Enantioselective α-Hydroxylation of 2-Arylacetic Acid Derivatives and Buspirone Catalyzed by Engineered Cytochrome P450 BM-3

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Biocatalytic processes are becoming increasingly important in organic synthesis[1] due to their unique selectivity advantages over conventional methods.[2,3] Common biocatalytic transformations include enantioselective transesterification,[4] reduction of ketones,[2] Baeyer−Villiger oxidation,[5] and epoxide opening.[6] Recently, biocatalytic hydroxylations have attracted considerable interest.[7,8] Here, we report a variant of cytochrome P450 BM-3 (BM-3; isolated from *Bacillus megaterium*) capable of efficient and highly enantioselective hydroxylation at the alpha position of certain carboxylic and peptide groups.

BM-3 is a well-studied, NADPH-dependent monooxygenase that hydroxylates long-chain fatty acids at the ω-1, ω-2, and ω-3 positions at high rates.[9] BM-3 has provided an evolvable protein framework for obtaining modified or new activities. Rational design and directed evolution approaches have created BM-3 variants with activity on medium-chain fatty acids,[10] high selectivity for 2-hydroxylation of *n*-alkanes such as hexane,[11] activity on aromatic compounds,[12] and the ability to oxidize ethane to ethanol.[13] BM-3 variants were also recently shown to yield human drug metabolites.[14]

2-Aryl-2-hydroxyacetic acid derivatives are pharmaceutical building blocks for semisynthetic penicillins, cephalosporin, and antiobesity agents.[15] Mandelic acid derivatives have been found to act as thrombin inhibitors and anticoagulants.[16] Due to the high level of interest in these compounds, a number of methods have been developed for their synthesis in the enantiomerically pure form.[17] These methods often use racemic substrates or intermediates for resolution or selective hydrolysis rather than direct enantioselective hydroxylation at the targeted position. No cytochrome P450 that accepts 2-arylacetic acids as substrates is reported in the literature. α-Hydroxylation of carboxylic acids has only been reported for P450 BSβ and SPα, both of which are active only on long-chain fatty acids (e.g., myristic acid) and only as peroxygenases.[18] We were therefore interested in whether BM-3 variants could hydroxylate small aromatic carboxylic acids.

Observations in our laboratory suggested that small, charged molecules such as carboxylic acids are unlikely to be accepted by the hydrophobic active site of BM-3. Recent studies have shown that protecting groups can strongly influence P450 activity.[8,19] Therefore, using different-sized ester groups to mask the acid, we investigated whether the chain length of the substrate influences binding and specificity of hydroxylation.

BM-3 shows low activity with moderate enantioselectivity on 2-phenylacetic acid esters (Table 1). For example, the methyl and propyl esters of (S)-mandelic acid were obtained in 90 and 82% enantiomeric excess (ee), respectively. The low total turnover

Table 1. Chiral Product Analysis of P450 Monooxygenase-Catalyzed Hydroxylation of the Indicated Phenylacetic Acid Esters

enzyme	substrate	TTN	% selectivity[a]	% ee (S)
BM-3	**1a** (Me)	105	44	90
	1b (Et)	92	29	70
	1c (Pr)	63	17	82
	1d (Bu)	20	6	71
BM-3-F87A	**1a** (Me)	25	25	43
	1b (Et)	234	86	74
	1c (Pr)	623	99	85
	1d (Bu)	396	88	75
9-10A	**1a** (Me)	102	57	77
	1b (Et)	63	63	52
	1c (Pr)	77	41	85
	1d (Bu)	86	25	95
9-10A-F87A	**1a** (Me)	27	12	39
	1b (Et)	115	75	56
	1c (Pr)	1640	88	93
	1d (Bu)	660	76	89

[a] Regioselectivity for mandelic acid esters, side products due to hydroxylation or hydrolysis of ester chain.

