Reaction Versus Subsite Stereospecificity of Peptidylglycine 
α-Monoxygenase and Peptidylamidoglycolate Lyase, 
the Two Enzymes Involved in Peptide Amidation*

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Carboxyl-terminal amidation, a required post-translational modification for the bioactivation of many neuropeptides, entails sequential enzymatic action by peptidylglycine α-monoxygenase (PAM, EC 1.14.17.3) and peptidylamidoglycolate lyase (PGL, EC 4.3.2.5). The monoxygenase, PAM, first catalyzes conversion of a glycine-extended pro-peptide to the corresponding α-hydroxyglycine derivative, and the lyase, PGL, then catalyzes breakdown of this α-hydroxyglycine derivative to the amidated peptide plus glyoxylic acid. We have previously established that PAM and PGL exhibit tandem reaction stereospecificities, with PAM producing, and PGL being reactive toward, only α-hydroxyglycine derivatives of absolute configuration (S). We now demonstrate that PAM and PGL exhibit dramatically different subsite stereospecificities toward the residue at the penultimate position (the P2 residue) in both substrates and inhibitors. Incubation of Ac-L-Phe-Gly, Ac- D-Phe- Gly, or (S)-O-Ac-mandelyl-Gly with PAM results in complete conversion of these substrates to the corresponding α-hydroxylated products, whereas the corresponding X-ω-Gly compounds undergo conversions of <1%. The Kᵦ of Ac-α-Phe-Gly is at least 700-fold higher than that of Ac- L-Phe-Gly, and the same pattern holds for other substrate stereoisomers. This S₂ subsite stereospecificity of PAM also holds for competitive inhibitors; thus, the Kᵦ of 45 μM for Ac- L-Phe-OCH₂CO₂H increases to 2.247 μM for the ω-Phe-enantiomer. In contrast, incubation of PGL with Ac-L-Phe-α-hydroxy-Gly, Ac- ω-Phe- α-hydroxy-Gly, (S)-O-Ac-mandelyl-ω-hydroxy-Gly, or (R)-O-Ac-mandelyl-ω-hydroxy-Gly results in facile enzymatic conversion of each of these compounds to their corresponding amide products. The simultaneous expression of high reaction stereospecificity and low S₂ subsite stereospecificity in the course of PGL catalysis was illustrated by a series of experiments in which enzymatic conversion of the diastereomers of Ac- ω-Phe- α-hydroxy-Gly and Ac- ω-Phe- α-hydroxy-Gly was monitored directly by HPLC. Kinetic parameters were determined for both substrates and potent competitive inhibitors of PGL, and the results confirm that, in sharp contrast to PAM, the configuration of the chiral moiety at the P₂ position has virtually no effect on binding or catalysis. These results illustrate a case where catalytic domains, which must function sequentially (and with tandem reaction stereochirality) in a given metabolic process, nevertheless exhibit sharply contrasting subsite stereospecificities toward the binding of substrates and inhibitors.

Tandem enzymatic reactions, which represent sequential steps along a metabolic pathway, are in many cases catalyzed by multifunctional proteins comprising two or more distinct catalytic domains on a single polypeptide chain (for a comprehensive treatment of early work, see Ref. 1; see also Refs 2–7). A case in point is carboxyl-terminal amidation, a required post-translational modification for the bioactivation of many neuropeptides (8), which entails sequential enzymatic action by peptidylglycine α-monoxygenase (PAM, EC 1.14.17.3) and peptidylamidoglycolate lyase (PGL, EC 4.3.2.5) (9–18). The monoxygenase, PAM, first catalyzes conversion of a glycine-extended pro-peptide to the corresponding α-hydroxyglycine derivative, and the lyase, PGL, then catalyzes breakdown of this α-hydroxyglycine derivative to the amidated peptide plus glyoxylic acid (9, 10, 19). The “amidating enzyme” gene in pituitary encodes a multifunctional protein, which contains the catalytic PAM domain at its NH₂ terminus followed by the adjacent catalytic PGL domain (8, 20, 21). Subsequent post-translational endoproteolytic processing of this multifunctional protein can give rise to various truncated forms of both PAM and PGL (8, 22–28).

