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Biohydroxylation Reactions Catalyzed by Enzymes and Whole-Cell Systems

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The biohydroxylation of a number of cyclic substrates (**3–24**) containing aromatic side chains was used to compare substrate specificity and selectivity of hydroxylation using microbial enzymes and whole-cell biocatalysts. In general, the regioselectivity of reaction was remarkably similar between the different catalysts in that little aromatic or benzylic, but significant aliphatic hydroxylation was observed. However, a more detailed investigation of isolated products showed complementary substrate specificity, functional group compatibility, and regioselectivity of hydroxylation. Substrate specificity and regioselectivity could be further modulated by small changes to the nature of the aromatic side chain, which appears to play an important role in substrate recognition.

The hydroxylation of nonfunctionalized hydrocarbon centers using biological systems is recognized as an important biotransformation (*1*). This reaction has traditionally been achieved using whole-cell systems, but as more isolated enzyme systems and their three-dimensional structure are now becoming available, enzymes can be used for catalysis and are particularly important for studying substrate recognition and regio- and stereoselectivity of hydroxylation. The regioselectivity of hydroxylation is of particular importance to these biotransformations, because substrates often have a number of potential hydroxylation sites and thus a very high degree of selectivity is required. In our studies, we have looked at a particular class of cyclic substrates bearing aromatic side chains (**3–24**) and compared the selectivity of hydroxylation in defined enzymatic systems with that of whole-cell systems.

BIOHYDROXYLATIONS USING ISOLATED ENZYMES

The best studied of the cell-free hydroxylase systems is the cytochrome P450cam monooxygenase from *Pseudomonas putida* which catalyzes the hydroxylation of camphor **1** to 5-*exo*-hydroxy camphor **2** (Scheme 1). This enzyme was first reported in the 1960s by Gunsalus and colleagues and has been widely used as a model system because it is easily obtained as a soluble protein from heterologous expression (*2*)

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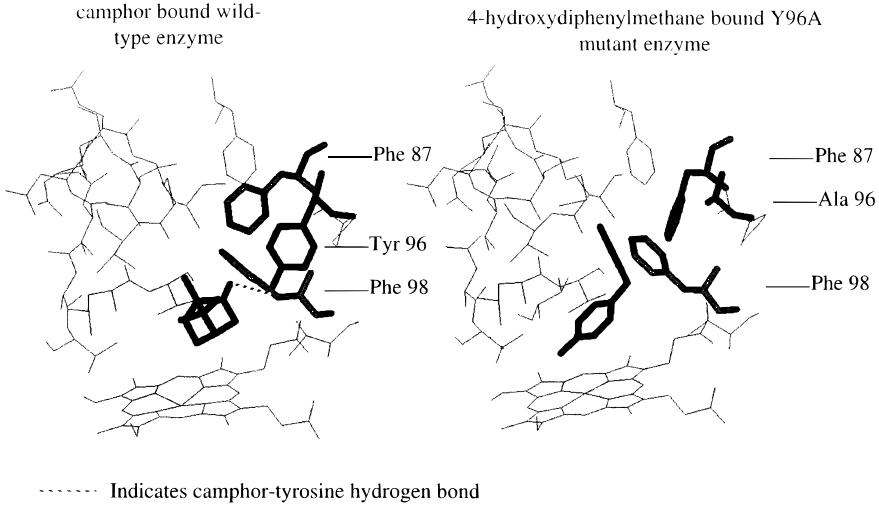
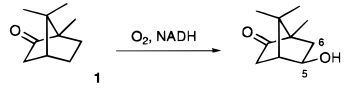


FIG. 1. Active-site residues of cytochrome P450cam monooxygenase. (Left) Wild-type enzyme with camphor bound to the active site (*3*); (right) mutant Y96A with 4-hydroxydiphenyl methane bound to the active site (*14*).

and a high-resolution crystal structure is available (*3*). The reaction is catalyzed with remarkable regio-and stereoselectivity in that only 1 of 10 possible hydroxylation products is formed. This selectivity is due to a combination of the shape of the active site which provides multiple van-der-Waals interactions between enzyme and substrate and a single hydrogen bond between the camphor carbonyl group and tyrosine 96 of the enzyme. The importance of the hydrogen bond has been established by mutagenesis of tyrosine 96 and studies with substrate analogues lacking the carbonyl group (*4*).

