, and the indicated concentrations of dansylated tripeptide. [d] Assays contained 2.5 μg/mL α-AE (specific activity 0.64 μmol min^{-1} mg^{-1}) and were incubated at 37 °C for 4 h. [e] Assays contained 72 μg/mL α-AE (specific activity 1.38 μmol min^{-1} mg^{-1}) and were incubated at 37 °C for 40 min. [f] Assays contained 1.2 mg/mL α-AE (specific activity 1.38 μmol min^{-1} mg^{-1}) and were incubated at 37 °C for 45 min.

Table II: Comparison of Substrate Consumption Rates to Product Generation Rates[a]

substrate/product	enzyme source	substrate consumption rate, nmol min^{-1} mg^{-1}	product generation rate, nmol min^{-1} mg^{-1}	ratio[b]
ascorbate/dansyl-Tyr-Val-NH_2[c]	C127	85.1 ± 6.0 (3)	105.3 ± 4.8 (3)	1.2 ± 0.1
ascorbate/dansyl-Tyr-Val-NH_2[d]	CHO	606.1 ± 85.5 (7)	490.9 ± 27.6 (4)	0.8 ± 0.1
O_2/dansyl-Tyr-Val-NH_2[e]	C127	421.3 ± 34.7 (4)	395.2 ± 69.3 (4)	0.9 ± 0.2

[a] Rates for substrate consumption and dansyl-Tyr-Val-NH_2 production were determined separately under similar conditions, as described in Materials and Methods. The number of determinations is shown in the parentheses, and the errors are the standard deviation. The error is propagated using the method of Morrison and Uhr (1966). [b] Ratio of product generation rates to substrate consumption rates. [c] Assays contained 26 μg/mL α-AE (specific activity 0.80 μmol min^{-1} mg^{-1}). [d] Assays contained 0.9 μg/mL α-AE (specific activity 2.64 μmol min^{-1} mg^{-1}). [e] Assays contained 13 μg/mL α-AE (specific activity 0.72 μmol min^{-1} mg^{-1}). Standard assay conditions were used (37 °C, 20 μM dansyl-Tyr-Val-Gly, and 10 mM ascorbate; see Materials and Methods) except that $CuSO_4$ was omitted to minimize background O_2 consumption. Rates were corrected for nonspecific O_2 consumption by running controls which lacked dansyl-Tyr-Val-Gly.

oxylate while amidation of dansyl-Tyr-Val-D-Ala should produce dansyl-Tyr-Val-NH_2 and pyruvate. Because glyoxylate is a poor substrate for LDH (Holbrook et al., 1975) and dansyl-Tyr-Val-D-Ala is a poor substrate for α-AE, quantification of glyoxylate or pyruvate continuously during amidation using LDH and NADH proved impractical. Instead, amidations of dansyl-Tyr-Val-Gly or dansyl-Tyr-Val-D-Ala were terminated by heat denaturation of α-AE and were analyzed for accumulated glyoxylate or pyruvate using LDH and NADH. The stoichiometry of glyoxylate formed/dansyl-Tyr-Val-Gly amidated or pyruvate formed/dansyl-Tyr-Val-D-Ala amidated was 1.0, within experimental error. The ratio of glyoxylate produced/dansyl-Tyr-Val-Gly amidated obtained with C127 α-AE varied from 0.9 ± 0.2 to 1.1 ± 0.1 while the ratio elucidated for the CHO α-AE was 0.9 ± 0.1 (Table I). The ratio of pyruvate/dansyl-Tyr-Val-D-Ala amidated was 0.9 ± 0.03 using the CHO enzyme (Table I).

One method to define the stoichiometric relationships between substrates and products is to compare rates of substrate consumption to rates of product formation. Jones et al. (1988) developed a convenient procedure for determining the rate of peptide-Gly amidation. Use of this assay in parallel with methods to measure rates of O_2 consumption or ascorbate oxidation showed that the formation of each mole of dansyl-Tyr-Val-NH_2 utilized 0.9 ± 0.2 mol of O_2 and 1.0 ± 0.2 mol of ascorbate (Table II). The dansyl-Tyr-Val-NH_2/ascorbate stoichiometry obtained using α-AE produced by either eukaryotic expression system was approximately the same: 1.2 ± 0.2 for C127 α-AE and 0.8 ± 0.1 for CHO α-AE.

