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Titration and Assignment of Residues that Regulate the Enantioselectivity of Phenylacetone Monooxygenase

Francesca Zambianchi,[a] Marco W. Fraaije,[b] Giacomo Carrea,[a] Gonzalo de Gonzalo,[c] Cristina Rodríguez,[c] Vicente Gotor,[c] and Gianluca Ottolina[a,*]

[a] Istituto di Chimica del Riconoscimento Molecolare, CNR, via Mario Bianco 9, 20131, Milano, Italy
Fax: (+39)-(0)22-890-1239; e-mail: gianluca.ottolina@icrm.cnr.it
[b] Laboratory of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
[c] Departamento de Química Orgánica e Inorgánica, Universidad de Oviedo, c/Julian Clavería, 33006, Oviedo, Spain

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Abstract: Phenylacetone monooxygense (PAMO) from *Thermobifida fusca* was employed for the asymmetric oxidation of thioanisole (sulfooxidation) and of racemic 2-phenylpropionaldehyde (Baeyer–Villiger oxidation). A pH dependence of enantioselectivity was observed in both cases. Two different residues, with pK_a values of 7.8 ± 0.2 and 9.2 ± 0.2, appeared to be responsible for the pH effects on PAMO enantioselectivity. The protonation of Arg337 and the FAD:C4a-hydroperoxide/FAD:C4a-peroxide equilibrium were identified as the major factors responsible for the fine-tuning of PAMO enantioselectivity in Baeyer–Villiger oxidation and sulfooxidation, respectively.

Keywords: biocatalysis; chirality; enantioselectivity; monooxygenase; oxidation; pH

In enzymatic reactions the proper protonation state of catalytically important residues is required for efficient catalysis to occur.[1] The role of these residues has been unveiled by, *inter alia*, structural studies combined with site-directed mutagenesis experiments. As an example, by means of NMR experiments, the histidine residue present in the catalytic dyad His-Asp of glucose 6-phosphate dehydrogenase of *Leuconostoc mesenteroides* has been clearly pinpointed and titrated, and its pK_a value simply obtained by fitting the chemical shift values with the Henderson–Hasselbach equation.[2] The protonation state might also influence the stereochemical outcome of the products. In fact, a pH dependence of stereoselectivity is conceivable for enzymes that have, in the active site, acidic or basic groups, which must be either charged or uncharged during the enzyme-catalyzed reactions. Since the protonation of these groups depends on their pK_a values, the variation of reaction pH would modify the active site microenvironment,[1] leading then to a variation of enantioselectivity.

Actually, a pH dependence of enantioselectivity has been described in several cases, but these studies have not given any indication on the residues involved in the modification of enzyme selectivity.[3] In other cases, models capable to correlate enantioselectivity and kinetic constants (K_M and V_{max}) at different pHs have been proposed for enzymatic transformations; however, fitting the experimental results has been quite cumbersome and problematic.[4]

Baeyer–Villiger monooxygenases are an interesting class of enzymes belonging to the family of flavin-dependent monooxygenases which are able to oxidize several compounds such as ketones, organic sulfides and amines.[5] These enzymes are gaining interest in synthetic organic chemistry since they are able to use environmentally benign molecular oxygen to catalyze the oxidation of substrates in an asymmetric manner.[6] Numerous microorganisms produce these enzymes, and amid them, the moderate thermophilic bacterium *Thermobifida fusca* expresses an appealing Baeyer–Villiger monooxygenase: phenylacetone monooxygense (PAMO; EC 1.14.13.92).[7] The structure of this monomeric enzyme, which was recently resolved (Figure 1, PDB 1W4X), displays some remarkable features: i) a two-domain architecture resembling that of the disulfide oxidoreductases and ii) an arginine residue that lays above the flavin ring in a position suited to stabilize the negatively charged flavin peroxide and Criegee intermediates.[8]

