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Incorporation of Aerobic Oxygen into the Hydroxyglycyl Intermediate during Formation of *C*-Terminal Peptide Amides by Peptidylglycine α-Amidating Monooxygenase (PAM)

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Incubation of D-phenylalanyl-L-phenylalanylglycine with peptidylglycine α-amidating monooxygenase (PAM) in the presence of $^{18}O_2$ generates the corresponding hydroxyglycyl peptide whose FAB mass spectrum shows high incorporation of ^{18}O into the hydroxy group; this suggests that the mechanism may involve direct hydroxylation of carbon.

Many important peptides in mammalian and invertebrate neural and endocrine systems have a primary amide functionality at the carboxy terminus.[1,2] Recent studies show that the production of such peptide amides from glycine-extended precursors is not mediated by a single enzyme as previously thought, but is promoted by two proteins with separable activities.[3–5] The first enzyme, peptidylglycine α-amidating monooxygenase (PAM; EC 1.14.17.3), oxidizes a *C*-terminal glycine residue to form an α-hydroxyglycine peptide in a process dependent upon ascorbate, copper and molecular oxygen (Scheme 1). In a second step, α-hydroxyglycine amidating dealkylase (HGAD) catalyses conversion of this product to the peptide amide and glyoxylate at neutral or acidic pH with no cofactor requirements.[3,4] However, the cleavage also occurs spontaneously at alkaline pH. Previous investigations show that PAM stereospecifically removes the *pro-S* hydrogen of the *C*-terminal glycine[6] (consistent with its ability to convert peptides terminating in D-alanine but not L-alanine residues[7]), and that the cleavage enzyme catalyses transformation of only one α-hydroxyglycine peptide isomer (absolute configuration not determined).[8] However, it remained uncertain whether the key hydroxyglycine intermediate forms by direct hydroxylation on carbon or *via* oxidative formation of an *N*-acyl imine followed by stereospecific addition of water.[9] In this work we report ^{18}O labelling results that support the direct hydroxylation mechanism.

PAM was purified from frozen pig pututaries (Pel-Freeze, Rogers, Arkansas) as previously described.[6] D-Phenylalanyl-L-phenylalanylglycine **1a** and D-phenylalanyl-L-phenylalanyl amide **3a** were prepared by standard solution methods,[10] and the corresponding *N-tert*-butoxycarbonyl derivative **3b**[10] was condensed with glyoxylic acid **4** in refluxing acetone to afford an epimeric mixture of hydroxyglycine compounds **2b**. Removal of the *tert*-butoxycarbonyl group with trifluoroacetic acid followed by HPLC purification [Chemcopak column, 4.6 × 250 mm, 5 μm C_{18} reverse phase (Dychrom, Sunnyvale, CA); mobile phase, A: 1 mmol dm^{-3} NH_4HCO_3, pH 9.0, B: MeCN; 0 to 40% B linear gradient over 20 min; flow rate 1.5 ml min^{-1}; UV detection at 214 nm] gave unlabelled compound **2a** as a mixture of isomers at the hydroxy-bearing carbon. A new assay for PAM was employed which is based, like our previous assay,[6] on detection of glyoxylate, the common product of peptide α-amidation, rather than on the variable peptide amide product. Thus the transformation of D-phenylalanyl-L-phenylalanyl[1,2-^{14}C]glycine **1a** (● = ^{14}C) (specific activity 113 μCi $μmol^{-1}$) by PAM is monitored by liberation of

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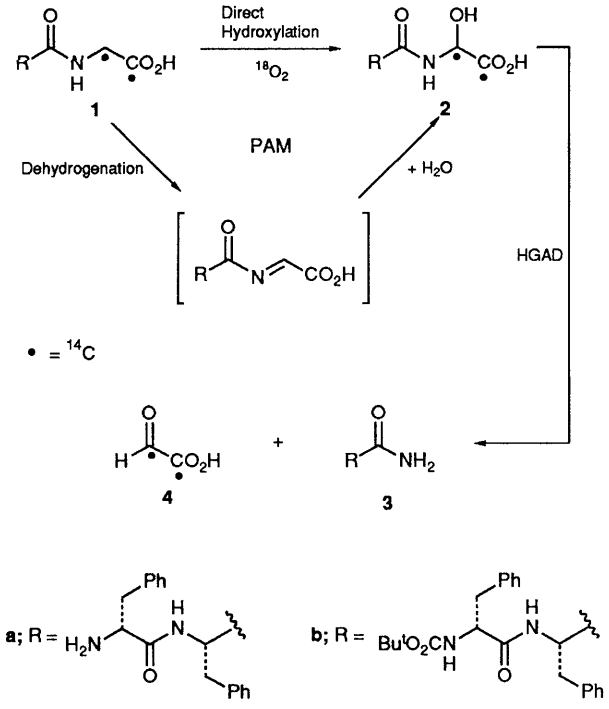

