Enzyme Mechanisms

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Extreme Rate Acceleration by Axial Thiolate Coordination on the Isomerization of Endoperoxide Catalyzed by Iron Porphyrin**

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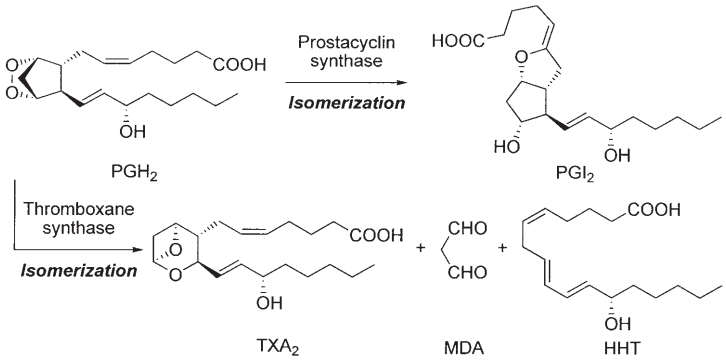
Cytochrome P450s catalyze various types of oxidative reactions involved in steroid biosynthesis, the metabolism of fatty acids, and the detoxification of xenobiotic compounds.[1] Most of the reactions are monooxygenase-type NADPH/NADH- and O_2-dependent ones, but when the endoperoxide (EP) isomerizing P450 works as a prostacyclin synthase or thromboxane synthase it is neither NADPH/NADH- nor O_2-dependent, and is known generally as prostaglandin H_2 (PGH_2) isomerase.[2] Malondialdehyde (MDA) is one of the major products from the isomerization of PGH_2 by thromboxane synthase, and the reaction is accompanied by the formation of 12-hydroxyheptadeca-5,8,10-trienoic acid (HHT, Scheme 1).[3] Prostacyclin (PGI_2) and thromboxane A_2



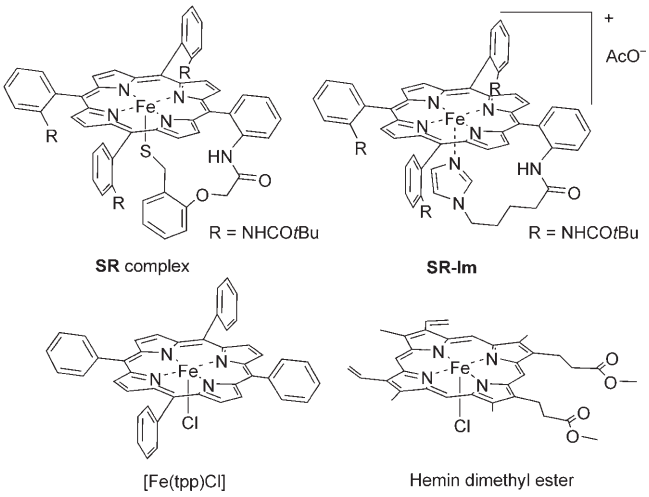
Scheme 1. Isomerization of PGH_2 with cytochrome P450 enzymes.

(TXA_2) are regulatory factors of platelet aggregation as well as of vaso- and bronchodilation and constriction. Both compounds are derived from the common precursor PGH_2 endoperoxide.

Hydroperoxides are widely used as oxidants by heme-containing peroxidases and P450. However, no metalloenzyme other than the P450-type one is known to catalyze reactions involving EPs that do not have active hydrogen atoms. It would be of interest to know why nature has selected the heme-thiolate structure, from the wide range of metal complexes, for the isomerization of EPs. Several proposed reaction mechanisms for the catalysis are based largely on the structure of the product and by analogy with the general mechanism of P450;[3a,4] more direct evidence is needed to test these ideas. PGH_2 isomerases contain the heme-thiolate structure in their active sites, whereas most peroxidases have imidazole-ligated heme structures. Therefore, we focused on the role of the thiolate ligand in the isomerase catalysis. Metalloporphyrins have been extensively investigated as chemical models of P450 and/or peroxidases to understand the catalysis by P450 and peroxidase in the presence of hydroperoxides as oxidants.[5] There have been two reports describing the isomerization of EPs in the presence of synthetic metalloporphyrins,[4,6] but the effect of the axial ligand on the isomerization was not considered.

We have already succeeded in the synthesis of the first synthetic heme thiolate (**SR** complex, Scheme 2), in which thiolate coordination is retained during the catalytic oxidation, and have observed several remarkable effects of the thiolate axial ligand.[7] We report here the first isomerization of an EP catalyzed by a stable synthetic heme-thiolate complex and the large enhancing effect of the thiolate ligand on the reaction.

A simple EP **1**, which is a partial structure of PGH_2, was prepared by modification of the reported method.[9] Reactions of **1** with iron porphyrins were monitored by ^{1}H NMR spectroscopic measurement of samples



Scheme 2. Structures of various heme complexes.