number (TTN; mol product/mol catalyst) for BM-3 likely reflects an active site evolved for the alkyl tails of fatty acids, which leads to increased uncoupling (consuming NADPH cofactor without oxidizing the substrate) for nonnatural substrates.
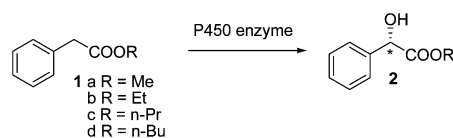
It is known that mutations at F87 can increase BM-3 activity toward small aromatic compounds.[20] We found that the F87A mutation increased the TTN nearly 10-fold and increased regioselectivity for the α-hydroxylated product to as high as 99% on the propyl ester. The ee for this reaction depends on the size of the ester group and varies from 43 to 85% ee, with the highest value for the propyl ester. We also tested variants of BM-3 that were evolved previously for high activity on medium-chain alkanes, 9-10A and 1-12G (differing by 14 and 16 mutations, respectively, from the wild-type sequence),[11] to see if they exhibit higher TTN on substrates **1a**−**d**. Bioconversions with 1-12G showed 5−20-fold less conversion and very low regioselectivity for the desired products in comparison to BM-3-F87A and was therefore excluded from further investigations (data not shown). Variant 9-10A, on the other hand, showed increased TTN on the propyl and butyl esters. We then constructed 9-10A with the F87A mutation and found that this 9-10A-F87A variant has the highest TTN (up to 1640) and gives propyl mandelate in 93% ee. Each 9-10A-F87A

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Table 2. Chiral Product Analysis of P450 Monooxygenase-Catalyzed Hydroxylation of Chlorinated Phenylacetic Acid Esters

enzyme	substrate	TTN	% selectivity[a]	% ee
9-10A-F87A	**3b** (Et)	180	70	57
	3c (Pr)	940	90	89
	3d (Bu)	700	89	94

[a] Regioselectivity for mandelic acid ester derivatives.

enzyme catalyzes the hydroxylation of 254 ± 29 molecules per minute with 25% coupling to cofactor consumption.

9-10A-F87A also hydroxylates substituted substrates such as *m*-chloro phenylacetic acid (Table 2). The ee values are comparable to those obtained for phenylacetic acid, although the highest ee was obtained for the butyl rather than the propyl ester.
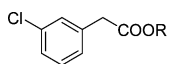

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3 b R = Et
  c R = n-Pr
  d R = n-Bu
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The reaction can be improved with a system that regenerates the expensive NADPH cofactor in situ.[21] The use of a regeneration system involving isocitrate dehydrogenase and isocitrate increased the TTN for the production of propyl mandelate by 9-10A-F87A from 1640 to over 5800 in a 3-h reaction with NADPH concentration maintained at 50 μM. Lower concentrations increased TTN; high concentrations of cofactor inhibit the wild-type enzyme[22] and possibly our variants. The ee and selectivity were unchanged. Using the regeneration system with 500 nM enzyme yielded 96% conversion of 15 mL of 1 mM propyl phenylacetate in a batch reaction after 7 h.

Motivated by the high enantioselectivity and activity of this variant, we decided to test its ability to hydroxylate the α-position of the peptide group of buspirone (Buspar, **4**). Buspirone is a known substrate of human CYP3A4,[23] and both it and its human metabolite, 6-hydroxybuspirone (**5**), are anti-anxiety agents.[24] Bioconversion on 0.5 mL scale with 50 nM 9-10A-F87A gave 3800 TTN and 8.9% conversion of a 2 mM buspirone solution. (*R*)-6-hydroxy-buspirone (**5**) was the sole product and was obtained with >99.5% ee. Interestingly, this *R* enantioselectivity is the opposite of that usually observed when buspirone is converted by natural microbial cultures.[24] A 7-h bioconversion with cofactor regeneration yielded up to 72% conversion. Thus, the bacterial P450 variant efficiently produces an authentic human metabolite of the drug.
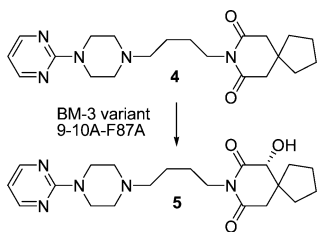
The F87A mutation likely carves out space in the BM-3 active site and allows a wider range of substrates to be bound with a defined orientation. By varying the chain length of the ester substrates, we can improve catalyst selectivity and probe the active site. Enantioselective hydroxylation in the α-position of carboxylic acid or peptide derivatives represents a novel reaction type by P450 BM-3 and opens up a new biocatalytic route to (*S*)-mandelic acid derivatives and (*R*)-6-hydroxybuspirone.