In the presence of trace transition metals and O_2, ascorbate is oxidized to dehydroascorbate (Khan & Martell, 1967), which has neglible absorbance at 250 nm [$\epsilon_{250,dehdroascorbate}/\epsilon_{250,ascorbate} \approx 0.004$; Herbert et al. (1933)]. If this side reaction occurred to any significant extent in our system, the dansyl-Tyr-Val-NH_2/ascorbate stoichiometries in Table II would be underestimated. To control for this side reaction, we assayed for the total concentration of dansyl-Tyr-Val-NH_2 produced at limiting ascorbate concentration with relatively high levels of α-AE (Table III). The ratio of dansyl-Tyr-Val-NH_2 produced/initial ascorbate concentration from the 10 determinations presented in Table III was 0.9 ± 0.04. The agreement between this result and the data in Table II suggested that the nonspecific oxidation of ascorbate during the time course of amidation was minimal.

Table III: Production of Dansyl-Tyr-Val-NH_2 at Limiting Ascorbate Using C127 α-AE[a]

expt	ascorbate, μM	dansyl-Tyr-Val-NH_2 produced,[b] μM	[dansyl-Tyr-Val-NH_2]/ [ascorbate]$_{initial}$
I[c,e]	5.2	4.5	0.9
	10.5	9.0	0.9
	13.1	12.1	0.9
	15.7	14.5	0.9
	20.9	19.0	0.9
	26.1	23.5	0.9
II[d,e]	5.2	4.0	0.8
	10.5	8.6	0.8
	15.7	14.3	0.9
	20.9	18.4	0.9

[a] The reactions were carried out in 100 mM MES, pH 6.0, 30 mM KCl, 1% (v/v) ethanol, 0.001% (v/v) Triton X-100, and the indicated initial concentrations of ascorbate. [b] Determined by HPLC as described in Materials and Methods. [c] Reactions initially containing 30 μM dansyl-Tyr-Val-Gly were incubated for 210 min at 37 °C in the presence of 80 μg/mL α-AE (specific activity 0.65 μmol min^{-1} mg^{-1}). The percent conversion to dansyl-Tyr-Val-NH_2 was unchanged after 100 min. [d] Reactions initially containing 48 μM dansyl-Tyr-Val-Gly and 30 mM KI were incubated for 120 min at 37 °C in the presence of 79 μg/mL α-AE (specific activity 0.80 μmol min^{-1} mg^{-1}). The percent conversion to dansyl-Tyr-Val-NH_2 was unchanged after 75 min. [e] Controls using heat-denatured α-AE (5 min at 100 °C) showed no formation of dansyl-Tyr-Val-NH_2.

Semidehydroascorbate: A Product of the Amidation Reaction. The results in Tables II and III show that each α-AE turnover required the input of two electrons from ascorbate. However, these electrons could have been supplied via one two-electron reduction or two one-electron reductions. If ascorbate underwent one two-electron oxidation, dehydroascor-

Table IV: Stoichiometry of the α-AE–Semidehydroascorbate Reductase Coupled Reaction as a Function of Semidehydroascorbate Reductase Concentration[a]

expt	[SDR], unit/mL	[α-AE][b] μg/mL	addition (μM)	NADH oxidation, μM/min	amidation rate, μM/min	ratio[c]	[oxid NADH]$_{total}$/ [dansyl-Tyr-Val-NH$_2$]$_{total}$
I	0.018	0	none	1.8 ± 0.3 (6)			
	0.018	0	(30) DHA[d]	2.1			
	0.018	0	(60) DHA	2.4 ● 0.4 (2)			
	0.018	10	none	9.4 ± 0.2 (6)	9.4 ± 0.7 (4)	0.8 ± 0.2	0.7 ± 0.01 (6)
	0	10	none	0			
II	0.036	0	none	2.7 ± 0.3 (11)			
	0.036	0	(60) DHA	3.6			
	0.036	10	none	12.4 ± 1.1 (9)	10.5 ± 0.2 (3)	0.9 ± 0.1	0.9 ± 0.04 (9)
	0	10	none	0			
III	0.033	0	none	2.5 ± 0.4 (9)			
	0.033	0	(60) DHA	3.1			
	0.033	10	none	11.9 ± 0.5 (6)	10.9 ± 0.7 (4)	0.9 ● 0.2	0.9 ± 0.09 (6)
	0	10	none	0			
IV	0.066	0	none	3.0 ± 0.3 (8)			
	0.066	0	(60) DHA	4.0			
	0.066	10	none	13.6 ± 0.8 (5)	11.5 ± 0.3 (4)	0.9 ± 0.1	1.0 ± 0.05 (5)