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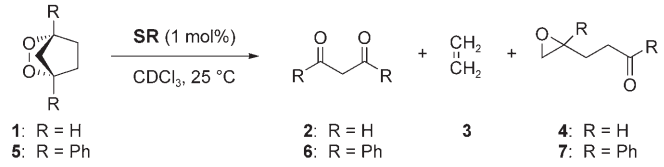
obtained after quenching the reaction by adding coordinative [D_5]pyridine to aliquots of the reaction mixture at regular intervals. In the presence of [Fe(tpp)Cl] or hemin dimethyl ester, **1** was completely unchanged in $CDCl_3$ at 25 °C for 24 h (Table 1). In contrast, **SR** showed extremely high catalytic activity for the isomerization/breakdown of **1** under the same conditions. Most of **1** was converted into malondialdehyde (**2**), ethylene (**3**), and epoxyaldehyde (**4**) within 10 s (Scheme 3). It was confirmed by checking the ESR spectrum of the reaction mixture that most of the **SR** remained intact after the reaction when 10 mol % of **SR** was used, whereas considerable decomposition of the **SR** complex was observed at a lower concentration (1.0 mol %). The **SR-Im** complex,[7b] which is a monoimidazole-ligated iron porphyrin (UV/Vis spectroscopic analysis has already confirmed that **SR-Im** is almost entirely in the monoimidazole form), showed much lower activity than the **SR** complex. The initial rate of isomerization/breakdown by **SR** was almost 1000-fold higher than that by **SR-Im**. The coordination of a chloride anion had no positive effect on the isomerization. This is the first example in which the axial ligand effect of a thiolate on the isomerization of an EP has been unambiguously evaluated. The redox potential (Fe^{III}/Fe^{IV} or the electronic isomer) of **SR** was lower than those of the other complexes. This is also the first report of the redox potential (Fe^{III}/Fe^{IV}) of synthetic heme-thiolate. The ready accessibility of the high-valent iron form of heme thiolate is considered critical for cleavage of the O–O bond of the EP, which has no active hydrogen atom.

Table 1: Comparison of reaction rates, product profiles, and redox potentials with various heme complexes.[a]

Catalyst	Ligand	Endoperoxide	Yields [%][b]			TOF [s^{-1}][c]	Redox potentials Fe^{III}/Fe^{IV} $E_{1/2}$ [V][g]
			2	**3**[d]	**4**		
SR[e]	R-S^-	**1**	40	17	56	10.5	0.69
SR-Im	imidazole	**1**	34	2	44	1.1×10^{-2}	0.86
[Fe(tpp)Cl]	Cl^-	**1**	no reaction				0.84
hemin dimethyl ester	Cl^-	**1**	no reaction				0.77
			6	**3**[d]	**7**		
SR (5.0 mol %)[f]	R-S^-	**5**	33	2	58	3.4×10^{-4}	0.69

[a] Reactions were carried out in $CDCl_3$ at 25 °C for 24 h under Ar. Substrate: 3.5 mM, catalyst: 1.0 mol %. [b] Determined by ^{1}H NMR spectroscopy. [c] Turnover frequency (TOF) was calculated from the initial rate of the reaction. [d] Yields of **3** would have been underestimated because ethylene was gradually released from the solution into the gas phase. [e] Reaction was carried out for 15 min. [f] 1 mol % **SR** isomerized **5** too slowly. [g] Iron porphyrin: 0.1 mM, tetra-*n*-butylammonium perchlorate: 100 mM, solvent: CH_2Cl_2, working electrode: Pt, reference electrode: Ag/AgCl.



Scheme 3. Isomerization/beakdown of EPs with **SR**.

Our previous study showed that the initial rate of 2,4,6-tri-*tert*-butylphenol (TBPH) oxidation with *m*CPBA catalyzed by **SR** was only 70-fold higher than that with **SR-Im**, while [Fe(tpp)Cl] could catalyze the oxidation with RCOOOH. Thus, the difference in the axial ligand effect on the isomerization of EPs is much greater than that on the oxidation with a peroxycarboxylic acid. The remarkable difference in the axial ligand effect provides unambiguous mechanistic insight into the cleavage of the O–O bond by heme (Fe^{III}). **SR** showed much lower activity for the isomerization of EP **5**. This result suggests that steric hindrance around the O–O bond has a major influence on the reaction, and that coordination of the oxygen atom of the EP is necessary for subsequent cleavage of the O–O bond, since the electronic state of the oxygen atoms of **5** would be similar to **1**. No clear solvent effect on the isomerization of **1** with **SR** was observed (see the Supporting Information). This result suggests that the reaction does not involve the formation of a polar intermediate in the rate-determining step. Furthermore, the presence of tetrahydrofuran (at four times the concentration of **1**), which would be more strongly coordinating than **1**, had little effect on the rate of isomerization of **1**. Therefore, it is likely that the rate-determining step is cleavage of the O–O bond, and the large difference in the reaction rate among the iron porphyrins should directly reflect the effect of the axial ligand on the cleavage of the O–O bond. The product profile in the case of **SR-Im** resembled that obtained with **SR**. Interestingly, the same type of conversion of **1** into **2** and **3** occurs in the reactions of PGH_2 with thromboxane synthase, prostacyclin synthase, and cytochrome $P450_{CAM}$.[3]