Supporting Information Available: Full experimental details (PDF), and full author list for ref 17d. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) de Meijere, A.; Diederich, F. *Metal-catalyzed Cross-Coupling Reactions*; Wiley-VCH: Weinheim, 2004.
(2) Bornscheuer, U. T.; Kaslauskas, R. J. *Hydrolases in Organic Synthesis*; Wiley-VCH: Weinheim, 1999.
(3) (a) Faber, K. *Biotransformations in Organic Chemistry*, 4th ed.; Springer: Berlin, 2000. (b) Drauz, K.; Waldmann, H.; Roberts, S. M. *Enzyme Catalysis in Organic Synthesis*; Wiley-VCH: Weinheim, 2002. (c) Koeller, K. M.; Wong, C. H. *Nature* **2001**, *409*, 232−240.
(4) (a) Hummel, W. *Adv. Biochem. Eng. Biotechnol.* **1997**, *58*, 145−184. (b) Martin-Matute, B.; Edin, M.; Bogar, K.; Kaynak, F. B.; Bäckvall, J. E. *J. Am. Chem. Soc.* **2005**, *127*, 8817−8825. (c) Pamies, O.; Bäckvall, J. E. *Curr. Opin. Biotechnol.* **2003**, *14*, 407−413.
(5) (a) Reetz, M. T.; Brunner, B.; Schneider, T.; Schulz, F.; Clouthier, C. M.; Kayser, M. M. *Angew. Chem., Int. Ed.* **2004**, *43*, 4075−4078. (b) Alphand, V.; Carrea, G.; Wohlgemuth, R.; Furstoss, R.; Woodley, J. M. *Trends Biotechnol.* **2003**, *21*, 318−323.
(6) Kroutil, W.; Mischitz, M.; Faber, K. *J. Chem. Soc., Perkin Trans.* **1997**, *1*, 3629−3636.
(7) (a) Cirino, P. C.; Arnold, F. H. *Angew. Chem., Int. Ed.* **2003**, *42*, 3299−3301. (b) Meunier, B.; de Visser, S. P.; Shaik, S. *Chem. Rev.* **2004**, 104, 3947−3980.
(8) (a) Munzer, D. F.; Meinhold, P.; Peters, M. W.; Feichtenhofer, S.; Griengl, H.; Arnold, F. H.; Glieder, A.; de Raadt, A. *Chem. Commun.* **2005**, 2597−2599. (b) Li, Z.; Chang, D. *Curr. Org. Chem.* **2004**, *8*, 1647−1658.
(9) (a) Narhi, L. O.; Fulco, A. J. *J. Biol. Chem.* **1986**, *261*, 7160-7169. (b) Schwaneberg, U.; Schmidt-Dannert, C.; Schmitt, J.; Schmid, R. D. *Anal. Biochem.* **1999**, *269*, 359−366.
(10) Li, Q. S.; Schwaneberg, U.; Fischer, M.; Schmitt, J.; Pleiss, J.; Lutz-Wahl, S.; Schmid, R. D. *Biochim. Biophys. Acta* **2001**, *1545*, 114−121.
(11) Peters, M. W.; Meinhold, P.; Glieder, A.; Arnold, F. H. *J. Am. Chem. Soc.* **2003**, *125*, 13442−13450.
(12) (a) Appel, D.; Lutz-Wahl, S.; Fischer, P.; Schwaneberg, U.; Schmid, R. D. *J. Biotechnol.* **2001**, *88*, 167−171. (b) Li, Q. S.; Schwaneberg, U.; Fischer, P.; Schmid, R. D. *Chem.−Eur. J.* **2000**, *6*, 1531−1536.
