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Inhibition of Peptidylglycine α-Amidating Monooxygenase by Exploitation of Factors Affecting the Stability and Ease of Formation of Glycyl Radicals

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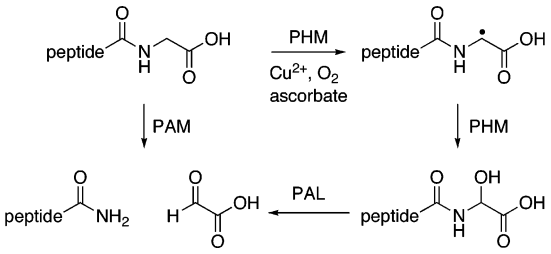
Abstract: Peptidylglycine α-amidating monooxygenase catalyzes the biosynthesis of peptide hormones through radical cleavage of the *C*-terminal glycine residues of the corresponding prohormones. We have correlated ab initio calculations of radical stabilization energies and studies of free radical brominations with the extent of catalysis displayed by peptidylglycine α-amidating monooxygenase, to identify classes of inhibitors of the enzyme. In particular we find that, in closely related systems, the substitution of glycolate for glycine reduces the calculated radical stabilization energy by 34.7 kJ mol^{-1}, decreases the rate of bromination with *N*-bromosuccinimide at reflux in carbon tetrachloride by a factor of at least 2000, and stops catalysis by the monooxygenase, while maintaining binding to the enzyme.

Introduction

Peptidylglycine α-amidating monooxygenase (PAM) catalyzes the biosynthesis of a wide variety of *C*-terminal peptide amides through oxidative cleavage of the corresponding glycine-extended precursors (Scheme 1). The products include mammalian peptide hormones,[1] as well as physiologically active peptides of other organisms such as insects[2,3] and cnidarians,[4,5] and the *C*-terminal amide moiety is vital to the activity of many of these compounds. Amidated peptide hormones are important in cellular communication, in particular as neuropeptides,[1] and are implicated in a broad range of pathological conditions, including asthma,[6] inflammation,[7] and cancers.[8−10] The biosynthesis of amides from glycine-extended precursors other than peptides, such as fatty acids,[11] bile acids,[12] nicotinic acid,[13] and aspirin,[14] has also been attributed to PAM. Fatty acid amides are known to affect a number of neurochemical communication pathways, including sleep regulation,[15,16] while the amidation of aspirin may be important in its metabolic processing.

Scheme 1



The importance of PAM in pathological conditions has led to interest in its regulation and the development of a number of inhibitors. One of the first was *trans*-4-phenylbut-3-enoic acid,[17] which is effective in vivo in reducing serum PAM activity[18] as well as showing anti-inflammatory and analgesic effects.[19]

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(1) Merkler, D. J. *Enzyme Microb. Technol.* **1994**, *16*, 450−456.
(2) Konopinska, D.; Rosinski, G.; Sobotka, W. *Int. J. Pept. Protein Res.* **1992**, *39*, 1−11.
(3) Schoofs, L.; Veelaert, D.; VandenBroeck, J.; DeLoof, A. *Peptides* **1997**, *18*, 145−156.
(4) Hauser, F.; Williamson, M.; Grimmelikhuijzen, C. J. P. *Biochem. Biophys. Res. Commun.* **1997**, *241*, 509−512.
(5) Williamson, M.; Hauser, F.; Grimmelikhuijzen, C. J. P. *Biochem. Biophys. Res. Commun.* **2000**, *277*, 7−12.
(6) Groneberg, D. A.; Springer, J.; Fischer, A. *Pulm. Pharmacol. Ther.* **2001**, *14*, 391−401.
(7) Wiesenfeld-Hallin, Z.; Xu, X. J. *Eur. J. Pharmacol.* **2001**, *429*, 49−59.
(8) Schally, A. V.; Comaru-Schally, A. M.; Nagy, A.; Kovacs, M.; Szepeshazi, K.; Plonowski, A.; Varga, J. L.; Halmos, G. *Front. Neuroendocrinol.* **2001**, *22*, 248−291.
(9) Du, J. L.; Keegan, B. P.; North, W. G. *Cancer Lett.* **2001**, *165*, 211−218.
(10) Jimenez, N.; Jongsma, J.; Calvo, A.; van der Kwast, T. H.; Treston, A. M.; Cuttitta, F.; Schroder, F. H.; Montuenga, L. M.; van Steenbrugge, G. J. *Int. J. Cancer* **2001**, *94*, 28−34.
(11) Wilcox, B. J.; Ritenour-Rodgers, K. J.; Asser, A. S.; Baumgart, L. E.; Baumgart, M. A.; Boger, D. L.; DeBlassio, J. L.; deLong, M. A.; Glufke, U.; Henz, M. E.; King, L.; Merkler, K. A.; Patterson, J. E.; Robleski, J. J.; Vederas, J. C.; Merkler, D. J. *Biochemistry* **1999**, *38*, 3235−3245.
(12) King, L.; Barnes, S.; Glufke, U.; Henz, M. E.; Kirk, M.; Merkler, K. A.; Vederas, J. C.; Wilcox, B. J.; Merkler, D. J. *Arch. Biochem. Biophys.* **2000**, *374*, 107−117.
(13) Merkler, D. J.; Glufke, U.; Ritenour-Rodgers, K. J.; Baumgart, L. E.; DeBlassio, J. L.; Merkler, K. A.; Vederas, J. C. *J. Am. Chem. Soc.* **1999**, *121*, 4904−4905.
(14) DeBlassio, J. L.; deLong, M. A.; Glufke, U.; Kulathila, R.; Merkler, K. A.; Vederas, J. C.; Merkler, D. J. *Arch. Biochem. Biophys.* **2000**, *383*, 46−55.
(15) Cravatt, B. F.; Lerner, R. A.; Boger, D. L. *J. Am. Chem. Soc.* **1996**, *118*, 580−590.
(16) Cravatt, B. F.; Prosperogarcia, O.; Siuzdak, G.; Gilula, N. B.; Henriksen, S. J.; Boger, D. L.; Lerner, R. A. *Science* **1995**, *268*, 1506−1509.
(17) Bradbury, A. F.; Mistry, J.; Roos, B. A.; Smyth, D. G. *Eur. J. Biochem.* **1990**, *189*, 363−368.
(18) Mueller, G. P.; Driscoll, W. J.; Eipper, B. A. *J. Pharmacol. Exp. Ther.* **1999**, *290*, 1331−1336.