Spin-trap experiments were then carried out to examine the formation of radical species. Compound **1** was treated with **SR** (2.5 mol %) for 0.5 s at 25 °C in benzene and then 3,3,5,5-tetramethylpyrroline-*N*-oxide (TMPO) was added to the mixture. The ESR spectrum of the reaction mixture clearly showed a sextet signal (Figure 1) that could be assigned to a product derived from an alkoxyl radical, since its hyperfine coupling constants (a_N: 1.32 mT, $a_{\beta H}$: 0.58 mT) were in good agreement with those of the TMPO-*tert*-butoxyl

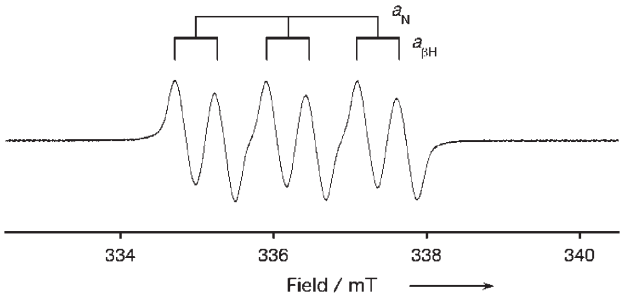
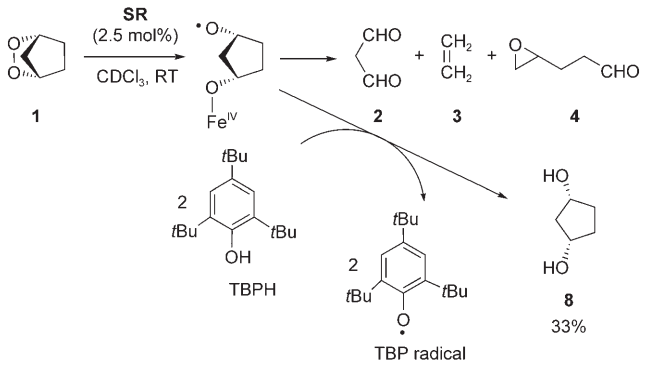


Figure 1. ESR spectrum of the radical adduct with TMPO. The reaction was carried out in benzene at 25 °C. Substrate **1**: 6.0 mM, **SR**: 2.5 mol %, TMPO: 60 mM.

radical adduct (a_N: 1.33 mT, $a_{\beta H}$: 0.58 mT).[10] This is the first direct evidence for the formation of an oxy radical intermediate in the isomerization of an EP catalyzed by heme.

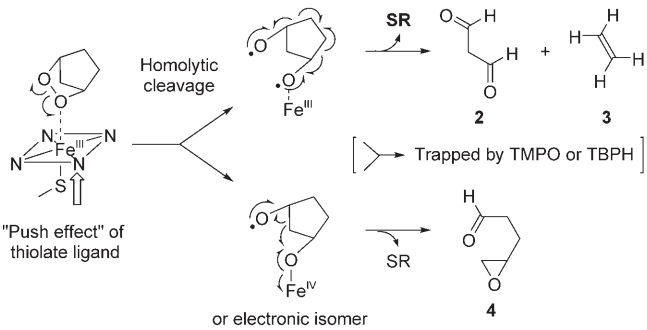
The presence of 10 equivalents of TBPH did not alter the product profile in the isomerization of **1** with **SR** (2.5 mol%). However, the reaction in the presence of 100 equivalents of TBPH afforded 1,3-cyclopentanediol **8** (33%) as well as **2**, **3**, and **4** (Scheme 4)



Scheme 4. Radical trapping with TBPH during the isomerization of **1**.

Differential UV/Vis spectroscopy indicated the formation of TBP$^{\bullet}$ ($\lambda_{max} = 630$ nm). The TBPH would thus reduce the O-radical intermediate and the high-valent iron species to give **8** and TBP$^{\bullet}$. The results suggest that 1) cleavage of the O–O bond occurs prior to cleavage of the C–C bond, and 2) the observed ESR signal is not derived from the radicals formed by direct oxidation of TMPO (only 10 equiv to **1**), because TBPH is a much better substrate for the oxidant than TMPO.