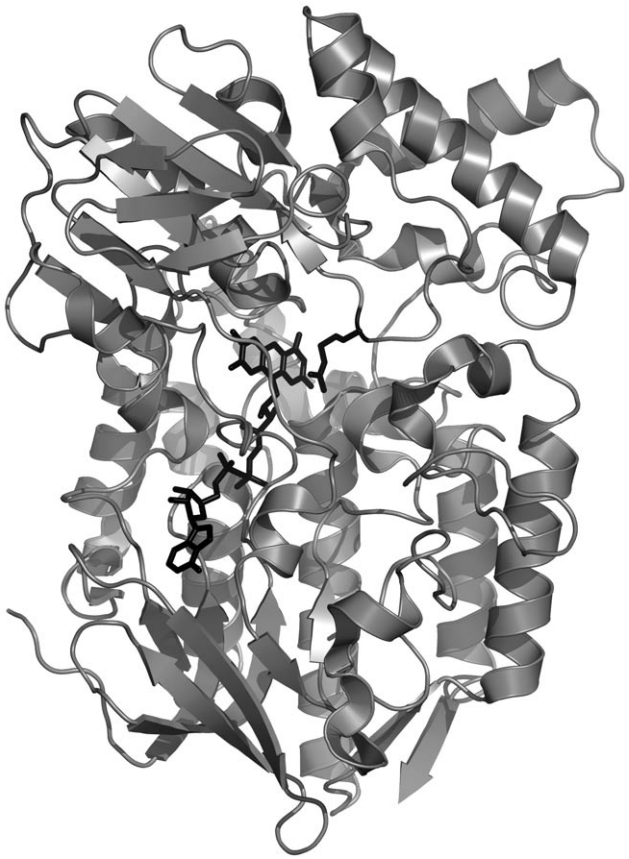
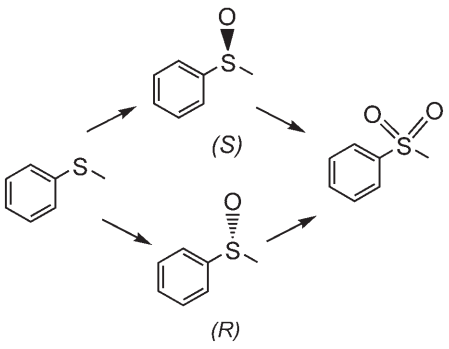
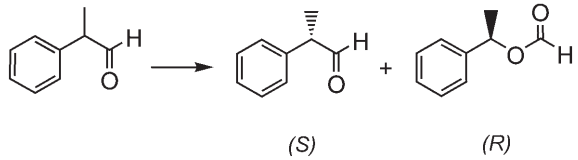


Figure 1. Model of the three-dimensional structure of PAMO (FAD and Arg337 in black).

As part of a long-term program aimed at exploiting the use of oxidative enzymes in synthetic organic chemistry, we recently became interested in the use of PAMO as a catalyst for the asymmetric oxidation of organic sulfides and ketones.[9] In the present work we have investigated the effect of pH on the enantioselectivity of PAMO, using as substrates thioanisole (methyl phenyl sulfide) (Scheme 1) and 2-phenylpropionaldehyde (Scheme 2). In the oxidation of thioanisole to the corresponding (*R*)-sulfoxide, the enantio-



Scheme 1. Pathway of the PAMO-catalyzed sulfoxidation of thioanisole to methyl phenyl sulfoxide and methyl phenyl sulfone.



Scheme 2. Enantioselective PAMO-mediated Baeyer–Villiger oxidation of racemic 2-phenylpropionaldehyde to (*S*)-formic-acid 1-phenylethyl ester.

meric excess (*ee*) values increased from 9.6 to 44.9% by raising the pH from 6 to 10 (Figure 2). The enantiomeric excess values were obtained from several

