

## **Supporting Information**

### **Catalytic Mechanism of Cytochrome P450 for 5'-Hydroxylation of Nicotine: Fundamental Reaction Pathways and Stereoselectivity**

Dongmei Li,<sup>a,b</sup> Xiaoqin Huang,<sup>b</sup> Keli Han,<sup>a,\*</sup> and Chang-Guo Zhan<sup>b,\*</sup>

*<sup>a</sup>State Key Laboratory of Molecular Reaction Dynamics, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, People's Republic of China and <sup>b</sup>Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 789 South Limestone Street, Lexington, Kentucky 40536*

**Running Title:** P450-catalyzed nicotine 5'-hydroxylation

#### **Correspondence:**

Chang-Guo Zhan, Ph.D.

Professor

Department of Pharmaceutical Sciences

College of Pharmacy

University of Kentucky

789 South Limestone Street

Lexington, KY 40536

TEL: 859-323-3943

FAX: 859-323-3575

E-mail: [zhan@uky.edu](mailto:zhan@uky.edu)

**Supporting Information Available:** More information about the energetic results and the detailed reaction pathways; complete citations of refs. 43 and 53. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

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\* Corresponding authors. E-mail: [zhan@uky.edu](mailto:zhan@uky.edu) and [klhan@dicp.ac.cn](mailto:klhan@dicp.ac.cn)

## More information about the energetic results and the detailed reaction pathways

Table S1. Computational binding free energies (in kcal/mol) according to the initial docking and final MM-PBSA calculations

	SR <sub>t</sub>	SR <sub>c</sub>	SS	SRH	SSH	SRHH	SSHH
Docking	-5.44	-4.43	-5.28	-5.32	-5.64	-5.22	-5.30
MM-PBSA	-6.86	-5.42	N/A	-2.04	-4.73	-6.11	-6.02

Table S2. Relative free energies (in kcal/mol) calculated at the QM/MM(B3LYP/B2:AMBER) level using the geometries optimized at the QM/MM(B3LYP/B1:AMBER) level with and without the FEP simulations. The free energies listed include the zero-point and thermal corrections for the QM subsystem.

	QM/MM(B3LYP/B2:AMBER)		QM/MM(B3LYP/B2:AMBER)- FEP	
	<i>trans</i> -5'-hydroxylation			
Structure	Quartet	Doublet	Quartet	Doublet
RC	0.0	0.0	0.0	0.0
TS <sub>H</sub>	15.8	15.4	16.9	14.1
IM	-1.2	0.2	-0.1	-2.0
TS <sub>reb</sub>	-1.2	1.1	0.0	-0.9
PC	-52.7	-47.2	-49.8	-49.4
	<i>cis</i> -5'-hydroxylation			
Structure	Quartet	Doublet	Quartet	Doublet
RC	0.0	0.0	0.0	0.0
TS <sub>H</sub>	14.7	13.0	16.5	14.4
IM	1.2	-1.1	3.3	1.5
TS <sub>reb</sub>	1.6	-0.4	3.6	2.4
PC	-54.4	-50.5	-52.2	-48.1

In the *trans*-5'-hydroxylation pathway, the potential energy surface which is determined by QM/MM reaction coordinate calculations at the B3LYP/B1:AMBER level clearly shows two transition states (TS<sub>H</sub> and TS<sub>reb</sub>). The geometries of the reactant complex, transition states, intermediates and product complexes were verified by the full geometry optimizations followed by harmonic normal mode calculations at the same QM/MM level (B3LYP/B1:AMBER). (*S*)-(-)-Nicotine and Cpd I initially form the reactant complex, <sup>4/2</sup>RC, in which the distance between the *trans*-5'-hydrogen and the oxygen of Cpd I is 2.74/2.63 Å. In the geometry of the first transition state, <sup>4/2</sup>TS<sub>H</sub>, the C-H distance is 1.30/1.26 Å, the O-H distance is 1.32/1.38 Å, and the C-H-O angle is 177.2°/176.2°. Thus, the transition state, <sup>4/2</sup>TS<sub>H</sub>, has a structure associated with hydrogen-transfer with partially broken C-H bond, partially formed O-H bond, and almost linear arrangement of the C-H-O portion. The high spin density ( $\rho_{\text{nic}} = 0.57/-0.50$  in <sup>4/2</sup>TS<sub>H</sub>) and low charge ( $Q_{\text{nic}} = 0.09/0.10$  in <sup>4/2</sup>TS<sub>H</sub>) on the (*S*)-(-)-nicotine moiety indicate that the transition state involves hydrogen-transfer of the radical type. In the geometry of intermediate <sup>4/2</sup>IM, where the *trans*-5'-hydrogen has been transferred to the oxygen of Cpd I, the distance between the hydrogen of the iron-hydroxo complex and the carbon of the (*S*)-(-)-nicotine moiety is 2.64/2.58 Å. It is apparent that, in the intermediate <sup>4/2</sup>IM, the (*S*)-(-)-nicotine moiety is still coordinated to the hydroxyl group of the iron-hydroxo complex. In <sup>4/2</sup>IM, the spin density of the (*S*)-(-)-nicotine moiety is 0.93/-0.90 and the charge is 0.06/0.09, which further indicates that the H-transfer step involves hydrogen-transfer of the radical type. The second transition state <sup>4/2</sup>TS<sub>reb</sub> is associated with the OH group rotation, *i.e.* the C-Fe-O-H dihedral angle increasing relative to the <sup>4/2</sup>IM species. The dihedral C-Fe-O-H of <sup>4/2</sup>IM is 21.3/5.7°, and it increases to 41.9/57.0° in <sup>4/2</sup>TS<sub>reb</sub>. In <sup>4/2</sup>TS<sub>reb</sub>, the distance from the hydrogen to the carbon of the (*S*)-(-)-nicotine moiety is 2.79/3.47 Å. Once the OH group snaps out of the weak OH-C interaction, the C-O bond between the (*S*)-(-)