Elucidation of the stereochemistry of carboxyl-terminal amidation is a critical issue for detailed mechanistic studies on the enzymology of this process and for the rational design of pseudosubstrates and inhibitors targeted at PAM and PGL. In this regard, we recently demonstrated that PAM and PGL exhibit tandem reaction stereospecificities in carrying out the two requisite steps in carboxyl-terminal amidation (29). Thus, PAM produces only α-hydroxyglycine derivatives of absolute configuration (S), and PGL is reactive only toward (S)-α-hydroxyglycines (Scheme 1). While these results elucidate the reaction stereospecificity of both PAM and PGL toward the COOH-terminal (S)-α-hydroxyglycine moiety, the quite distinct question of whether PAM and PGL differ with respect to subsite stereospecificity remains unresolved.

We report here a series of experiments, which demonstrate that PAM and PGL indeed exhibit dramatically different subsite stereospecificities toward the residue at the penultimate position (the P₂ residue) of their respective substrates. In addition, we introduce new competitive inhibitors for PAM and PGL, and we demonstrate that the distinctive difference in S₂ subsite stereospecificities for substrates is also reflected in the

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binding of competitive inhibitors targeted at either enzyme, respectively. Finally, we illustrate the simultaneous expression of high reaction stereoselectivity and low $S_2$ substrate stereospecificity in the course of PGL catalysis by a series of experiments in which enzymatic conversion of all four diastereomers of Ac-Phe-$\alpha$-hydroxy-Gly was monitored directly by HPLC. Thus, these results provide a clear example of a case where catalytic domains, which must function sequentially (and with tandem reaction stereochemistry) in a given metabolic process, nevertheless exhibit contrasting substrate stereospecificities toward the binding of both substrates and inhibitors.

**EXPERIMENTAL PROCEDURES**

**Materials**

Frozen bovine pituitaries were purchased from Pel-Freez Biologicals (Rogers, AR). Ac-L-Phe-amide was purchased from Bachem (Philadelphia, PA). (S)-(+)-O-Acytylemidic acid, (R)-(−)-O-acetylemidic acid, Ac-D-phenylalanine, Ac-DL-phenylalanine, Ac-L-phenylalanine, Ac-L-leucine, and Ac-D-phenylalanine were purchased from Sigma. Acetylating agents: tert-($\alpha$)-2-phenylbutyric acid (1.0 g). \( \text{m} \; \text{z} = 238 \) (M$^+$; 85/20, 0.2% phosphoric acid). Nearly complete hydrolysis was achieved in about 2 h, after which the pH of the solution was adjusted to neutrality with dilute HCl. The mixture was extracted with methylene chloride (2 × 30 ml) to remove any unreacted Ac-D-Phe-OEt. The pH of the solution p$\text{H}$ was then adjusted to about 3.0 and extracted with ethyl acetate (2 × 30 ml). The ethyl acetate layer was dried over MgSO$_4$ and evaporated to dryness to give Ac-D-Phe-OEt (0.3 g).$^1$H NMR (Me$_2$SO-$\text{d}_6$, 300 MHz): $\delta$ 8.20 (d, 1H, CO$_2$H), 7.15–7.30 (m, 5H, Ph), 4.50 (m, 1H, NHCHCONH), 3.85 (d, 2H, NHCONH$_2$), 3.05 (dd, 1H, PhCHCH$_2$), 2.73 (dd, 1H, PhCHCH$_2$), 1.75 (s, 3H, CH$_2$CO). Mass spectroscopy (electron impact): m/z 264 (M$^+$, molecular ion).

Method II—This compound was synthesized using the solid-phase method, with the procedure being analogous to the synthesis of Ac-Oct-Phe-Gly.

Synthetic Ac-Oct-Phe-Gly has a specific rotation value of $\pm 11.8^\circ$ in methanol.