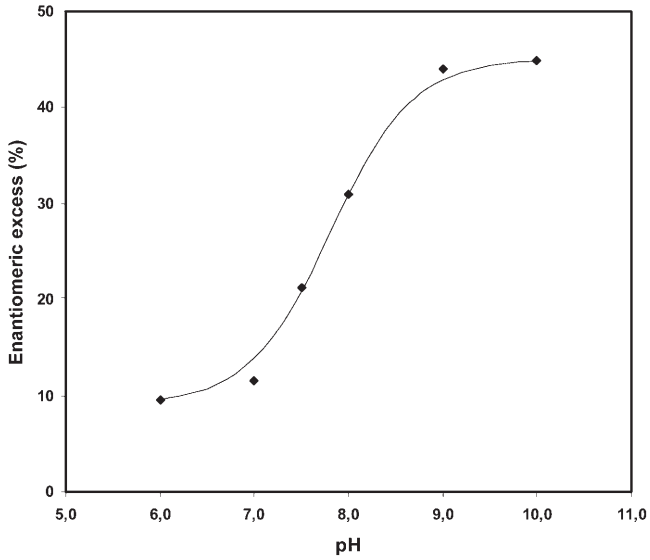


Figure 2. pH dependence of enantiomeric excess (*ee*) of the product (*R*)-methyl phenyl sulfoxide in the PAMO oxidation of thioanisole. The pH values remained constant throughout the entire experimental period. The correlation coefficient of the curve was 0.996.

measurements, at different degrees of conversion (5–100%). It should be mentioned that the asymmetric oxidation of thioanisole to sulfoxide was accompanied by the overoxidation to achiral sulfone (Scheme 1). The second oxidation step was not enantioselective as demonstrated by: i) the *ee* values of the (*R*)-sulfoxide did not change as a function of conversion degree, and ii) no kinetic resolution was observed starting from racemic sulfoxide (see Supporting Information). The pH profile of the *ee* values resembled a simple acid/base titration curve (Figure 2) and, therefore, the data were fitted in the acid dissociation equilibrium equation (Henderson–Hasselbach). The pK_a value was determined by the three-parameter fit of the following equation:

$$ee_{obs} = \frac{ee_{HA} + ee_{A^-} \times 10^{pH-pK_a}}{1 + 10^{pH-pK_a}}$$

where ee_{HA} is the enantiomeric excess in the acidic pH regime and ee_A- the enantiomeric excess at basic pH. The fitted pK_a value was found to be 7.8 ± 0.2.

The second studied conversion refers to the PAMO-mediated Baeyer–Villiger oxidation of racemic 2-phenylpropionaldehyde to (*S*)-formic-acid 1-phenylethyl ester which leaves the (*R*)-2-phenylpropionaldehyde mostly unreacted (Scheme 2). Enzyme enantioselectivity, expressed as enantiomeric ratio,[10] showed with the aldehyde substrate an opposite trend with respect to the sulfide substrate as it decreased from 26.5 to 16.7 on raising the pH from 7 to 10 (Figure 3). The data resembled again an acid/base titration curve, and, therefore the pK_a was determined as already described, giving a value of 9.2 ± 0.2.

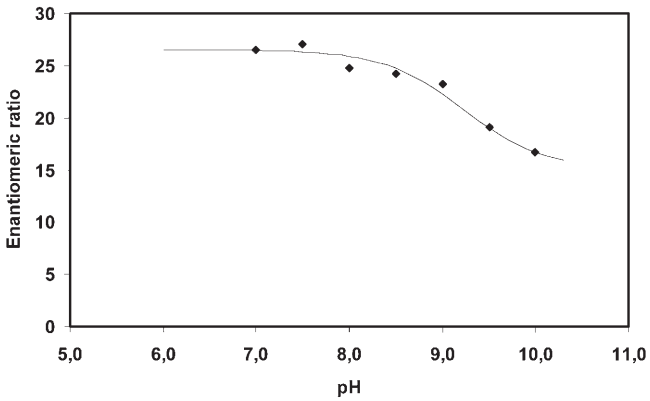


Figure 3. pH dependence of enantioselectivity, expressed as enantiomeric ratio of PAMO in the oxidation of 2-phenylpropionaldehyde. The pH values remained constant throughout the entire experimental period. The correlation coefficient of the curve was 0.986.

Although the two reactions were performed in the same conditions, two different residues seem to be responsible for the pH effects on PAMO enantioselectivity in the oxidation of an electron-rich substrate (sulfide) or an electron-poor substrate (aldehyde).