[a] NADH oxidation and amidation were carried out at 37 °C in 100 mM Tris-HCl, pH 7.3, 30 μM dansyl-Tyr-Val-Gly, 10 mM ascorbate, 100 μM NADH, and 50 μM NADH, and 50 μM $CuSO_4$/unit of SDR. The numbers in the parentheses are the number of determinations, and the errors are the standard deviation. The errors were propagated as described (Morrison & Uhr, 1966). [b] The specific activity of the CHO α-AE used was 2.58 μmol^{-1} min^{-1} mg^{-1}. [c] The ratio of α-AE-dependent NADH oxidation minus the NADH oxidation in the absence of α-AE divided by the rate of dansyl-Tyr-Val-NH_2 production. [d] DHA, dehydroascorbate.

bate would be the product of peptidyl-Gly amidation while two one-electron oxidations would produce 2 mol of semidehydroascorbate/α-AE turnover. In principle, the use of electron spin resonance to follow α-AE turnover could resolve this question (Klaus & Filby, 1981; Dhariwal et al., 1991). However, this technique would not easily provide ratios for dansyl-Tyr-Val-Gly consumption/semidehydroascorbate formation since the subsequent disproportionation of two semidehydroascorbate molecules produces ascorbate and dehydroascorbate (Bielski et al., 1975). Another approach to this problem was to couple the amidation reaction to semidehydroascorbate reductase. SDR catalyzes the NADH-dependent reduction of semidehydroascorbate to ascorbate [eq 2; Oehler et al. (1972)]. If two molecules of semidehydroascorbate were formed during amidation, each α-AE turnover would result in the quantitative oxidation of 1 mol of NADH when sufficient SDR is included in the reaction mix.

$$2 \text{ semidehydroascorbate} + NADH + H^+ \rightarrow 2 \text{ ascorbate} + NAD^+ \quad (2)$$

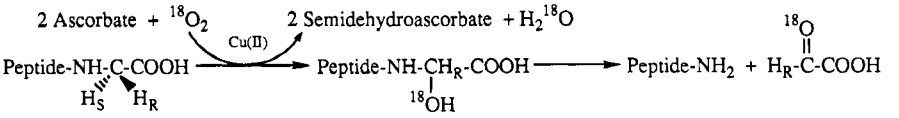
In the absence of α-AE, partially purified SDR showed a low background rate of NADH oxidation ranging from 1.8 ± 0.3 to 3.0 ± 0.3 μM/min (Table IV). When α-AE was added, the NADH oxidation rate increased 4.5–5.2-fold at all SDR concentrations. The α-AE-dependent increase in the NADH oxidation rate was not due to the initial formation of dehydroascorbate which then reacted with the remaining ascorbate to generate semidehydroascorbate (Klaus & Filby, 1981) because addition of 60 μM dehydroascorbate (twice the initial dansyl-Tyr-Val-Gly concentration) only marginally increased the NADH oxidation rate (1.2–1.3-fold). In the absence of SDR, there was no observable α-AE-dependent NADH oxidation. The ratio of the α-AE-dependent NADH oxidation rate minus the background rate to the dansyl-Tyr-Val-NH_2 production rate varied from 0.8 ± 0.2 at 0.018 unit/mL SDR to 0.9 ± 0.1 at 0.066 unit/mL SDR. In addition, the ratio of total NADH oxidized to total dansyl-Tyr-Val-NH_2 produced ranged from 0.7 ± 0.01 at 0.018 unit/mL SDR to 1.0 ± 0.05 at 0.066 unit/mL SDR. The results of the experiments which coupled amidation to NADH oxidation using SDR are summarized in Table IV.

DISCUSSION

The purpose of this study was to (1) establish the substrate/product stoichiometries for peptidyl-Gly amidation, (2) characterize the oxidation product of ascorbate, and (3) determine the source of the α-carbonyl oxygen of glyoxylate. Recognition that bioactive peptides were C-terminally amidated began in the early 1950s with the characterization of oxytocin (Du Vigneaud et al., 1953; Tuppy & Michl, 1953). The formation of the C-terminal amide was not understood until Bradbury et al. (1982) described an enzyme, α-AE, which converted C-terminal, glycine-extended peptides to peptidyl-NH_2 plus glyoxylate. Eipper et al. (1983) later showed that amidation required O_2, ascorbate, and copper. A more complete understanding of this enzymic reaction has been thwarted by the limited quantities of homogeneous enzyme available for study. Given the availability of milligram quantities of recombinant α-AE, we are in position to elucidate the molecular details of the amidation reaction.