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Others include α,β-unsaturated acids,[20−22] a peptide terminating in α-vinylglycine,[23] and diastereomers of a peptide terminating in an α-styrylglycine.[24] These are all mechanism-based inhibitors in that they show turnover-dependent inactivation of the enzyme. Inhibitors of other types have also been reported, such as inorganic sulfite,[25] benzyl hydrazine,[26] and *N*-formylamides,[27] as well as derivatives of β-mercaptostyrene[28] and homocysteine.[29]

PAM consists of two functional subunits, peptidylglycine α-hydroxylating monooxygenase (PHM, E.C. 1.14.17.3) and peptidylamidoglycolate lyase (PAL, E.C. 4.3.2.5.) (Scheme 1). PHM catalyzes the copper-, molecular oxygen-, and ascorbate-dependent hydroxylation of a *C*-terminal glycine residue of a peptide substrate. The product hydroxyglycine is then hydrolyzed to the corresponding amide and glyoxylate, a process that is catalyzed by PAL at physiological pH. The determination of the crystal structure of PHM in both reduced and oxidized forms,[30−32] and kinetic[33−35] and mutagenesis studies,[36] have resulted in a detailed picture of the mechanism of action of this enzyme. In particular, it has been concluded that, in the first step, a copper-bound superoxide radical abstracts the pro-*S* hydrogen from the glycine residue, to give a glycyl radical.

In the present work we have sought to exploit factors affecting the formation of such radicals in order to design analogues of the substrates of PAM that competitively bind to, but are not processed by, the enzyme and therefore inhibit reaction of the substrates. To this end, we have compared the results of ab initio calculations and studies of relative reaction rates in free radical brominations, which identify factors affecting the stability and ease of formation of glycyl and related radicals,[37,38] with the kinetic parameters defining the interactions of analogous compounds with PAM.

Publication of our results is very timely in the light of a quite recent paper by Prigge et al.,[32] in which crystallography of frozen protein soaked with a slowly reacting substrate was used to identify and characterize the precatalytic complex of PHM with copper, oxygen, and the substrate. That study was the first to delineate the role of copper in the activation of dioxygen in this or any other enzyme system. The authors also drew a correlation between the reactivity of PHM substrates and the stability of the corresponding radical intermediates. They reported that our earlier theoretical studies[37] showed a peptide α-carbon-centered alanyl radical to be 9.1 kJ mol^{-1} less stable than a corresponding glycyl radical and noted that, even so, *N*-acetyl-(*S*)-tryptophanyl-(*R*)-alanine is still processed by the enzyme during X-ray diffraction. They also predicted, on the basis of our calculations with related amino acids, that an α-carbon-centered threonyl radical would be even less stable than an alanyl radical, and accordingly found that a peptide containing (*R*)-threonine instead of (*R*)-alanine at the *C*-terminus was less effectively turned over by the enzyme. Our calculations of glycyl and alanyl radical stability[37] were based on the radicals **3c** and **5d** (Chart 1). Contrary to the above discussion, their relative radical stabilization energies (RSEs), which correspond to the negative of the relative bond dissociation energies of the corresponding closed-shell molecules, actually showed the alanyl radical **5d** to be less stable than the glycyl radical **3c** by only 1.6 kJ mol^{-1}. We have not studied threonine derivatives, but Rauk et al.[39] used two different methods to calculate that an α-carbon-centered threonyl radical is destabilized relative to a glycyl radical by only 7−14 kJ mol^{-1}. Consequently, the destabilization of alanyl and threonyl radicals appears to have been somewhat over-estimated by Prigge et al.,[32] with the result that the relationship between the stabilization energies of radicals and the ease of their formation through PAM catalysis warrants further investigation. We also include in the present study a further comparison with the relative rates of formation of radicals in conventional brominations.