**Ac-L-Phe**

This compound was synthesized by the same procedures used for the synthesis of Ac-Oct-Phe-Gly.

Synthetic Ac-L-Phe-Gly has a specific rotation value of 11.9° in methanol.

(R)-O-Ac-mandelyl-Gly and (S)-O-Ac-mandelyl-Gly

Both (R)- and (S)-O-acetylmandelylesterstereoselectively were synthesized by the synthetic procedure used for the synthesis of Boc-Oct-Phe-Gly ester ethyl ester. Cleavage of the tert-buty group by trifluoroacetic acid (5 × molar excess) gave the desired products.

Ac-D-Phe-amide, (S)-O-Ac-mandelamic acid, and (R)-O-Ac-mandelamic acid

These compounds were synthesized according to a previously published procedure (32). The amide products exhibit the following specific rotation in methanol: Ac-D-Phe-amide, $[\alpha]_D = 26.1^\circ$; (S)-O-Ac-mandelamic acid, $[\alpha]_D = 146.7^\circ$; (R)-O-Ac-mandelamic acid, $[\alpha]_D = 146.8^\circ$.

**Ac-L-Phe-$\alpha$-hydroxy-Gly, Ac-O-Phe-$\alpha$-hydroxy-Gly, (R)-O-Ac-mandelony-$\alpha$-hydroxy-Gly, and (S)-O-Ac-mandelony-$\alpha$-hydroxy-Gly**

These compounds were synthesized according to a previously published method (11). The two diastereomers of Ac-L-Phe-$\alpha$-hydroxy-Gly (with the configurations (S,S) and (S,R)) and the two diastereomers of Ac-Phe-$\alpha$-hydroxy-Gly (with the configurations (R,S) and (R,R)) were separated on a C8 RP-HPLC column with an isotropic solution of H$_2$O/CH$_3$CN (98/2, 0.2% phosphoric acid).

(R)-2-Phenylbutyl-$\alpha$-hydroxy-Gly

(R)-2-Phenylbutyl-$\alpha$-hydroxy-Gly (1.0 g, 6.09 mmol) was converted to its acid chloride by reaction with cyanuric chloride, according to a published procedure (33). The acid chloride was then dissolved in anhydrous ether (20 ml) and treated with anhydrous ammonia gas to form the corresponding amide. The insoluble ammonium chloride salt was filtered off. The filtrate was washed twice with aqueous sodium bicarbonate solution, dried over MgSO$_4$, concentrated under reduced pressure, and recrystallized from ether/hexane. $^1$H NMR (CDCl$_3$, 300 MHz): $\delta$ 7.26–7.38 (m, 5H, Ph), 5.31 (bs, 2H, NH$_2$), 3.29 (t, 1H, COCH$_2$), 1.80–2.25 (m, 2H, CH$_2$CH$_2$CO), 0.90 (t, 3H, CH$_3$).

(R)-2-Phenylbutyramide (0.28 g, 1.70 mmol) was then reacted with glyoxylic acid in acetic acid, as described in the literature (34), to give (R)-2-Phenylbutyl-$\alpha$-hydroxy-Gly (0.21 g, $[\alpha]_D = -29.48^\circ$ in methanol).

Ac-L-Phe-$\alpha$-hydroxy-Gly (0.8 g) was obtained by acetylation of (S)-Phe-OEt (1.0 g) in a mixture of 10 ml of pyridine and 4 ml of acetic anhydride for 30 min at room temperature.

CS$_2$(OH)$_4$N$_2$$\text{O}_4$

Calculated: C 60.75 H 6.38 N 5.90 Found: C 60.60 H 6.53 N 5.87

(S)-2-Phenylbutyl-$\alpha$-hydroxy-Gly

This compound (0.20 g) was synthesized as described for (R)-2-Phenylbutyl-$\alpha$-hydroxy-Gly, starting from (S)-2-phenylbutyric acid (1.0 g).
Ac-Gly-OCH$_2$CO$_2$H