Scheme 1

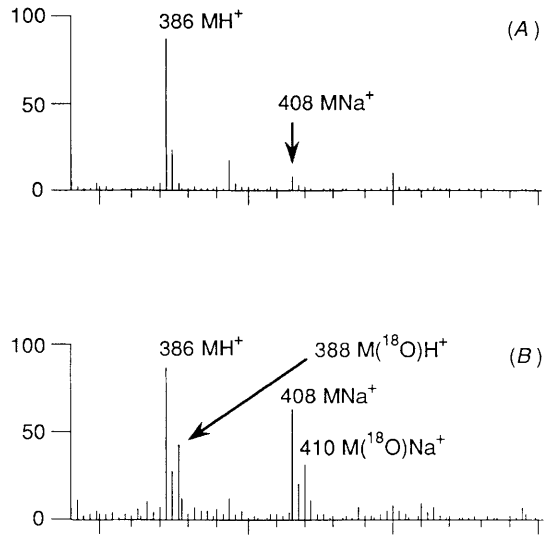

Fig. 1 Positive ion FAB mass spectra of (*A*): unlabelled D-phenylalanyl-L-phenylalanyl-α-hydroxyglycine **2a**; (*B*): D-phenylalanyl-L-phenylalanyl-α-hydroxyglycine isolated from the incubation of D-phenylalanyl-L-phenylalanylglycine and PAM under an $^{18}O_2$ atmosphere

[1,2-^{14}C]glyoxylate **4** (● = ^{14}C) upon base treatment. Typically, a 25 μl aliquot is removed from a 125 μl incubation mixture containing 1 mmol dm^{-3} ascorbate, 5 μmol dm^{-3} copper sulphate, 25 mmol dm^{-3} potassium iodide, 0.125 mg ml^{-1} catalase, 1.25 μmol dm^{-3} radioactive **1a**, and PAM in 50 mmol dm^{-3} sodium phosphate buffer at pH 6.8. The aliquot is mixed in a microcentrifuge tube with 10 μl of 2 mol dm^{-3} NaOH and incubated at 37 °C for 5 min to cleave the α-hydroxyglycine peptide. The sample is acidified with 25 μl of 1 mol dm^{-3} HCl and applied to a column of cation exchanger (Bio-Rad AG 50W-X8, 0.5 × 4 cm, 200–400 mesh, H^+ form). Glyoxylate is washed from the column with 4.5 ml of water and collected in a scintillation vial. Unconverted substrate is eluted with 4.5 ml of 15% NH_4OH and collected separately, and the amount of ^{14}C in both samples is determined by liquid scintillation counting.

To determine the source of the hydroxy oxygen in the enzyme-catalysed process, unlabelled **1a** was incubated with purified PAM under an ^{18}O enriched atmosphere. Degassed buffer solutions[6] were aerated with $^{18}O_2$ (50% isotopic purity; Cambridge Isotope Laboratories, Woburn, Massachusetts) prior to addition of the ascorbate, catalase and PAM. The solutions were blanketed with an atmosphere of 50% $^{18}O_2$, sealed, and incubated for 8 h at 37 °C with gentle shaking. The mixtures were concentrated and separated by HPLC. Fractions corresponding to the hydroxylated product (determined by comparison with elution profiles of synthetic **2a**) were acidified with trifluoroacetic acid, lyophilized and analysed by fast atom bombardment mass spectrometry (FAB MS) using a formic acid–glycerol matrix. Absolute intensities of the peaks at *m/z* 386 (MH^+) and 388 [$M(^{18}O)H^+$] were normalized by comparison with corresponding spectra of completely unlabelled material (Fig. 1). The MNa^+ peaks, which are common in FAB MS, were at *m/z* 408 and 410 and were analysed similarly. The results show that the hydroxy oxygen of **2a** obtained by PAM oxidation of **1a** contains 30% ^{18}O and demonstrate that aerobic oxygen is the primary source of the hydroxy group. Although a mechanism involving generation of 'sequestered' [^{18}O]water in the enzyme active site which then adds to an enzyme-bound *N*-acyl imine intermediate cannot be rigorously excluded, the highly reactive nature of glyoxylate-derived *N*-acyl imines[9,11] and the large amounts of unlabelled water present in the mixture strongly suggest a direct carbon hydroxylation mechanism. Further studies on the stereochemistry, mechanism and inhibition of PAM are in progress.

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