Results

The natural substrates of PAM all have in common an *N*-acylated glycine, and they are therefore comprised of acyl, amido, methylene, and carboxyl groups. Since the carboxyl group of a substrate is known to be important for binding to PAM,[31] we have not examined alternatives to this moiety in the present study. However, the effect of modifying each of the other groups was explored. A range of *N*-acyl substituents was investigated, since these are known to affect the stability and ease of formation of glycyl radicals[38,40] and to be tolerated by PAM. Although derivatives of α-substituted amino acids tend not to bind to PAM, small α-alkyl substituents, such as the methyl group of (*R*)-alanine,[32,41] the vinyl moiety of (*R*)-vinylglycine,[42] and the hydroxyethyl group of (*R*)-threonine,[32] are accommodated. The incorporation of a trifluoromethyl group was therefore studied since β,β,β-trifluoroalanine derivatives are known to be resistant to α-carbon-centered radical formation.[37] The effect of replacing the amido group with an ester or ketone using derivatives of glycolate or γ-keto acids instead of *N*-acylglycines was also explored. Glycolate inhibitors of PAM have been previously reported[22,42] in studies of broad ranges of

(19) Ogonowski, A. A.; May, S. W.; Moore, A. B.; Barrett, L. T.; O'Bryant, C. L.; Pollock, S. H. *J. Pharmacol. Exp. Ther.* **1997**, *280*, 846−853.
(20) Feng, J.; Shi, J.; Sirimanne, S. R.; Mounier-Lee, C. E.; May, S. W. *Biochem. J.* **2000**, *350*, 521−530.
(21) Moore, A. B.; May, S. W. *Biochem. J.* **1999**, *341*, 33−40.
(22) Katopodis, A. G.; May, S. W. *Biochemistry* **1990**, *29*, 4541−4548.
(23) Zabriskie, T. M.; Cheng, H. M.; Vederas, J. C. *J. Am. Chem. Soc.* **1992**, *114*, 2270−2272.
(24) Andrews, M. D.; O'Callaghan, K. A.; Vederas, J. C. *Tetrahedron* **1997**, *53*, 8295−8306.
(25) Merkler, D. J.; Kulathila, R.; Francisco, W. A.; Ash, D. E.; Bell, J. *FEBS Lett.* **1995**, *366*, 165−169.
(26) Merkler, D. J.; Kulathila, R.; Ash, D. E. *Arch. Biochem. Biophys.* **1995**, *317*, 93−102.
(27) Klinge, M.; Cheng, H. M.; Zabriskie, T. M.; Vederas, J. C. *J. Chem. Soc., Chem. Commun.* **1994**, 1379−1380.
(28) Casara, P.; Ganzhorn, A.; Philippo, C.; Chanal, M. C.; Danzin, C. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 393−396.
(29) Erion, M. D.; Tan, J.; Wong, M.; Jeng, A. Y. *J. Med. Chem.* **1994**, *37*, 4430−4437.
(30) Prigge, S. T.; Kolhekar, A. S.; Eipper, B. A.; Mains, R. E.; Amzel, L. M. *Nat. Struct. Biol.* **1999**, *6*, 976−983.
(31) Prigge, S. T.; Kolhekar, A. S.; Eipper, B. A.; Mains, R. E.; Amzel, L. M. *Science* **1997**, *278*, 1300−1305.
(32) Prigge, S. T.; Eipper, B. A.; Mains, R. E.; Amzel, L. M. *Science* **2004**, *304*, 864−867.
(33) Francisco, W. A.; Merkler, D. J.; Blackburn, N. J.; Klinman, J. P. *Biochemistry* **1998**, *37*, 8244−8252.
(34) Francisco, W. A.; Knapp, M. J.; Blackburn, N. J.; Klinman, J. P. *J. Am. Chem. Soc.* **2002**, *124*, 8194−8195.
(35) Francisco, W. A.; Blackburn, N. J.; Klinman, J. P. *Biochemistry* **2003**, *42*, 1813−1819.
(36) Bell, J.; El Meskini, R.; D'Amato, D.; Mains, R. E.; Eipper, B. A. *Biochemistry* **2003**, *42*, 7133−7142.
(37) Croft, A. K.; Easton, C. J.; Radom, L. *J. Am. Chem. Soc.* **2003**, *125*, 4119−4124.
(38) Croft, A. K.; Easton, C. J.; Kociuba, K.; Radom, L. *Tetrahedron: Asymmetry* **2003**, *14*, 2919−2926.
(39) Rauk, A.; Yu, D.; Taylor, J.; Shustov, G. V.; Block, D. A.; Armstrong, D. A. *Biochemistry* **1999**, *38*, 9089−9096.
(40) Easton, C. J. *Chem. Rev.* **1997**, *97*, 53−82.
(41) Landymore-Lim, A. E. N.; Bradbury, A. F.; Smyth, D. G. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 289−293.
(42) Ping, D.; Mounier, C. E.; May, S. W. *J. Biol. Chem.* **1995**, *270*, 29250−29255.