In the context of applications to biocatalysis, P450cam suffers from very narrow substrate specificity. It therefore provides an ideal target for redesign by site-directed mutagenesis. Our goal was to broaden the substrate specificity but at the same time retain regioselectivity of hydroxylation. We were particularly interested in aromatic substrates, such as polyaromatic compounds and substrates with aromatic side chains, because of potential applications in bioremediation (*5*) and in the synthesis of chiral intermediates (*6–10*), respectively.

Investigation of the active site of P450cam (Fig. 1, left) suggests that removal of tyrosine 96 would both enlarge the substrate pocket and perhaps even provide an aromatic binding site, since tyrosine 96 in the wild-type structure is flanked by two phenylalanine residues (Phe87 and Phe98, Figure 1), which might provide aromatic



SCHEME 1. Hydroxylation of camphor using P450cam monooxygenase from *Pseudomonas putida.*

stacking interactions. With this in mind, a number of single-site mutants at position 96 were generated, of which the alanine mutant Y96A was the most interesting and hence was studied in most detail (*11*,*12*).

First, simple aromatic compounds such as naphthalene and diphenylmethane (**3**) were tested as substrates. The hydroxylation of naphthalene was much more efficient with the Y96A mutant than with wild-type enzyme (*13*), and diphenylmethane **3** was almost exclusively converted to 4-hydroxy diphenylmethane (Table 1) by Y96A, whereas no hydroxylation was observed with wild-type enzyme (*11*). No benzylic hydroxylation was observed, which emphasizes that the mode of binding of the substrate to the enzyme active site can be more important for selectivity than C–H bond strength.

Thus, the type of substrate binding we observed with the Y96A mutant (Table 1) suggested to us that removal of the tyrosine aromatic side chain had indeed created a binding pocket for aromatic rings as predicted, where the substrate now takes the place of the former protein tyrosine side chain. To verify this, we conducted crystallographic studies on the Y96A mutant. We have recently obtained the structure of the mutant Y96A with 4-hydroxy diphenylmethane bound to the active site to 1.9 Å resolution in collaboration with Malcolm Walkinshaw at Edinburgh University (*14*). These preliminary studies have revealed that, perhaps not surprisingly, some significant rearrangement of active-site residues occurs. Comparison of the wild-type with the Y96A mutant shows that most active-site residues remain in the same place, but very importantly that the phenylalanine 87 side chain has moved into the original site of tyrosine 96 (Fig. 1, right). Thus, the mutation has increased the size of the substrate binding pocket which now appears to be lined at the bottom, away from the active site, with aromatic side chains (Phe 87 and 98). Thus, rather than, as predicted, providing an aromatic pocket at the previous site of tyrosine 96, Phe 98 appears to be stacking against aromatic side chains of the substrate, resulting in regioselectivity of hydroxylation. We are currently investigating cocrystals of Y96A with other substrates to see if this change of active residue orientation is generally observed.

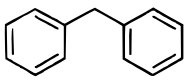
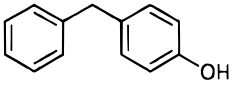
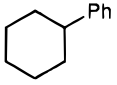
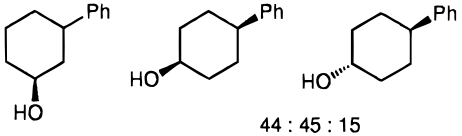
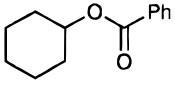
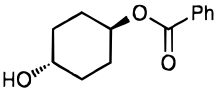
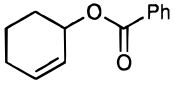
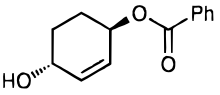
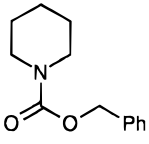
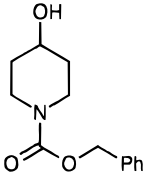
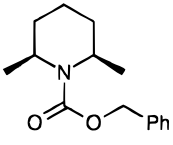
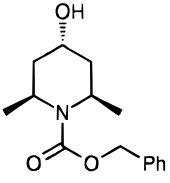
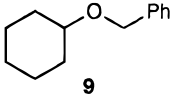
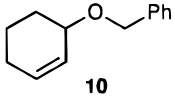
Further evidence for this aromatic stacking interaction comes from studies of the mutant Y96A with substrates **4–9** (Table 1), where no aromatic hydroxylation was observed, but good regioselectivity of hydroxylation of the aliphatic part of the molecule occurred. However, this can also be explained by favorable van-der-Waals interactions of the aliphatic part of the substrate with aliphatic protein side chains (Val 244, Leu 247, Val 295) closer to the active site.