)-nicotine moiety and the hydroxyl group of the iron-hydroxo complex gradually forms, and the Fe-O bond gradually breaks. In the product complex  ${}^{4/2}\text{PC}$ , the hydroxyl group is covalently bonded with the carbon atom at the *trans*-5'-position of  $\text{SR}_t$  while the Fe-O bond no longer exists.

As in the *trans*-5'-hydroxylation pathway, the potential energy surface in the *cis*-5'-hydroxylation process also shows two transition states ( $\text{TS}_H$  and  $\text{TS}_{\text{reb}}$ ). In the optimized reactant complex  ${}^{4/2}\text{RC}$ , the *cis*-5'-hydrogen of  $\text{SR}_c$  is 2.21/2.21 Å from the oxygen of Cpd I. The transition state  ${}^{4/2}\text{TS}_H$  involves a partially broken C-H bond ( $R_{\text{C-H}} = 1.28/1.24$  Å) and a partially formed H-O bond ( $R_{\text{H-O}} = 1.31/1.38$  Å), with a C-H-O angle of 175.7/175.6°. The  ${}^{4/2}\text{TS}_H$  structure involves a hydrogen transfer of the radical type, as evidenced from the large spin density ( $\rho_{\text{nic}} = 0.58/-0.48$ ) and the small charge ( $Q_{\text{nic}} = 0.06/0.06$ ) on the (*S*)-(-)-nicotine moiety. This is consistent with the finding that the spin density of the (*S*)-(-)-nicotine moiety is 0.94/-0.88 and the charge is 0.06/0.10 in the formed intermediate  ${}^{4/2}\text{IM}$ . In  ${}^{4/2}\text{IM}$ , the hydrogen atom forms a covalent bond with the oxygen of Cpd I, and the (*S*)-(-)-nicotine moiety is still coordinated to the hydroxyl group of the iron-hydroxo complex as the distance from the hydrogen of the iron-hydroxo complex to the carbon of (*S*)-(-)-nicotine moiety is 2.29/2.18 Å. In the rebound transition state,  ${}^{4/2}\text{TS}_{\text{reb}}$ , the distance from the hydrogen to the carbon of (*S*)-(-)-nicotine moiety is 2.40/2.33 Å. The C-Fe-O-H dihedral angle increased from 16.6/12.4° in  ${}^{4/2}\text{IM}$  to 34.2/30.7° in  ${}^{4/2}\text{TS}_{\text{reb}}$ . After the rebound transition state, the (*S*)-(-)-nicotine moiety and the hydroxyl group of the iron-hydroxo complex gradually forms a C-O bond, while the Fe-O bond gradually breaks. Finally, the product *cis*-5'-hydroxynicotine is formed.

Summarized in Table S2 are the free energy barriers (the zero-point and thermal corrections for the QM subsystem) calculated for (*S*)-(-)-nicotine 5'-hydroxylation at the QM/MM(B3LYP/B2:AMBER) level with and without FEP simulations. The data in Table S2

reveal that the calculated free energy barriers without the FEP simulations favor the *cis*-5'-hydroxylation reaction by 2.4 kcal/mol. With the FEP simulations, the calculated free energy barrier of the *trans*-5'-hydroxylation is very close to that of the *cis*-5'-hydroxylation. The QM/MM-FEP-calculated free energy barriers are 14.1 kcal/mol (*trans*-5'-hydroxylation) and 14.4 kcal/mol (*cis*-5'-hydroxylation). Thus, the dynamic effects of the protein environment exert a remarkable effect on the free energy barriers for the catalytic 5'-hydroxylation reaction processes.

### Complete citations of refs. 43 and 53

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