Chart 1

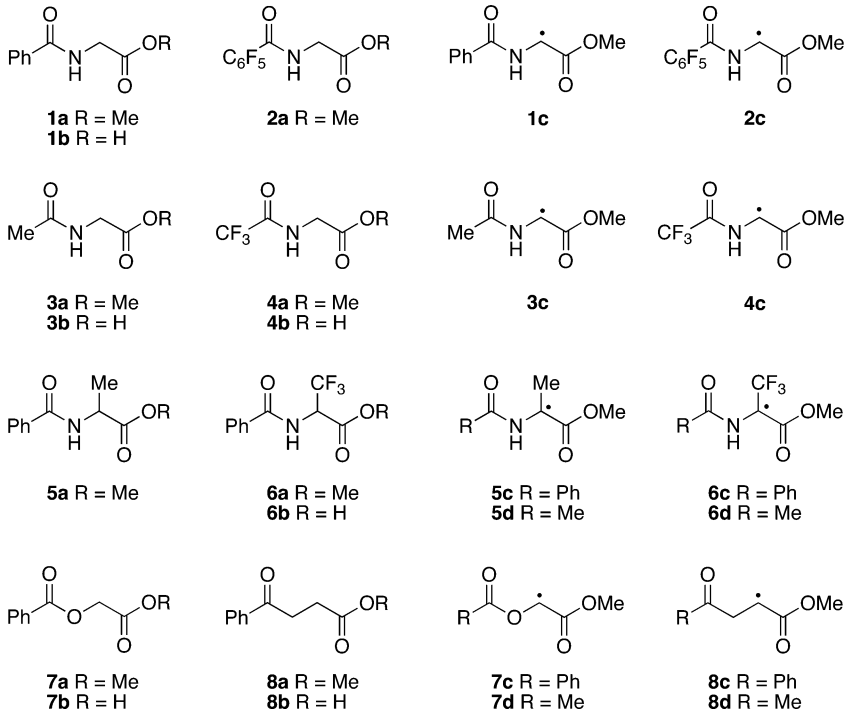


Table 1. Correlation of Studies of Free Radical Brominations and ab Initio Calculations of Radical Stabilization Energies with the Extent of Catalysis Displayed by Peptidylglycine α-Amidating Monooxygenase

	PART A relative rates of reaction of **1a**–**8a** with *N*-bromosuccinimide to give the corresponding radicals **1c**–**8c**		**PART B** radical stabilization energies [0 K, RMP2/G3large] of derivatives of glycyl and related radicals		**PART C** kinetic parameters for interactions of various substrates and inhibitors with peptidylglycine α-amidating monooxygenase				
						kinetic parameters			
glycine derivative or substitute	compd	relative rate of reaction[a]	radical	RSE[b]	compd	$V_{M,app}$ (μmol min^{-1} mg^{-1})	$K_{M,app}$ (mM)	K_I (mM)	IC_{50}[c] (mM)
N-benzoyl	**1a**	1.0[d]			**1b**	6.5[e]	1.3[e]		
N-pentafluorobenzoyl	**2a**	0.25[f]							
N-acetyl	**3a**	1.2[f]	**3c**	79.1	**3b**	6.4[e]	9.3[e]		
N-trifluoroacetyl	**4a**	0.05[f]	**4c**	69.9	**4b**	1.4[e]	4.1[e]		
other *N*-acyl					**10a**	12.6[e]	0.1[e]		
					11a		0.03		
					12a	3.3[g]	0.0012[g]		
					13a	8.2[e]	0.096[e]		
					14a	5.6[g]	0.0079[g]		
α-methyl	**5a**	0.33[h]	**5d**	78.8					
α-trifluoromethyl	**6a**	<0.0005[i,j]	**6d**	39.9	**9a**	inhibitor			5
					9b	inhibitor			5
glycolate	**7a**	<0.0005[j]	**7d**	44.4	**7b**	inhibitor			0.25
					10b	inhibitor			0.04
					11b	inhibitor			0.5
					12b	inhibitor			0.05
					13b	inhibitor		0.0598[g]	
					14b	inhibitor		0.0452[g]	
γ-keto acid	**8a**	<0.0005[j]	**8d**	34.9	**15**	inhibitor			6
					16	inhibitor			3

[a] From mixtures of substrates, in carbon tetrachloride at reflux. [b] Radical stabilization energies (RSEs) were calculated as the energy change in the isodesmic reaction $R^{\bullet} + CH_4 \rightarrow RH + {}^{\bullet}CH_3$. The RSEs correspond to the differences between the bond dissociation energies (BDEs) of methane and RH,[45] and reflect the stability of $R^{\bullet}$ compared with ${}^{\bullet}CH_3$, relative to the corresponding closed-shell systems. [c] Corresponds to loss of 50% of the catalytic activity of PAM in processing the substrate (*R*)-tyrosyl-(*S*)-valylglycine at a concentration of 0.1 mM, under conditions where $K_{M,app}$ for the substrate is 0.2 mM. Although $K_{M,app}$, K_I, and IC_{50} values are not directly comparable as measures of enzyme binding affinity, they are adequate as used in the present work, mainly to establish that compounds interact with PAM. [d] Assigned as unity. [e] Data from ref 11. [f] Data from ref 38. [g] Data from ref 42. [h] Data from ref 44. [i] Data from ref 37. [j] No detectable bromination.