To a solution of bromoacetic acid (4.5 g, 32 mmol) in ethyl acetate (500 ml) were added phenacyl bromide (33 g, 0.17 mol) and triethyl amine (4.5 ml, 32 mmol) (35). The reaction mixture was stirred overnight at room temperature and filtered to remove the insoluble triethylamine hydrobromide salt. The filtrate was washed with saturated NaHCO$_3$ solution until no more bromoacetic acid was observed on TLC (eluting solvent: EtOAc), then washed with water twice. The organic layer was dried over Na$_2$SO$_4$ and concentrated under reduced pressure. Recrystallization from ethyl acetate/hexane gave pure Ac-Gly phenacyl acetate (4.74 g). Melting point: 76–78°C; $^1$H NMR (CDCl$_3$, 300 MHz) $\delta$: 8.27 (d, 1H, CO$_2$), 4.34 (q, 1H, CH$_2$CH$_2$), 3.01 (s, 2H, CO$_2$CH$_2$CO$_2$), 2.03 (s, 3H, CH$_3$CO); mass spectroscopy (FAB-MS): m/z 272 (M$^+$ + 1).

Ac-Gly-OCH$_2$CO$_2$H

This compound (0.45 g) was synthesized by the same procedure used for Ac-Gly-OCH$_2$CO$_2$H, starting from Ac-Phe (1.40 g). [$\alpha$]$_D$ = −37.8° in methanol; $^1$H NMR (D$_2$O, 300 MHz) $\delta$: 8.17 (d, 1H, CO$_2$H), 7.20–7.28 (m, 2H), 5.24 (s, 2H, CO$_2$CH$_2$CO$_2$), 4.01 (s, 2H, BrCH$_2$CO$_2$). To a solution of Ac-glycine (1.0 g, 8.54 mmol) in 30 ml of acetonitrile was added 2-bromophenyl acetate (2.2 g, 8.56 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (1.3 ml, 8.69 mmol) (36). The reaction mixture was stirred at room temperature overnight. Water was then added (50 ml) and the mixture extracted with ethyl acetate (2 × 50 ml). The combined organic extracts were washed with (50 ml) water, then dried over Na$_2$SO$_4$, and concentrated under reduced pressure. Recrystallization from EtOAc/hexane gave Ac-L-Phe-OCH$_2$CO$_2$H (0.29 g) as a very hygroscopic powder. $^\circ$C: 25.16° in methanol; $^1$H NMR (CDCl$_3$, 300 MHz) $\delta$: 8.37 (d, 1H, CO$_2$H), 7.20–7.28 (m, 5H, Ph), 4.61 (d, 2H, CO$_2$CH$_2$CO$_2$H), 4.36 (m, 1H, NHCH$_2$CO$_2$), 3.10 (dd, 1H, NHCH$_2$CO$_2$H), 1.76 (s, 3H, CH$_3$CO); mass spectroscopy (FAB-MS): m/z 266 (M$^+$ + 1).

Ac-OH$_3$CO$_2$H

The reaction mixture was stirred at room temperature overnight. Water was then added (50 ml) and the mixture extracted with ethyl acetate (2 × 50 ml). The combined organic extracts were washed with water (50 ml), dried over Na$_2$SO$_4$, and concentrated under reduced pressure. Recrystallization from EtOAc/hexane gave Ac-Phe-OCH$_2$CO$_2$H (1.40 g). [$\alpha$]$_D$ = −40.2° in methanol; $^1$H NMR (D$_2$O, 300 MHz) $\delta$: 8.27 (d, 1H, CO$_2$H), 7.20–7.28 (m, 5H, Ph), 4.61 (d, 2H, CO$_2$CH$_2$CO$_2$), 4.36 (m, 1H, NHCH$_2$CO$_2$), 3.10 (dd, 1H, NHCH$_2$CO$_2$H), 2.86 (dd, 1H, NHCH$_2$CO$_2$H), 1.76 (s, 3H, CH$_3$CO); mass spectroscopy (FAB-MS): m/z 266 (M$^+$ + 1).
abolished by the presence of a D-amino acid residue, or of the result that substrates reactivity toward PAM is virtually
and (O-Ac-mandelyl-Gly. Thus, it is clearly evident from -inhibitors, PAM substrates is also reflected in the potency of competitive
termine whether the S2 subsite stereospecificity observed with
position.
Incubation of synthetic Ac-L-Phe-Gly is transparent to our assay, it causes the meas-
were determined for both Ac-L-Phe-Gly and its non-reactive
-O-hydroxy-Gly, or (O-Ac-mandelyl-NH2 are
Ac-D-Phe-OCH2CO2H 2.25
Ac-L-Leu-OCH2CO2H 5.98 \times 10^{-2}
Ac-o-Leu-OCH2CO2H 2.11