The data in Tables II and III indicate that 1 mol of peptidyl-NH_2 is formed per mole of ascorbate oxidized. For three different sets of experiments, the stoichiometries of dansyl-Tyr-Val-NH_2 formed/ascorbate oxidized were 0.8 ± 0.1, 0.9 ± 0.04, and 1.2 ± 0.1. An earlier report of 1.4 mol of D-Tyr-Glu-NH_2 formed per mole of ascorbate consumed during the amidation of D-Tyr-Glu-Gly is consistent with our data (Murthy et al., 1987). A dansyl-Tyr-Val-NH_2/ascorbate stoichiometry of unity requires the input of two electrons per α-AE turnover. The quantitative coupling of amidation to the NADH-dependent reduction of semidehydroascorbate using *N. crassa* semidehydroascorbate reductase (Table IV) showed that the two electrons per α-AE turnover are supplied as two one-electron reductions. Thus, semidehydroascorbate, and not dehydroascorbate, is the product of ascorbate oxidation during peptidyl-Gly amidation. In the absence of SDR, the two semidehydroascorbate molecules formed per turnover disproportionate to ascorbate (which recycles as substrate) and dehydroascorbate (Bielski et al., 1975), accounting for the dansyl-Tyr-Val-NH_2/ascorbate stoichiometries of Tables II and III. Dopamine β-hydroxylase (DβH), an enzyme that catalyzes the O_2, ascorbate, and copper-dependent hydroxylation of dopamine to norepinephrine, also undergoes two

Scheme II: The Reaction Catalyzed by Bifunctional α-AE



one-electron reductions producing semidehydroascorbate (Skotland & Ljones, 1980; Diliberto & Allen, 1981).

The amidation reaction catalyzed by α-AE involves the oxidative cleavage of peptidyl-Gly or peptidyl-D-Ala to peptidyl-NH_2 and glyoxylate or pyruvate. The amidation of peptidyl-D-Ala is probably of no biological significance because D-alanine-extended peptides are not likely to exist in eukaryotic systems. This reaction dictates that the ratio of dansyl-Tyr-Val-NH_2 formed/dansyl-Tyr-Val-Gly consumed should be 1.0. Jones et al. (1988) verified this stoichiometry when developing the HPLC assay for α-AE. Similarly, the ratio of glyoxylate formed/dansyl-Tyr-Val-Gly amidated or pyruvate formed/dansyl-Tyr-Val-D-Ala amidated should be unity. As shown in Table I, the stoichiometry of glyoxylate formed/dansyl-Tyr-Val-Gly amidated ranged from 0.9 ± 0.1 to 1.1 ± 0.1 for four different determinations. The ratio of pyruvate formed/dansyl-Tyr-Val-D-Ala amidated was also near unity, 0.9 ± 0.03 (Table I). Consistent with our results, Katopodis et al. (1990) reported that the stoichiometry of glyoxylate formed/peptidyl-α-hydroxyglycine consumed is 1.08 ± 0.05 using purified bovine PAL.