compounds, but there has been no analysis of, nor explanation for, their behavior. One γ-keto acid has been previously investigated, but it was found not to interact with PAM, either as a substrate or an inhibitor.[43]

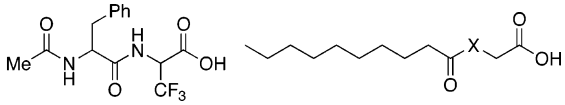
Compiled in Table 1 (part A) are the relative rates of reaction of the acylated glycine derivatives **1a**−**4a**, as well as the derivatives of alanine **5a**, trifluoroalanine **6a**, glycolate **7a**, and γ-ketopropionate **8a**, with *N*-bromosuccinimide at reflux in carbon tetrachloride. They were derived by measuring the

(43) Tamburini, P. P.; Jones, B. N.; Consalvo, A. P.; Young, S. D.; Lovato, S. J.; Gilligan, J. P.; Wennogle, L. P.; Erion, M.; Jeng, A. Y. *Arch. Biochem. Biophys.* **1988**, *267*, 623−631.

relative rates of consumption of **1a**−**8a** from mixtures.[37,38,44] Since the reactions involve radical bromination, with hydrogen atom transfer from the carbon adjacent to the ester group of each substrate determining the relative rate at which that compound is brominated, the relative rates of reaction correspond to the relative ease of formation of the radicals **1c**−**8c**.

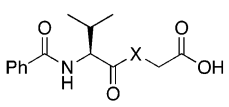
For comparison with the relative rates of bromination of **1a**−**8a**, and the relative ease of formation of the radicals **1c**−**8c** in those reactions, RSEs for the radicals **3c**, **4c**, and **5d**−**8d** were determined (Table 1, part B). The RSEs relate the stability of the radicals in question to that of methyl radical (relative to the corresponding closed-shell species), with a more stable radical having a more positive RSE.[45] The geometries and zero-point vibrational energies were determined at the B3-LYP/6-31G(d) level, while improved relative energies were obtained by carrying out single-point calculations on these optimized structures at the RMP2 level with the 6-311+G(2df,p) and G3large basis sets.[46] The results quoted in Table 1, part B, correspond to RMP2/G3large//B3-LYP/6-31G(d) RSEs at 0 K. The radicals **1c**, **2c**, and **5c**−**8c** were not studied because their aryl groups would substantially increase the complexity of the calculations. The acetamides **5d**−**8d** were examined instead of the benzamides **5c**−**8c**. The effect of different acyl groups was examined with only **3c** and **4c**, and not **1c** and **2c**. The level of theory used in these calculations is significantly higher than that used in our earlier studies.[37,38] Nevertheless, the results are in good agreement where comparisons are possible. In particular, they show the RSEs of the radicals **3c** and **5d** to differ only slightly (by 0.3 kJ mol^{-1}), consistent with our previous work (which gave a difference of 1.6 kJ mol^{-1}).

Also shown in Table 1 (part C) are kinetic parameters for the interactions of the glycine derivatives **1b**, **3b**, **4b**, and **10a**−**14a**, the trifluoroalanine derivatives **9a** and **9b**, the glycolates **7b** and **10b**−**14b**, and the γ-keto acids **15** and **16** with PAM. The data for the glycine derivatives **1b**, **3b**, and **4b**, and the glycolate **7b**, allow for a direct comparison with the rates of bromination of the analogous esters **1a**, **3a**, **4a**, and **7a**, and the stability and ease of formation of the corresponding radicals **1c**, **3c**, **4c**, and **7c**. The properties of the glycine derivatives **10a**−**14a** and the glycolates **10b**−**14b** allow for further analysis of the relationships between these two classes of compounds. It was not feasible to examine the interaction of either the trifluoroalanine derivative **6b** or the γ-keto acid **8b** with PAM, for comparison with the glycine derivative **1b**, due to their poor enzyme-binding affinities. Instead, the effect of these substitutions was explored using the more tightly binding trifluoroalanine derivatives **9a** and **9b**, and the γ-keto acids **15** and **16**, and relating their behavior to that of the analogous glycinated dipeptide derivatives **13a** and **14a**. The data for **1b**, **3b**, **4b**, **10a**, **12a**−**14a**, **13b**, and **14b** have been previously reported.[11,22,42] The trifluoroalanine derivatives **9a**,**b**, the glycolates **7b** and **10b**−**12b**, the dipeptide **11a**, and the γ-keto acids **15** and **16** were prepared as described in the Supporting Information. Their interactions with PAM were assayed using modified literature procedures.[19,22,28] There was no evidence of enzyme-catalyzed reaction for **7b**, **9a**,**b**, **10b**−**12b**, **15**, or **16**. A double-reciprocal plot of 1/*V* vs 1/[S] was used to determine the $K_{M,app}$ value for **11a**, and Dixon plots of 1/*V* vs [I] were used to determine the IC_{50} values for **7b**, **9a**,**b**, **10b**−**12b**, **15**, and **16**.