TABLE II
Inhibition constants for PAM inhibitors

| Compound                  | \( K_i \) (M) |
|---------------------------|--------------|
| Ac-Gly-OCH2CO2H           | 1.25         |
| Ac-L-Phe-OCH2CO2H         | 4.52 \times 10^{-2} |
| Ac-o-Phe-OCH2CO2H         | 2.25         |
| Ac-DL-Phe-OCH2CO2H        | 0.522        |
| Ac-L-Leu-OCH2CO2H         | 5.98 \times 10^{-2} |
| Ac-o-Leu-OCH2CO2H         | 2.11         |

conversion of each of these compounds to their corresponding amide products. The time courses of the PGL-catalyzed conversion of Ac-D-Phe-hydroxy-Gly to Ac-D-Phe-NH2 and of (R)-O-Ac-mandelyl-\( \alpha \)-hydroxy-Gly to (R)-O-Ac-mandelyl-NH2 are shown in Fig. 2. It is evident from these time courses that enzymatic turnover terminates prior to complete conversion of the substrates to amide products by PGL. Therefore, prolonged incubation experiments with PGL were carried out to quanti-
tate the maximal percentage conversion of Ac-L-Phe-\( \alpha \)-hydroxy-Gly, Ac-Phe-\( \alpha \)-hydroxy-Gly, (S)-O-Ac-mandelyl-\( \alpha \)-hydroxy-Gly, and (R)-O-Ac-mandelyl-\( \alpha \)-hydroxy-Gly to their corresponding amide products. As shown in Table III, maximal conversion in all cases is about 50%. To further confirm this result, a double addition experiment was carried out wherein Ac-L-Phe-\( \alpha \)-hydroxy-Gly and Ac-Phe-\( \alpha \)-hydroxy-Gly were each incubated overnight with PGL then analyzed by HPLC to confirm 50% conversion, after which the enzyme was removed by ultrafiltration. Each filtrate was then again incubated with a large quantity of PGL, and monitored by HPLC. As expected, no additional enzymatic conversion of unreacted substrate to amide products occurred for either substrate.

**Synthetic preparations of Ac-L-Phe-\( \alpha \)-hydroxy-Gly and (S)-O-Ac-mandelyl-\( \alpha \)-hydroxy-Gly actually consist of equimolar mixtures of their respective (S,S) and (R,R) diastereomers; similarly, synthetic Ac-D-Phe-\( \alpha \)-hydroxy-Gly and (R)-O-Ac-mandelyl-\( \alpha \)-hydroxy-Gly actually consist of equimolar mixtures of the respective (R,S) and (R,R) diastereomers. Previously, we demonstrated that PGL is stereospecific toward the terminal \( \alpha \)-hydroxyglycine moiety (29), i.e. PGL reacts only with \( \alpha \)-hydroxyglycine moieties of the (S) configuration. Accordingly, if PGL exhibits the same \( S_2 \) subsite stereospecificity as PAM, we would expect 50% conversion only for Ac-L-Phe-\( \alpha \)-