Nowadays it is possible to predict with good approximation the pK_a of every amino acid in proteins and, among the many methodologies that have been proposed, we have chosen the PROPKA method to calculate pK_a values.[11] A survey on mutations that influence enzyme enantioselectivity has revealed that in most cases mutations closer to the active site are more effective than distant ones.[12] Mutations distant more than 10 Å (Cα) from the key active site atom are generally poorly effective in perturbing enantioselectivity. The same should hold for the protonation states of amino acids: the closer variations might be more effective than the distant ones in perturbing enzyme enantioselectivity. Table 1 provides an inventory of the amino acids with a theoretical pK_a close to the values determined from the titration curves

Table 1. PAMO amino acids with theoretical pK_a values[a] close to 7.8 ± 0.2 or 9.2 ± 0.2.

Amino acid	pK_a	ΔpK_a	Cα-FAD:C_{4a} [Å]
Arg10	7.72	0.08	30.2
His137	7.53	0.27	31.8
Glu341	7.35	0.45	13.0
Cys73	8.31	0.51	11.2
Lys239	9.29	0.09	22.4
Lys336	9.29	0.09	11.0
Cys528	9.34	0.14	23.7
Arg337	9.36	0.16	7.5
Tyr56	8.86	0.34	12.2

[a] The pK_a values were calculated with the PROPKA method[11] (see text).

(smaller ΔpK_a) and their distance[8] from the flavin (Cα-FAD:C_{4a}). For the pK_a 7.8 ± 0.2, Arg10 and His137 are too far away from the flavin (more than 30 Å); instead, Glu341 and Cys73, which are closer to the flavin, have ΔpK_a values that deviate by more than double the standard deviation. Furthermore, Cys73 is on the *si* side of the flavin, which is opposite to the active site, with a distance Cα-FAD:C_{4a} as high as 11.2 Å. Moreover, the side chain of this amino acid is pointing away from the FAD:C_{4a}, with a sulfur-FAD:C_{4a} distance of 13.0 Å.[8] The data suggest that the variation of enzyme enantioselectivity in the oxidation of thioanisole cannot be linked to a specific protonation state of an amino acid close to the active site. However, it should be emphasized that there is another functional group in the active site that can be influenced by variations of pH, that is, the FAD cofactor or, more precisely, the FAD:C4a-hydroperoxide/FAD:C4a-peroxide derivatives. For the closely related enzyme cyclohexanone monooxygenase from *Acinetobacter* sp. NCIB9871, these two intermediates can be interconverted by changing the pH. The interconversion showed a pK_a of 8.4 ± 0.2.[13] This value is quite close to pK_a 7.8 ± 0.2. Therefore, it is tempting to conclude that the observed pK_a of PAMO related to enantioselectivity in sulfoxidations reflects the pK_a of the PAMO FAD:C4a-hydroperoxide/FAD:C4a-peroxide equilibrium.

For the observed pKa of 9.2 ± 0.2, Arg337, which has pKa of 9.36 and is only at 7.53 Å from the flavin, appears of special interest (Table 1). All other amino acids (Lys239, Lys336, Cys528 and Tyr56), are too far from the active site and, thus, unable to influence enzyme selectivity. In PAMO, Arg337 is indispensable for catalysis as demonstrated by the finding that Arg337Ala and Arg337Lys replacements completely suppress the oxygenating activity of the enzyme[14] This residue is a strictly conserved and essential for activity for the other Baeyer–Villiger monooxygenases.[15] As already reported, Arg337 is located inside the active site, on the *re* side of the flavin.[8] Judging

from the crystal structure, Arg337 appears to be able to directly interact with the Criegee intermediate.[8] Furthermore, Arg337 can adopt two alternate conformations, which underlines an inherent flexibility that can be functionally important.[8]