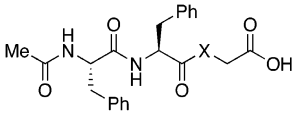


9a diastereomer 1
9b diastereomer 2

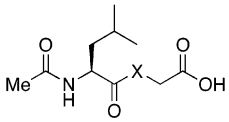
10a X = NH
10b X = O



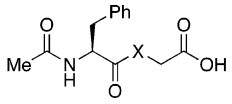
11a X = NH
11b X = O



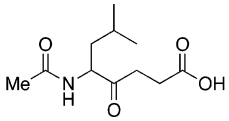
12a X = NH
12b X = O



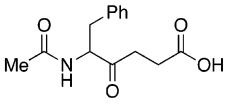
13a X = NH
13b X = O



14a X = NH
14b X = O



15



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Discussion

N-Benzoyl-, pentafluorobenzoyl-, acetyl-, and trifluoroacetylglycine methyl esters **1a**−**4a** differ only in their *N*-acyl substituents. In their reactions with *N*-bromosuccinimide, the benzamide **1a** is 4 times more reactive than the pentafluorobenzamide **2a**, and the acetamide **3a** is 24 times more reactive than the trifluoroacetamide **4a** (Table 1, part A). These relative reactivities can be attributed to differences between the π-electron-donating abilities of the various amido groups, to stabilize and therefore facilitate formation of the corresponding radicals **1c**−**4c**. The inductively electron-withdrawing fluorines of the pentafluorobenzamide **2a** and the trifluoroacetamide **4a** decrease the extent of resonance stabilization and rates of formation of the radicals **2c** and **4c**, relative to those radicals **1c** and **3c** derived from the non-halogenated precursors **1a** and **3a**, respectively. This effect of the fluorines is reflected in the calculated RSEs in the cases of the radicals **3c** and **4c**, where the trifluoroacetamide **4c** is less stable than the acetamide **3c** by 9.2 kJ mol^{-1} (Table 1, part B). The fluorines also exert an inductive effect that decreases the pK_as of the carboxylic acid analogues of the amido groups, due to stabilization of the corresponding carboxylate anions (the pK_as of benzoic acid, pentafluorobenzoic acid, acetic acid, and trifluoroacetic acid are 4.2, 1.5, 4.8, and 0.6, respectively[47]). As a result, there is a strong correlation between the effect of the fluorines on the relative

(44) Burgess, V. A.; Easton, C. J.; Hay, M. P. *J. Am. Chem. Soc.* **1989**, *111*, 1047−1052.
(45) See, for example: (a) Parkinson, C. J.; Mayer, P. M.; Radom, L. *Theor. Chem. Acc.* **1999**, *102*, 92−96. (b) Parkinson, C. J.; Mayer, P. M.; Radom, L. *J. Chem. Soc., Perkin Trans. 2* **1999**, 2305−2313. (c) Henry, D. J.; Parkinson, C. J.; Mayer, P. M.; Radom, L. *J. Phys. Chem.* **2001**, *105*, 6750−6756.
(46) These levels of theory have been found to be suitable for the calculation of radical stabilization energies: see ref 45c.
(47) Krygowski, T. M.; Guilleme, J. *J. Chem. Soc., Perkin Trans. 2* **1982**, 531−534.

reactivity of the glycine derivatives (**1a**−**4a**), the relative acidity of the acids, and the RSEs of the radicals **3c** and **4c**. The effect of the fluorines on the reactivity of **1a**−**4a** and the acidity of the corresponding carboxylic acids is greatest with the trifluoroacetamide **4a** and trifluoroacetic acid, relative to the acetamide **3a** and acetic acid, respectively.

In analogous systems, the fluorines have a related impact on the interactions of *N*-acetylglycine **3b** and *N*-trifluoroacetylglycine **4b** with PAM. They decrease the overall rate of turnover ($V_{M,app}$) of **4b** relative to **3b**, via the corresponding glycyl radicals, by a factor of 4.5 (Table 1, part C). Consequently, the correlation of the differences between the RSEs of the *N*-acetyl- and trifluoroacetyl-glycyl radicals **3c** and **4c**, the relative rates of bromination of *N*-acetyl- and trifluoroacetyl-glycine methyl esters **3a** and **4a**, and the pK_as of acetic and trifluoroacetic acids extends to the relative rates of the enzyme-catalyzed reactions of *N*-acetyl- and trifluoroacetyl-glycine **3b** and **4b**. The fluorines reduce the RSE of the radical **4c** (by 9.2 kJ mol^{-1}), the rate of bromination of the ester **4a** (by a factor of 24), the pK_a of trifluoroacetic acid (by 4.2 units), and the rate of turnover of the acid **4b** by the enzyme (by a factor of 4.5). By way of comparison, replacing the acetyl substituent of *N*-acetylglycine methyl ester **3a**, *N*-acetylglycine **3b**, and acetic acid with the benzoyl group of *N*-benzoylglycine methyl ester **1a**, *N*-benzoylglycine **1b**, and benzoic acid has relatively little effect on any of these properties. It decreases the rate of bromination of the ester **1a** by a factor of only 1.2, decreases the pK_a of benzoic acid by only 0.6, and changes the rate of the enzyme-catalyzed reaction of the acid **1b** by less than 2%.