In general, substrates **3–9** in Table 1 demonstrate that the substrate range for hydroxylation has been greatly expanded by the single mutation of tyrosine 96, in particular since none of these compounds bind to wild-type enzyme. The aromatic side chain that is necessary for good binding to the enzyme does not need to be part of the desired substrate structure, but can be a protecting group as in **5–9**, which can be cleaved off after hydroxylation. This allows for the enzyme to catalyze the hydroxylation of functionalized cyclohexane (**5,6,9**) and piperidine (**7,8**) derivatives, which themselves are not substrates.

Another advantage for using aromatic protecting groups in these biotransformations is that they can be used as a further tool to manipulate regioselectivity of hydroxylation.

TABLE 1

Hydroxylation Products Isolated from Incubation of Substrates with the Y96A Mutant of P450cam Monooxygenase from *Pseudomonas putida*

SUBSTRATE	MAJOR PRODUCT(S)
 **3**	
 **4**	 44 : 45 : 15
 **5**	
 **6**	
 **7**	
 **8**	
 **9**	Mixture of products identified by gas chromatography, but not isolated
 **10**	No products observed by gas chromatography

Note. The relative configuration of the stereocenters in products is as indicated. However, absolute configurations have not been determined.

For example, the benzoyl ester of cyclohexanol **5** is selectively oxidized to the 4-hydroxyl derivative, whereas the benzyl ether **9** leads to a mixture of products, or the benzoyl protected cyclohexenol **6** gives the allylic hydroxylation product, whereas incubation of the benzyl ether **10** with Y96A resulted in recovered starting material. It appears that a certain distance between the aromatic side chain and the hydroxylation site is important in these substrates to ensure good selectivity of hydroxylation as comparison of **4** and **5** shows, where **4** gives 44% of 3-hydroxylated product and **5** gives almost exclusively 4-hydroxylated material.

A great practical problem with using isolated enzyme systems for biotransformations is the requirement for coenzymes and cofactors. For example, in the case of cytochrome P450cam monooxygenase discussed here, it is necessary to add the corresponding ferredoxin, ferredoxin reductase, and NADH as a source of electrons. These reactions are therefore only being conducted on a milligram scale and it would be expensive to scale them up much further. In the future, it may be possible to bypass electron transport proteins by driving the reaction electrochemically (*15*), but in the meantime the use of whole-cell systems, whether recombinant (*16*) or in the natural microorganism, is much more practical for preparative-scale biohydroxylations. A few whole-cell systems which we have investigated in our group are discussed below.

BIOHYDROXYLATIONS USING *B. bassiana*

The entomopathogen *Beauveria bassiana* ATCC 7159 (redesignated from *Sporotrichum sulfurescens*) has been used for a number of biotransformations. Of particular interest to us were studies by Fonken and Johnson at Upjohn Company (*6,10*) on the selective hydroxylation of *N*-benzoyl alkylpiperidines to give some optically active hydroxy alkylpiperidines. This work led to the proposal of a stereochemical model for hydroxylation of these amides, which placed particular importance on the distance between the amide oxygen and the hydroxylation site (*1*). Interestingly, our studies with the isolated enzyme, the cytochrome P450cam mutant Y96A, had given similar products of hydroxylation of the Cbz protected piperidine derivatives **7** and **8** to those observed by Johnson, but modeling studies of the substrates at the active site of the mutant using the available structural data (*17*) would suggest that no protein side chain is suitably placed to form a hydrogen bond with the carbonyl oxygen of the Cbz group. Rather, the size and flexibility of the aromatic substrate protecting group appear to determine regioselectivity of hydroxylation. The comparison of *N*-carboxybenzoyl protected piperidine derivatives with benzoyl protected piperidines using the *B. bassiana* whole-cell hydroxylation system would therefore give us more information on the importance of the aromatic side chain, since the carbonyl group in both substrate classes is placed at the same distance to the different potential hydroxylation sites of the ring. In addition, we were interested to see if the Cbz group was more compatible with whole-cell biotransformations, since it is a much more convenient protecting group for amines than the *N*-benzoyl group.

A comparison of the hydroxylated products from both series of substrates (Cbz and benzoyl protected) is shown in Table 2 (*17*). The protected parent piperidine derivatives **7** and **11** gave both 4-hydroxylated products, but with several of the alkyl substituted piperidines a significant change in the regioselectivity of hydroxylation was observed. Thus, while biohydroxylation of *N*-benzoyl 4-methyl piperidine **13**