(13) Meinhold, P.; Peters, M. W.; Chen, M. M.; Takahashi, K.; Arnold, F. H. *ChemBioChem* **2005**, *6*, 1765−1768.
(14) Otey, C. R.; Bandara, B.; Lalonde, J.; Takahashi, K.; Arnold, F. H. *Biotechnol. Bioeng.* **2006**, *93*, 494−499.
(15) (a) Saravanan, P.; Singh, V. K. *Tetrahedron Lett.* **1998**, *39*, 167−170. (b) Furlemmeier, A.; Quitt, P.; Vogler, K.; Lanz, P. U.S. Patent 3,957,758, 1976. (c) Mill, J.; Schmiegel, K. K.; Sha, W. N. U.S. Patent 4,391,826, 1983. (d) Coppola, G. M.; Schuster, H. F. *alpha-Hydroxy Acids in Enantioselective Syntheses*; Wiley-VCH: Weinheim, 1997.
(16) (a) Inghardt, T.; Johansson, A.; Svensson, A. PCT Int. Appl. WO 2002044145, 2002. (b) Inghardt, T.; Nyström, J. E. PCT Int. Appl. WO 2000042059, 2000.
(17) (a) Campbell, R. F.; Fitzpatrick, K.; Inghardt, T.; Karlsson, O.; Nilsson, K.; Reilly, J. E.; Yet, L. *Tetrahedron Lett.* **2003**, *44*, 5477−5481. (b) Yamamoto, K.; Oishi, K.; Fujimatsu, I.; Komatsu, K. I. *Appl. Environ. Microbiol.* **1991**, *57*, 3028−3032. (c) Huang, H. R.; Xu, J. H.; Xu, Y.; Pan, J.; Liu, X. *Tetrahedron: Asymmetry* **2005**, *16*, 2113-2117. (d) DeSantis, G.; et al. *J. Am. Chem. Soc.* **2002**, *124*, 9024−9025. (e) Huerta, F. F.; Laxmi, Y. R.; Bäckvall, J. E. *Org. Lett.* **2000**, *2*, 1037−1040. (f) Wang, P. Y.; Tsai, S. W. *Enzyme Microb. Technol.* **2005**, *37*, 266−271.
(18) (a) Lee, D. S.; Yamada, A.; Sugimoto, H.; Matsunaga, I.; Ogura, H.; Ichihara, K.; Adachi, S.; Park, S. Y.; Shiro, Y. *J. Biol. Chem.* **2003**, *278*, 9761−9767. (b) Matsunaga, I.; Sumimoto, T.; Ueda, A.; Kusunose, E.; Ichihara, K. *Lipids* **2000**, *35*, 365−371.
(19) Lussenburg, B. M.; Babel, L. C.; Vermeulen, N. P.; Commandeur, J. N. *Anal. Biochem.* **2005**, *341*, 148−155.
(20) Sulistyaningdyah, W. T.; Ogawa, J.; Li, Q. S.; Maeda, C.; Yano, Y.; Schmid, R. D.; Shimizu, S. *Appl. Microbiol. Biotechnol.* **2005**, *67*, 556−562.
(21) Schwaneberg, U.; Otey, C.; Cirino, P. C.; Farinas, E.; Arnold, F. H. *J. Biomol. Screening* **2001**, *6*, 111−117.
(22) Murataliev, M. B.; Feyereisen, R. *Biochemistry* **2000**, *39*, 12699−12707.
(23) Zhu, M.; Zhao, W.; Jimenez, H.; Zhang, D.; Yeola, S.; Dai, R.; Vachharajani, N.; Mitroka, J. *Drug. Metab. Dispos.* **2005**, *33*, 500−507.
(24) Hanson, R. L.; Parker, W. L.; Brzozowski, D. B.; Tully, T. P.; Liu, M.; Kotnois, A.; Patel, R. N. *Tetrahedron: Asymmetry* **2005**, *16*, 2711−2716.

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