We propose that the mechanism for the isomerization of an EP by heme-thiolate is as follows (Scheme 5). Rapid homolytic cleavage of the O–O bond of the EP coordinated to the iron atom occurs with the assistance of the potent electronic "push effect" of the thiolate ligand.[7a,11] The biradical then cleaves to give products **2** and **3**. An alternative pathway involves rearrangement of the oxy radical coordinated to the heme to afford unsymmetrical product **4**. Heterolysis of the O–O bond would also be a possible reaction pathway for the formation of **4**. The remarkable effect of an axial thiolate ligand on the isomerization of EPs probably arises because cleavage of the O–O bond of the EPs without any active hydrogen atoms is purely dependent on the donation of π electrons from the axial ligand.



Scheme 5. Proposed mechanism for the isomerization of an EP.

In summary, we have found an extremely strong enhancing effect of an axial thiolate ligand on the isomerization of EPs catalyzed by heme in hydrophobic media. This axial ligand effect is the largest found in heme–peroxide reactions. The thiolate ligand is suggested to play a critical role in cleavage of the O–O bond in the absence of an active hydrogen atom, which hydroperoxides have. The present results provide strong evidence for the critical role played by the heme-thiolate structure for the effective function of PGH_2 isomerase in a hydrophobic environment. Further investigation using PGH_2 is in progress.

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[1] a) *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 3rd ed. (Ed.: P. R. Ortiz de Montellano), Kluwer Academic/Plenum Publishers, New York, **2004**; b) M. Sono, M. P. Roach, E. D. Coulter, J. H. Dawson, *Chem. Rev.* **1996**, *96*, 2841–2888; c) I. G. Denisov, T. M. Makris, S. G. Sligar, I. Schlichting, *Chem. Rev.* **2005**, *105*, 2253–2278.

[2] a) V. Ullrich, L. Castle, P. Weber, *Biochem. Pharmacol.* **1981**, *30*, 2033–2036; b) D. L. DeWitt, W. L. Smith, *J. Biol. Chem.* **1983**, *258*, 3285–3293.

[3] a) M. Hecker, V. Ullrich, *J. Biol. Chem.* **1989**, *264*, 141–150; b) S. Hara, A. Miyata, C. Yokoyama, H. Inoue, R. Brugger, F. Lottspeich, V. Ullrich, T. Tanabe, *J. Biol. Chem.* **1994**, *269*, 19897–19903.

[4] V. Ullrich, *Arch. Biochem. Biophys.* **2003**, *409*, 45–51.

[5] a) B. Meunier, A. Robert, G. Pratviel, J. Bernadou in *The Porphyrin Handbook*, *Vol. 4* (Eds.: K. M. Kadish, K. M. Smith, R. Guilard), Academic Press, San Diego, **2000**, pp. 119–187; b) J. T. Groves, K. Shalyaev, J. Lee in *The Porphyrin Handbook*, *Vol. 4* (Eds.: Kadish, K. M. Kadish, K. M. Smith, R. Guilard), Academic Press, San Diego, **2000**, pp. 17–40.

[6] K. Takahashi, M. Kishi, *Tetrahedron* **1988**, *44*, 4737–4746.

[7] a) T. Higuchi, S. Uzu, M. Hirobe, *J. Am. Chem. Soc.* **1990**, *112*, 7051–7053; b) T. Higuchi, K. Shimada, N. Maruyama, M. Hirobe, *J. Am. Chem. Soc.* **1993**, *115*, 7551–7552; c) Y. Urano, T. Higuchi, M. Hirobe, T. Nagano, *J. Am. Chem. Soc.* **1997**, *119*, 12008–12009; d) N. Suzuki, T. Higuchi, Y. Urano, K. Kikuchi, H. Uekusa, Y. Ohashi, T. Uchida, T. Kitagawa, T. Nagano, *J. Am. Chem. Soc.* **1999**, *121*, 11571–11572; e) T. Ohno, N. Suzuki, T. Dokoh, Y. Urano, K. Kikuchi, M. Hirobe, T. Higuchi, T. Nagano, *J. Inorg. Biochem.* **2000**, *82*, 123–125; f) N. Suzuki, T. Higuchi, T. Nagano, *J. Am. Chem. Soc.* **2002**, *124*, 9622–9628; other synthetic heme-thiolate complexes are reviewed in reference [8].

[8] W.-D. Woggon, *Met. Ions Life Sci.* **2007**, *3*, 27–55.

[9] a) W. Adam, H. J. Eggelte, *J. Org. Chem.* **1977**, *42*, 3987–3988; b) D. J. Coughlin, R. G. Salomon, *J. Am. Chem. Soc.* **1977**, *99*, 655–657.

[10] G. J. Edward, V. S. Raghav, M. K. Susan, *Can. J. Chem.* **1981**, *59*, 756–758.

[11] J. H. Dawson, *Science* **1988**, *240*, 433–439.

