Stereochemistry of the Peptide α-Amidation Reaction involving Two Enzymes, Peptidylglycine α-Hydroxylating Monooxygenase (PHM) and Peptidylhydroxyglycine N–C Lyase (PHL)

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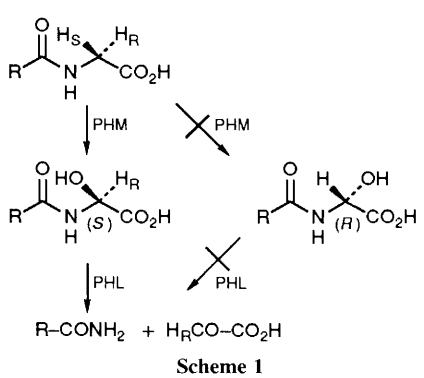
Peptidylglycine α-hydroxylating monooxygenase (PHM), the first enzyme involved in the peptide α-amidation, has been shown to convert *N*-benzoylglycine, a model substrate, stereoselectively to *S*-*N*-benzoyl-α-hydroxyglycine, whereas peptidylhydroxyglycine N–C lyase (PHL), the second enzyme, has been found to react exclusively with *S*-form of the intermediate.

Many bioactive peptides are amidated at the C-terminus. The amide structure is derived from the C-terminal glycine residue of glycine-extended precursor peptides.[1,2] Although this conversion was originally thought to be catalysed by a single enzyme called peptidylglycine α-amidating monooxygenase (PAM), recent work has shown that the conversion is a two-step process involving two distinct enzymes, *i.e.* PHM and PHL.[3–6] PHM catalyses α-hydroxylation of the C-terminal glycine residue of a precursor peptide to form the α-hydroxyglycine residue in a reaction requiring L-ascorbate and molecular oxygen. PHL, on the other hand, catalyses the cleavage of the N–C bond in the C-terminal α-hydroxyglycine residue to give rise to a C-terminally amidated peptide and glyoxylate. The two-step conversion can thus be expressed as in eqn (1), where X–CO– stands for a peptidyl moiety. Both PHM and PHL have been separately purified from the skin of the frog *Xenopus laevis*.[3,7] In certain cases the two activities were found to be due to a bi-functional protein,[8] which had earlier been recognized as PAM.

$$\text{X–CO–NH–CH}_2\text{–CO}_2\text{H} \xrightarrow{\text{PHM}} \text{X–CO–NH–CH(OH)–CO}_2\text{H} \xrightarrow{\text{PHL}} \text{X–CO–NH}_2 + \text{HCO–CO}_2\text{H} \quad (1)$$

Several stereochemical studies of the amidation reaction have so far been reported. Ramer *et al.*[9] stereospecifically tritium-labelled each one of the two α-hydrogens of the C-terminal glycine residue of a glycine-extended peptide. Using these peptides as substrates for the enzymatic amidation, they showed that the *pro-S* hydrogen on the α-carbon is selectively removed during the PAM-catalysed α-amidation. This stercoselectivity of PAM in the first step of the reaction is consistent with the observation that PAM converts peptidyl-D-alanines, but not peptidyl-L-alanines, to the C-terminal amide structures.[10] Moreover, Young and Tamburini[11] prepared and separated diastereoisomers of a peptidyl-α-hydroxyglycine and showed that only one of the two diastereoisomers is converted into the peptidyl amide by PAM. However, no assignment of the absolute configuration of the reactive diastereoisomer has as yet been made. In this study, we show that the absolute configuration of the α-hydroxyglycine residue produced by PHM is *S*-form and that PHL reacts only with the *S*-hydroxyglycine (but not *R*-hydroxyglycine) residue.



Scheme 1

N-Benzoylglycine **1** was used as a model for peptidylglycine substrates and subjected to hydroxylation by using a recombinant PHM preparation[3] as enzyme.† The product *N*-benzoyl-α-hydroxyglycine **2a** was purified and shown to be optically pure by HPLC on a chiral column.‡ Purified **2a** was then allowed to form the salt **3** with *S*-2-aminobutan-1-ol **4**, an optically active amine, to facilitate the determination of the absolute configuration of **2a** (as *S*-form) by an X-ray crystallographic method.§

We next examined the stereospecificity of PHL. For this purpose, racemic *N*-benzoyl-α-hydroxyglycine was resolved by HPLC on the chiral column in a preparative scale. It was thus found that PHL, purified from the frog skin,[7] converted **2a** (*S*-form), but not **2b** (*R*-form), into benzamide.

These results, coupled with previous findings, indicate that the stereochemistry of α-amidation of glycine-extended peptides can be illustrated as shown in Scheme 1. In the first step, PHM removes the *pro-S*-α-hydrogen of the C-terminal glycine

† Compound **1** (716 mg) was aerobically incubated with PHM [2.7 mg, 8.1×10^6 u (1 u = 16.67 nkat)] under the previously described conditions[7] with slight modifications. After incubation at 30 °C for 3 days, hydrochloric acid was added to the reaction mixture to pH 1 to acidify the product **2a**. The acidified product **2a** was extracted from the solution with ethyl acetate and purified by reversed phase HPLC [YMC-PACK 120A ODS, 20 × 250 mm (Yamamura): mobile phase, 12% acetonitrile–0.1% trifluoroacetic acid: flow rate, 5.0 ml min^{-1}: UV detection at 228 nm]. Pure **2a** was obtained by lyophilization of **2a**-containing fractions.

‡ Optical isomers (**2a** and **b**) of *N*-benzoyl-α-hydroxyglycine were separated from each other by HPLC on a chiral column [Chiralcel OF, 4.6 × 250 mm (Daicel): mobile phase, 50% propan-2-ol–50% hexane–0.1% trifluoroacetic acid: flow rate, 1.0 ml min^{-1}: UV detection at 228 nm]. Retention times of **2a** and **b** were 65 and 91 min, respectively.

§ Compound **3**, prepared by mixing equimolecular amounts of **2** and **4** in ethanol, was recrystallized twice from the ethanol solution by cooling gradually to −20 °C.

Crystal data for **3**: $C_{13}H_{20}N_2O_5$, M = 284.31, monoclinic, $P2_1$, a = 5.035(3), b = 8.706(3), c = 15.926(5) Å, β = 90.85(5), V = 698.0(5) $Å^3$, z = 2, D_c = 1.35 g cm^{-3}, λ = 0.71069 Å, $5 < 2\theta < 60°$, $R(R_w)$ = 0.0397 (0.0452). The structure was solved by using the TEXSAN structure solving system. The non-hydrogen atoms were refined anisotropically, and the positions of the hydrogen atoms were refined by using isotropic thermal parameters. Atomic coordinates, bond lengths and angles, and thermal parameters have been deposited at the Cambridge Crystallographic Centre. See Notice to Authors, Issue No. 1.

residue and adds a hydroxy group to the α-carbon from the same direction as the hydrogen removed, yielding the *S*-α-hydroxyglycine residue. In the second step, PHL cleaves the N–C bond in the *S*-α-hydroxyglycine residue stereoselectively.

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