**Subsite Stereospecificity of PGL toward Peptide Substrates**—Incubation of synthetic Ac-L-Phe-\( \alpha \)-hydroxy-Gly, Ac-o-Phe-\( \alpha \)-hydroxy-Gly, (S)-O-Ac-mandelyl-\( \alpha \)-hydroxy-Gly, or (R)-O-Ac-mandelyl-\( \alpha \)-hydroxy-Gly with PGL resulted in facile enzymatic conversion of each of these compounds to their corresponding amide products. The time courses of the PGL-catalyzed conversion of PGL was used to calculate both the concentration of Ac-L-Phe-\( \alpha \)-hydroxy-Gly derived from Ac-L-Phe-Gly, and the concentration of the new product derived from Ac-o-Phe-Gly.
hydroxy-Gly and (S)-O-Ac-mandelyl-α-hydroxy-Gly, while Ac-D-Phe-α-hydroxy-Gly and (R)-O-Ac-mandelyl-α-hydroxy-Gly should have been unreactive toward PGL. Therefore, our finding that 50% conversion actually occurs with all these substrates (Table III) is a strong indication that, unlike PAM, PGL reacts with substrates possessing residues of either the (S) or (R) configuration at the P2 position.

In order to demonstrate directly this simultaneous expression of high reaction stereospecificity and low S2 subsite stereospecificity in the course of PGL catalysis, the series of HPLC experiments illustrated in Fig. 3 was carried out. Panel A shows the HPLC elution profile of Ac-L-Phe-α-hydroxy-Gly produced from Ac-L-Phe-Gly by PAM. Since PAM catalysis produces only α-hydroxyglycines of the (S) configuration, this peak represents the retention time of the (S,S) diastereomer of Ac-L-Phe-α-hydroxy-Gly. Panels B and C show the elution profiles of synthetic Ac-L-Phe-α-hydroxy-Gly and Ac-D-Phe-β-hydroxy-Gly, respectively. It is evident from panels B and C that the two diastereomers of synthetic Ac-L-Phe-α-hydroxy-Gly (with the configurations (S,R) and (S,S)) and the two diastereomers of synthetic Ac-D-Phe-α-hydroxy-Gly (with the configurations (R,S) and (R,R)) are well separated on a C8 RP-HPLC column using a mobile phase of 2% CH3CN, 98% H2O, 0.2% H3PO4. Panels D and E show the elution profiles obtained after reaction of Ac-L-Phe-α-hydroxy-Gly and Ac-D-Phe-α-hydroxy-Gly, respectively, with PGL. It is quite clear from panel D that reaction of PGL with synthetic Ac-L-Phe-α-hydroxy-Gly results in conversion of only one substrate diastereomer to Ac-L-Phe-NH2. Since the reactive diastereomer has retention time identical to that of Ac-L-Phe-α-hydroxy-Gly (with (S,S) configuration) produced by PAM (Fig. 3, panel A), it is clear that this reactive diastereomer has the (S,S) configuration. Correspondingly, as shown in panel E, reaction of PGL with synthetic Ac-D-Phe-α-hydroxy-Gly also results in conversion of only one diastereomer of this substrate to Ac-D-Phe-NH2. From a comparison with the previous panels, it is quite clear that the reactive diastereomer of this substrate has the (R,S) configuration. Taken together, these experiments unequivocally confirm the sharp contrast between the reaction and the subsite stereospecificities of PGL; while PGL is reactive only toward an α-hydroxyglycine moiety of the (S) configuration, this lyase reacts readily with substrate diastereomers of either the (S) or (R) configuration at the P2 position.

Kinetic parameters for the two pairs of diastereomeric PGL substrates are listed in Table IV. It is apparent that the Vmax values are virtually unaffected by whether the residue at the P2 position has the (S) or (R) configuration, whereas small effects are apparent on the Km values. Both pairs of compounds are comparable in reactivity to N-benzoyl-α-hydroxy-Gly.

