Computational Studies for Structural and Functional Analysis of Cytochrome P450 Enzyme

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授与学位	博士（工学）
学位授与年月日	平成１９年９月１２日
学位授与の根拠法規	学位規則第４条第１項
研究科，専攻の名称	東北大学大学院工学研究科（博士課程）応用化学専攻
学位論文題目	Computational Studies for Structural and Functional Analysis of Cytochrome P450 Enzyme (計算化学手法によるシトクロム P450 の構造・機能解析)
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論文内容要旨

The cytochromes P450 (CYPs) form a large enzyme family, and are found in all living organisms and take part in the biosynthesis and metabolism of both endogenous and exogenous compounds. They are primarily monooxygenases and perform a wide range of reactions. Because of their wide distribution in living organisms and their extreme important role in biochemistry, pharmacology and toxicology, CYP have been extensively studied. Hydroxylation of inert C–H bonds catalyzed by CYP is an important reaction, which can influence the bioavailability of pharmaceuticals, transform a pro-drug to its active form, or equally produce toxic metabolites. Analyzing how these biological catalysts work will be vital for understanding the biological processes at the molecular level, and also ensure technological benefits in the form of genetic analysis, catalytic processes and development of new drugs. In this thesis, the main aim is to analyze the structural and functional role of CYP in metabolism of biologically important compounds. It span over predicting the reaction pathway for metabolism, investigating the major metabolites, studying the hydroxylation reaction in full protein, stability and reactivity, and effect of ligand binding on CYP. All these properties are extremely difficult to study using experimental methods, however computational approaches can give first answers.

Different theoretical methods were used. Quantum chemical calculations were carried out using density functional theory (DFT) and solvent effect was considered by applying the COSMO solvation model. Molecular dynamics simulation (MD) was carried out using an in-house program New-Ryudo. MD coupled with chemical reaction function was carried out using the New-Ryudo-CR program. Effect of flexibility and rigidity on ligand binding or the protein-protein interaction was studied using RIGIX program.

The CYP3A4 catalyzed metabolism of (S)-N-1-(3-morpholin-4ylphenyl) ethyl]-3-phenyl acrylamide (Substrate 1) was investigated using DFT assisted with MD simulation. In this chapter, the hydroxylation at phenyl ring was studied. Experimental studies found that CYP3A4 metabolizes substrate 1 and finally it forms a reactive intermediate. The aim was to investigate hydroxylation reaction pathway and the effect of substituent on this particular reaction.

Hydroxylation reaction of substrate 1 occurs through electrophilic attack of the phenyl ring to form a σ-complex. It is concluded that during the σ-complex formation, the morpholine N donates its lone pair of electron. Without loosing the aromaticity this compound forms a strongly bound σ-complex (Fig. 1), it results in lowering of the energy barrier for the σ-complex formation. Thus, enhancing or suppressing the drug metabolism should concentrate on this fragment. Two types of σ-complexes were found. A perpendicular σ-complex, which undergoes further rearrangement to produce a proton-shuttle intermediate and leads to the formation of the alcohol or the ketone products. While the parallel σ-complex leads solely to a ketone product. Based on DFT and by considering protein environment from MD results, it was found that phenyl ring remains perpendicular to the porphyrin plane. The alcohol formation as the major product was confirmed.

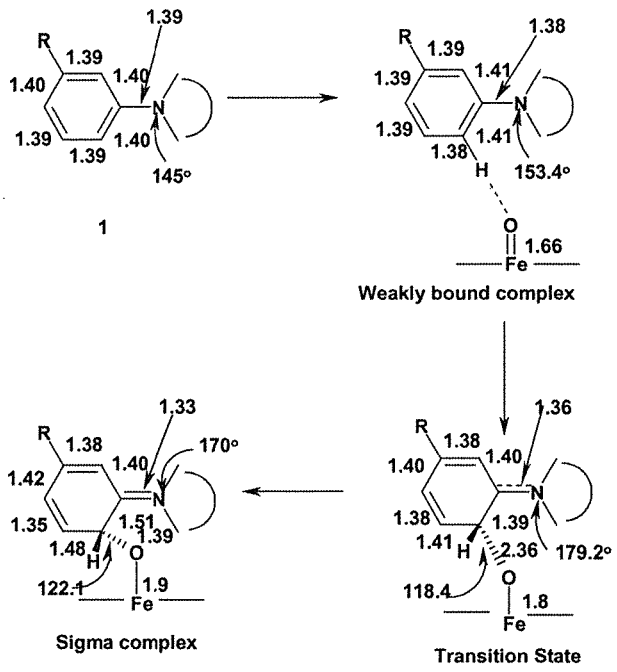

Figure 1. Proposed mechanism for the formation of σ-complex. Distances are in the unit of Å.