The effect of the fluorines, although reducing the rate of turnover, is clearly insufficient to prevent the PAM-catalyzed reaction of *N*-trifluoroacetylglycine **4a**. On the basis of the calculations with the *N*-acetyl- and trifluoroacetyl-glycyl radicals **3c** and **4c**, this indicates that a reduction in the RSE of 9.2 kJ mol^{-1} is not sufficient to stop glycyl radical formation by the enzyme. This is consistent with reductions in the RSEs of alanyl and threonyl radicals relative to those of the corresponding glycyl radicals, of 0.3 and 7−14 kJ mol^{-1}, respectively,[37,39] being too small to block the enzyme-catalyzed reactions of alanine and threonine derivatives.[32] The effect of the trifluoromethyl substituent on the stability of the trifluoroalanyl radical **6d** is much larger, reducing the calculated RSE relative to that of the corresponding glycyl radical **3c** by 39.2 kJ mol^{-1} (Table 1, part B). This substituent also prevents bromination of *N*-benzoyl-*β*,*β*,*β*-trifluoroalanine methyl ester **6a** (Table 1, part A) and the processing of the trifluoroalanine-containing dipeptides **9a**,**b** by PAM (Table 1, part C). *N*-Benzoyl-*β*,*β*,*β*-trifluoroalanine methyl ester **6a** was inert on treatment with *N*-bromosuccinimide, either alone or as a mixture with *N*-benzoylglycine methyl ester **1a**, that nevertheless reacted smoothly to give the corresponding α-bromoglycine derivative. There was no evidence of reaction of either of the trifluoroalanine-containing dipeptides **9a** or **9b** being catalyzed by PAM, even though competitive experiments with (*R*)-tyrosyl-(*S*)-valylglycine established that both **9a** and **9b** bind to the enzyme, albeit with IC_{50} values of around 5 mM (Table 1, part C). It is therefore apparent that the decrease in the RSE brought about by introducing the trifluoromethyl group is sufficient to prevent radical formation by PAM. However, this does not give effective enzyme inhibitors, as the trifluoromethyl substituent prevents tight binding to the enzyme. This is seen from the IC_{50} value of 5 mM for each of the trifluoroalanine derivatives **9a** and **9b**. As an indirect comparison, the $K_{M,app}$ value of the corresponding glycine derivative **14a** is 7.9 μM.

In a manner similar to the effect of introducing the trifluoromethyl group, replacing the glycine moiety with glycolate, which is a simple substitution of the glycine NH by O, reduces the RSE of the glycolyl radical **7d** compared with that of the corresponding glycyl radical **3c** by 34.7 kJ mol^{-1} (Table 1, part B). This can be attributed to the decreased π-electron-donating and increased σ-electron-withdrawing ability of the acetoxy group of **7d** relative to the acetamido substituent of **3c**, as reflected in the RSEs of the radicals $MeCONHCH_2^{\bullet}$ and $MeCO_2CH_2^{\bullet}$ of 41.3 and 17.1 kJ mol^{-1}, respectively.[48] The effect is magnified in the captodatively stabilized[49,50] glycyl and glycolyl radicals **3c** and **7d**, where the extent of the synergy displayed by the acetamido and acetoxy substituents in combination with the carboxyl group corresponds to 17.6 and 7.1 kJ mol^{-1}. These values are based on the differences between the RSEs of $MeCONHCH_2^{\bullet}$ (41.3 kJ mol^{-1}), $^{\bullet}CH_2CO_2Me$ (20.2 kJ mol^{-1}), and **3c** (79.1 kJ mol^{-1}) and $MeCO_2CH_2^{\bullet}$ (17.1 kJ mol^{-1}), $^{\bullet}CH_2CO_2Me$, and **7d** (44.4 kJ mol^{-1}), respectively. The substitution of glycine by glycolate prevents bromination of methyl O^{α}-benzoylglycolate **7a** (Table 1, part A). It also blocks catalysis of the reactions of the glycolates **7b** and **10b**−**14b** by PAM (Table 1, part C). The glycine derivatives **1b** and **10a**−**14a** are all turned over by the enzyme, but there is no evidence of reaction of any of the corresponding glycolates **7b** and **10b**−**14b**. In this case, the substitution does not severely disrupt binding to the enzyme. The IC_{50} and K_I values of the glycolates **7b** and **10b**−**14b** are all in the 0.04−0.5 mM range. The corresponding glycine derivatives **1b** and **10a**−**14a** have $K_{M,app}$ values between 1 μM and 1 mM. Therefore, the glycolates constitute a general class of inhibitors of the enzyme because they do not readily undergo hydrogen atom transfer, yet they bind effectively to PAM.