Two different mechanisms for peptidyl-Gly amidation have been discussed by Ramer et al. (1988) (Scheme I): direct hydroxylation at the α-carbon of the C-terminal glycine residue followed by conversion to products or *N*-acylimine formation followed by hydrolysis to products. These different mechanisms can be differentiated by the source of ^{18}O incorporated as the α-carbonyl oxygen of pyruvate upon amidation of dansyl-Tyr-Val-D-Ala in $H_2{}^{18}O$ or $^{18}O_2$. The source of oxygen for the hydroxylation mechanism would be $^{18}O_2$, while $H_2{}^{18}O$ would provide the oxygen for the *N*-acylimine mechanism. When dansyl-Tyr-Val-[α-^{13}C]-D-Ala was amidated in a 50:50 mixture of $H_2{}^{16}O/H_2{}^{18}O$ containing LDH and NADH ($^{16}O_2$ atmosphere), a single resonance was observed in the ^{13}C NMR spectrum with the same chemical shift as that of authentic [α-^{13}C,^{16}O]lactate (Figure 1B). Thus, no ^{18}O is incorporated from $H_2{}^{18}O$. In contrast, amidation of dansyl-Tyr-Val-[α-^{13}C]-D-Ala in $^{16}O_2/^{18}O_2$ (30:70), again in the presence of LDH and NADH ($H_2{}^{16}O$ solvent), resulted in a pair of resonances for [α-^{13}C]lactate (Figure 1A)[5]. The resonance of higher intensity is shifted 22 ppb upfield relative to the second resonance. For a series of secondary alcohols, Risley and Van Etten (1990) reported that ^{18}O induces a 18–29 ppb upfield shift in the ^{13}C NMR of the carbon bonded to the oxygen-18. The pair of resonances shown in Figure 1A results from an ^{18}O isotopic shift in the [α-^{13}C]lactate NMR spectrum, establishing that the α-hydroxy oxygen of lactate and, hence, the α-carbonyl oxygen of pyruvate, must come from O_2. These results also suggest that the α-carbonyl oxygen of glyoxylate produced during the amidation of C-terminal glycine-extended peptides is also derived from O_2. Therefore, peptidyl-Gly amidation must occur by direct hydroxylation of the C-terminal glycine by an activated O_2 species. A previous report showing the partial incorporation of ^{18}O (30%) into D-Phe-Ala-α-hydroxyglycine from $^{16}O_2/^{18}O_2$ (50:50) supports the hydroxylation mechanism but does not absolutely eliminate the possiblity of some *N*-acylimine intermediate formation (Zabriskie et al., 1991).

A mechanism for bifunctional α-AE that is consistent with the data presented in this report and other publications is shown in Scheme II (Young & Tamburini, 1989; Katopodis et al., 1990). Ramer et al. (1988) have shown that the *pro-S* proton of glycine is abstracted during amidation, consistent with the observation that D-alanine-extended peptides, and not L-alanine-extended peptides, are alternate substrates for α-AE (Landymore-Lim et al., 1983). As originally proposed by Glembotski et al. (1984), α-AE is now shown to be a monooxygenase. The reduction of O_2 during amidation formally requires four electrons: two electrons are supplied by the oxidation of two molecules of ascorbate to two molecules of semidehydroascorbate and two electrons come from the oxidation of the α-carbon of the C-terminal glycine. The similarities between the PGH mechanism of α-AE and DβH are evident. Alignment of the α-AE and DβH amino acid sequences shows regions of extensive homology and, in particular, two proposed His-X-His copper-binding motifs are conserved (Southan & Kruse, 1989). The two one-electron reductions per turnover suggest that α-AE, like DβH (Ash et al., 1984; Klinman et al., 1984), has two bound coppers per active site.[6] Miller & Klinman (1985) have shown that DβH forms a substrate radical during catalysis. By analogy, α-AE may form an α-carbon-centered glycine or alanine radical during catalysis. Evidence for the formation of α-carbon-centered glycyl or alaninyl radicals is provided by radiolytic fragmentation studies of glycine and alanine (Garrison, 1964) and photochemical modifications of glycine and alanine (Schwarzberg et al., 1973; Easton & Hay, 1986; Burgess et al., 1989). Recently, Wagner et al. (1992) characterized an α-carbon-centered glycyl radical in pyruvate formate-lyase that has a half-life of several days at room temperature.

The results presented here provide the foundation required for further studies to establish the exact chemical and kinetic mechanisms of bifunctional peptidylglycine α-amidating enzyme. Of particular interest will be the stoichiometry of copper bound to the enzyme. These studies are currently underway.

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NOTE ADDED IN PROOF

Recently, Noguchi et al. (1992) reported the incorporation of ^{18}O into the hydroxyl oxygen of D-Tyr-Val-α-hydroxyglycine from $^{18}O_2$ and not $H_2{}^{18}O$ using rat brain PGH, in agreement with our results. Ping et al. (1992) have shown that PGH forms (*S*)-peptidyl-α-hydroxyglycine and that only (*S*)-peptidyl-α-hydroxyglycine is a substrate for PGL.

[6] Preliminary EPR and atomic absorption results indicate that rat 75-kDa α-AE contains 1–3 type II coppers per mole of α-AE (Freeman, J., Villafranca, J. J., and Merkler, D. J., unpublished results).

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