The hydroxylation reaction at the morpholine ring of substrate 1 and its difluoro analogue, (S)-N-[1-(4-fluoro-3-morpholin-4-ylphenyl)ethyl]-3-(4-fluorophenyl) acryl amide (substrate 2), were investigated using DFT and MD methods. Experimental evidence found that the CYP3A4 enzyme metabolizes substrate 1 while the substrate 2 was devoid of metabolism. The aim was to find out the reason behind the structurally closely related compounds but different reactivity towards CYP. The energy barrier for the hydroxylation of substrate 1 was found to be 7.01 kcal/mol and 19.53 kcal/mol in gas phase and solvent phase, respectively. Similarly, the energy barrier for substrate 2 was found to be 11.07 kcal/mol in gas phase while it increases negligibly in the COSMO phase up to the value of 12.99 kcal/mol. The energy barrier for morpholine αC hydroxylation was higher than the phenyl ring hydroxylation in substrate 1. It confirmed that aliphatic hydroxylation occurs at higher energy barrier as compared to arene hydroxylation. Based on results of chapter 3 and 4, it is confirmed that metabolism site on phenyl position is preferred over morpholine and hence alcohol is the major product. Results are consistent with experimental finding.

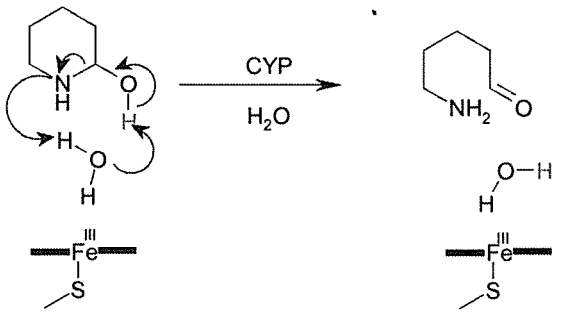

Figure 2. Water-assisted cleavage of C-N bond in the rate-determining step.

The details of the biodegradation reaction of morpholine by CYP were studied in detail for the first time using theoretical method. Morpholine is a simple heterocyclic compound with a great

industrial importance and biodegradation of morpholine has not emerged so far. Using DFT, the hydroxylation reaction on morpholine by CYP studied in two electronic states, the doublet and the quartet state. The hydroxylation reaction barrierless for the doublet state, while in the quartet state, the reaction occurs in two steps, hydrogen abstraction and rebound mechanism. In the subsequent step, the intramolecular hydrogen transfer occurs through hydroxyl (O-H) group to nitrogen of the hydroxymorpholine group leading to the formation of 2-(2-aminoethoxy)acetaldehyde. The energy barrier for the C-N bond breaking is 24.45 kcal/mol (in COSMO 26.73 kcal/mol) in the doublet state while it is 32.20 kcal/mol (34.51 kcal/mol) in the quartet state. Finally, in an attempt to reduce the energy barrier for C-N bond breaking step, a water molecule is introduced in the reaction pathway (Fig. 2). It was found that water mediated reaction greatly enhances the reaction by reducing the height of the energy barrier.