The pH dependence of PAMO stereoselectivity seems to point i) to the equilibrium FAD:C4a-hydroperoxide/FAD:C4a-peroxide and ii) to the protonation state of Arg337 as the two crucial factors for fine-tuning of enzyme stereochemical outcome. This hypothesis is supported by the fact that PAMO carries out the S-oxidation and the Baeyer–Villiger reaction with two distinct mechanisms, that is, electrophilic attack to the heteroatom (S-oxidation) and nucleophilic attack to the carbonyl group (Baeyer–Villiger reaction). The electrophilic attack to electron-rich substrates is the typical reaction of another flavoprotein monooxygenase: 4-hydroxybenzoate 3-monooxygenase, where the protonation of the distal oxygen of the peroxide moiety increases the electrophilic reactivity.[16] In S-oxidation of dimethyl sulfide by lumiflavin, theoretical studies indicated that the lowest activation energy of the transition state was obtained with the complex formed with FAD:C4a-hydroperoxide-water, which suggests that the electrophilic attack is preferred over the nucleophilic one.[17] We think that among FAD:C4a-hydroperoxide, protonated Arg337 and aqueous buffer, FAD:C4a-hydroperoxide mostly influenced the geometry of electrophilic attack to the sulfur atom, and therefore, the stereochemical arrangement of the transition state. Instead, in the Baeyer–Villiger oxidation of 2-phenylpropionaldehyde the stereochemical arrangement of the transition state is mainly influenced by the presence of protonated Arg337.

In conclusion, this study shows for the first time that is possible to take advantage of the enantioselective properties of an enzyme to titrate and assign some residues important for catalysis. The protonation of Arg337 and the FAD:C4a-hydroperoxide/FAD:C4a-peroxide equilibrium were identified as the two major factors responsible for the fine-tuning of PAMO enantioselectivity.

Experimental Section

Recombinant histidine-tagged phenylacetone monooxygenase was overexpressed and purified according to previously described methods.[7] Oxidation reactions were performed using the purified enzyme. One unit of phenylacetone monooxygenase oxidizes 1.0 μmol of thioanisole to methyl phenyl sulfoxide per minute at pH 9 and 25 °C in the presence of NADPH. Glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides* was obtained from Fluka-BioChemika. Glucose 6-phosphate and NADPH were purchased by Sigma–Aldrich. Chiral HPLC analysis: Jasco HPLC instrument (model 880-PU pump, model 870-UV/VS detector) equipped with a Chiracel OD (Daicel) chiral column. Chiral GC analysis: Hewlett–Packard 6890 Series gas chromatograph equipped with a CP-Chirasil-DEX-CB chiral column.

Enzymatic Oxidation of Thioanisole

The reactions were carried out at 27 °C in 1 mL of 50 mM Tris/HCl buffer, pH 6–10, containing 1 mg of thioanisole, 1 mg of glucose 6-phosphate, 0.5 U of PAMO and 10 U of glucose 6-phosphate dehydrogenase. The mixture was shaken at 250 rpm in a rotatory shaker for the times established. The reactions were then stopped, worked up by extraction with dichloromethane (3×0.5 mL), dried over Na_2SO_4 and analyzed by chiral HPLC[6b] in order to determine the conversion and the enantiomeric excesses of the sulfoxide. Standard deviations (average $\geq$ 7 measurements) were below $\pm 1.5\%$. The pK_a value was obtained by non-linear least-square fit of values according to the Henderson–Hasselbach equation.

Enzymatic Oxidation of 2-Phenylpropionaldehyde

The reactions were carried out at 27 °C in 1 mL of 50 mM Tris/HCl buffer, pH 6–10, containing 1 mg of 2-phenylpropionaldehyde, 1 mg of glucose 6-phosphate, 0.5 U of PAMO and 10 U of glucose-6-phosphate dehydrogenase. The mixture was shaken at 250 rpm in a rotatory shaker for the times established. The reactions were then stopped, worked up by extraction with dichloromethane (3×0.5 mL), dried over Na_2SO_4. The *ee* values of the product, from which the enantiomeric ratios were derived,[10] were determined by chiral GC.[18] Standard deviations (average $\geq$ 5 measurements) were below ± 1.5. The pK_a value was obtained by non-linear least-square fit of values according to the Henderson–Hasselbach equation.

Acknowledgements

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