Subsite Stereospecificity of PGL toward Inhibitors—A systematic specificity study revealed that the substrate analog, phenylacetyl-α-OH-Gly, is an inhibitor of PGL with a Ki value of 770 μM; inhibition is purely competitive as evidenced by Dixon plots. Therefore, in order to determine the effect of stereoenzyme configuration at the P2 position on the potency of PGL inhibitors, we synthesized (R)-2-phenylbutyl-α-hydroxy-Gly and (S)-2-phenylbutyl-α-hydroxy-Gly, analogs of phenylacetlyl-α-OH-Gly each possessing a chiral center of opposite configuration at the penultimate position. As is the case for the PGL substrates discussed above, synthetic (R)-2-phenylbutyl-α-hydroxy-Gly actually consists of an equimolar mixture of the (R,S) and (R,R) diastereomers; likewise, synthetic (S)-2-phenylbutyl-α-hydroxy-Gly is an equimolar mixture of (S,S) and (R,R) diastereomers.

Both (R)-2-phenylbutyl-α-hydroxy-Gly and (S)-2-phenylbutyl-α-hydroxy-Gly were found to indeed be PGL inhibitors. We also observed that they both undergo very slow enzymatic
conversion to the corresponding phenylbutyrasides (as confirmed by HPLC analysis). The substrate activity (V/K) of these compounds is ~10,000 times slower than that of our normal PGL assay substrate, TNP-o-Tyr-L-Val-α-hydroxy-Gly; therefore, inhibition kinetics are readily performed in the usual manner. Both compounds are highly potent competitive inhibitors, and the Kᵢ values obtained are 280 μM and 310 μM for (R)-2-phenethyl-α-hydroxy-Gly and (S)-2-phenethyl-α-hydroxy-Gly, respectively (Table V). It is thus clear that, in sharp contrast to PAM, the configuration of the chiral moiety at the P₂ position has no effect on the potency of these PGL inhibitors.

Table V also lists the kinetic constants measured for the very slow PGL-catalyzed reaction of these two compounds. As expected, Vₘₐₓ and Kₘ are identical for (R)- and (S)-2-phenethyl-α-hydroxy-Gly. Moreover, the measured Kᵢ values are identical to the Kᵢ values obtained in the inhibition experiments against TNP-o-Tyr-L-Val-α-hydroxy-Gly, thus confirming that the Kᵢ values represent true binding constants.

Taken together, the results reported here establish that despite their tandem reaction stereospecificities with respect to the COOH-terminal (S)-α-hydroxycarboxy moiety, PAM and PGL exhibit sharply contrasting S₂ subsite stereospecificities in the binding of both substrates and inhibitors. Physiologically, these two enzymatic steps must occur sequentially in order to convert a given glycine-derived pro-peptide to the corresponding mature amidated peptide (9, 11). Thus, from a metabolic viewpoint, the stereospecificities of these catalytic domains would have been expected to correlate. From a mechanistic perspective, there is, of course, a vast difference between the catalytic site of a metallo-monooxygenase on the one hand, which entails participation of both active site copper and an electron donor in catalysis, and that of a lyase on the other. Indeed, the contrasting stereospecificities we report here are likely reflections of corresponding differences in the active site topographies of PAM and PGL.

To our knowledge, this is the first demonstration of a case where catalytic domains that must function sequentially nevertheless exhibit contrasting binding stereospecificities. Most likely, the possibility that stereospecificities of such domains might differ has not been addressed since, of course, metabolites flow through the catalytic domains in sequence; a given domain would never “see” a metabolite of altered chirality such that it would have been precluded from binding at the preceding domain. Thus, inspection of the stoichiometric configurations of metabolic intermediates does not reveal possible differences in the subsite stereospecificities of the domains; stereochemical studies using substrate analogs and inhibitors of deliberately altered chirality are required for this purpose. For example, in the case of chorismate mutase-prephenate dehydrogenase, a multifunctional protein catalyzing sequential reactions that has been the subject of much recent interest (41–43), the relative stereochemistry at the ring position para to the site of enzymatic reaction clearly affects reactivity of substrate analogs in the dehydrogenase reaction. However, a comparison of the effect of commensurate changes in substrate stereochemistry on binding and turnover for the individual catalytic domains has not been carried out, and, unfortunately, synthetic approaches to the synthesis of individual substrate analog stereoisomers that might be used in such investigations have been unsuccessful (41–43).

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