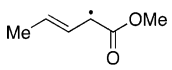
Replacing the acylglycine with a γ-keto acid or ester, through substitution of the glycine NH by CH_2, has effects similar to the swapping of glycine for glycolate. The RSE of the keto ester radical **8d** is less than that of the analogous glycyl radical **3c** by 44.2 kJ mol^{-1} (Table 1, part B). The γ-keto ester **8a** is inert to bromination (Table 1, part A), and the γ-keto acids **15** and **16** are not processed by PAM (Table 1, part C), even though, unlike the example reported previously,[43] these keto acids **15** and **16** do bind to some extent to the enzyme, with IC_{50} values of 6 and 3 mM, respectively. Again it is apparent that a reduction in the RSE of around 35−45 kJ mol^{-1} is sufficient to stop both the bromination with *N*-bromosuccinimide and the catalysis by the monooxygenase.

From the $K_{M,app}$ values of the glycine derivatives **13a** and **14a**, 96 and 7.9 μM, respectively, the K_I values of the corresponding glycolates **13b** and **14b**, 59.8 and 45.2 μM, and the IC_{50} values of the analogous γ-keto acids **15** and **16**, 6 and 3 mM (Table 1, part C), it appears that the replacement of an acylglycine with a γ-keto acid has a more adverse effect on the

(48) RSE values quoted in the text, in addition to those listed in Table 1, were also calculated using the RMP2/G3large level of theory.
(49) Welle, F. M.; Beckhaus, H.-D.; Rüchardt, C. *J. Org. Chem.* **1997**, *62*, 552−558.
(50) Schulze, R.; Beckhaus, H.-D.; Rüchardt, C. *Chem. Ber.* **1993**, *126*, 1031−1038.

binding to PAM than does the substitution of glycine by glycolate. One explanation for this is that both acylglycines and glycolates can form hydrogen bonds with the enzyme, through NH and the corresponding O, respectively, to facilitate binding, but this is not possible with the methylene of an analogous γ-keto acid. An alternative explanation for the less effective binding of the γ-keto acids relates to their greater conformational freedom in free solution. As a result of conjugation of the amide NH of an acylglycine and the analogous ester O of an acylglycolate, in each case there is easy rotation only around the bonds to the α-carbon. By contrast, with a γ-keto acid there is easy rotation around the bonds to both the α- and β-carbons. Assuming that the acylglycines, glycolates, and keto acids all bind to PAM with similar orientations, and that the planarity of the amide and ester groups of the acylglycine and acylglycolate is maintained, there is therefore a greater degree of conformational constraint and loss of entropy on binding of a γ-keto acid.

In any event, it is clear that introducing an α-trifluoromethyl substituent into the glycine residue of a PAM substrate, or replacing the acylglycine of a substrate with either a glycolate or a γ-keto-acid, stops catalysis by the enzyme, because it reduces by around 35−45 kJ mol^{-1} the RSE of the radical that would be formed. By contrast, swapping the acylglycine for a β,γ-unsaturated acid is likely to increase the RSE of the radical intermediate by around 21.1 kJ mol^{-1}, based on the values calculated for the acetamido and propenyl radicals **3c** and **17**, 79.1 and 100.2 kJ mol^{-1}, respectively. Thus, it is not surprising that, as outlined in the Introduction, β,γ-unsaturated acids are processed by PAM.[17,23,24]



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Conclusion

In summary, there are strong correlations between calculated radical stabilization energies (RSEs), the ease of formation of glycyl and related radicals in free radical brominations, and the extent of catalysis of the reactions of analogous compounds by peptidylglycine α-amidating monooxygenase (PAM). A decrease in the RSE of around 10 kJ mol^{-1} relative to that of a normal peptide glycyl radical slows but does not stop the rate of radical formation through either bromination or enzyme catalysis. However, reducing the RSE by around 35−45 kJ mol^{-1}, either through introduction of a trifluoromethyl substituent at the α-position or by replacing the acylglycine NH with a glycolate O or a γ-keto acid CH_2, stops both bromination and enzyme processing. Of these changes, only the glycolate substitution does not adversely affect the enzyme binding affinity. Consequently, glycolates are a general class of PAM inhibitors because they do not readily undergo hydrogen atom transfer, yet they bind effectively to the enzyme. We are now beginning to study the associated in vivo effects of this class of compounds, and already we have found that both the glycolates **10b** and **12b** actively reduce the levels of the peptide hormone Substance P produced by rat DRG cells over a 6 h period.[51]

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Supporting Information Available: Details of the procedures and methods used to determine the relative rates of reaction of **1a**−**8a** with *N*-bromosuccinimide, the methods used for the ab initio calculations, the Gaussian archive entries for the UB3-LYP/6-31G(d) optimized structures of derivatives of glycyl and related radicals, calculated RMP2/G3large and RMP2/6-311+G(2df,p) total energies and corresponding RSEs; protocols for the syntheses of **7b**, **9a**,**b**, **10b**, **11a**,**b**, **12b**, **15**, and **16** with studies of their interactions with PAM. This material is available free of charge via the Internet at http://pubs.acs.org.

JA046204N

(51) Easton, C. J.; Inoue, A.; Wright, A. Unpublished results.