The stereoselective and regioselective hydroxylation of camphor leading to the formation of 5-exo-hydroxycamphor was studied using the New-Ryudo-CR program. The methodology in New-Ryudo-CR is combination of molecular dynamics method and chemical reaction function. It was used to observe the bond breaking and bond formation process during the dynamic movement of the protein. Hydroxylation of camphor occurs in two steps, the hydrogen abstraction and rebound mechanism. The hydrogen abstraction and radical recombination in camphor occurs at 250 fs and 110 fs (Fig. 3), respectively. Experimental results indicate that radical recombination occurs at 70-100 fs, hence results are comparable with experimental results. In addition rate of reaction was calculated for the rate-determining step (hydrogen abstraction). Calculated rate of reaction is of the value of 0.64 s^{-1} which is also close to the experimental value of ~ 1 s^{-1}. Hence New-Ryudo-CR is successfully applied to study the CYP enzyme catalyzed reaction and also the validation was carried out with experimental results.

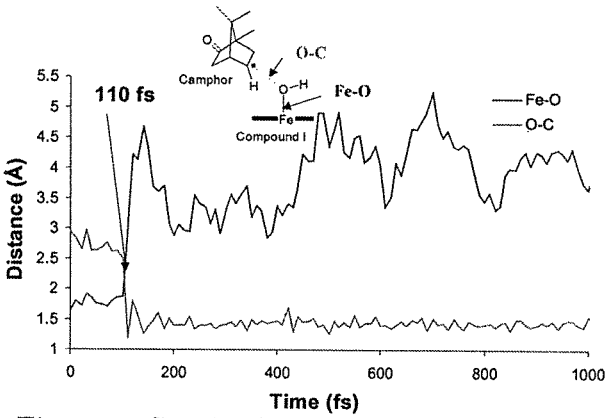

Figure 3. Graph of change of O-C and Fe-O distance with respect to time.

In order to study the role of CYP enzyme on ligand binding, rigidity and flexibility of CYP was studied. New methodology based on graph theoretical approach in RIGIX program has been applied to several protein-protein and protein-ligand complexes. Results were compared with MD result. After successful validation of method for several protein complexes, it was applied to study the role of flexibility in CYP. After analysis of the substrate 1 and CYP3A4 complex using RIGIX, it was found that the substrate 1 was surrounded by rigid amino acids, which restrict movement of ligand and overall effect that C4 site of phenyl ring become accessible for

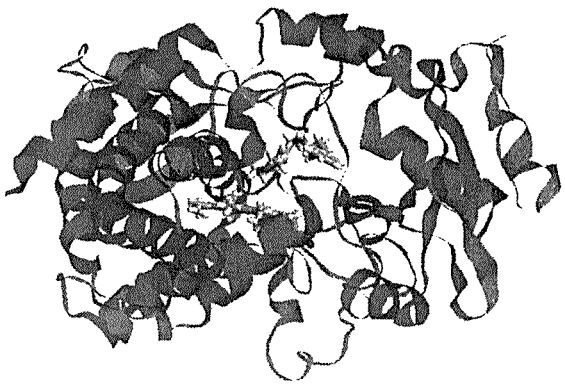
Figure 4. CYP3A4 complexed with substrate 1. Rigid residues are shown with dark color and flexible residues are shown with light gray.

metabolism (Fig. 4). Good correlation was found between Flex index obtained from RIGIX and the experimental B-factor. Several other applications were shown, where the CYP changes conformation after ligand binding and CYP-CYP binding.

Structural and functional role of CYP was studied using theoretical methods. CYP catalyzed reaction has been studied using DFT method assisted with molecular mechanical methods. Metabolism of KCNQ2 potassium channel opener was investigated. It has been found that the electron donating ortho substituent on arene ring facilitates the rate-determining step by donating the lone pair of N. Subsequent investigation confirmed the alcohol as the major product in this reaction. Further studies were carried out to show the biodegradation pathway for morpholine, one of the environmentally hazardous compound. The first step of reaction was hydroxylation. The second step was breaking of C-N bond to form 2-(2-aminoethoxy)acetaldehyde. The cleavage of C-N bond was found to be rate-determining step. Water-mediated breakdown of C-N bond lowers the energy barrier of the rate-determining step. In order to study the reaction in protein environment, new methodology developed in our laboratory was applied to study the hydroxylation reaction. The time required to break and form the bond, rate of reaction was evaluated. To consider the structural effect on CYP during ligand binding and on metabolism reaction, flexibility studies was carried out using RIGIX program. Unique combination of approach has been applied to study this important enzyme catalyzed reaction. Effect of substituent on reaction, detailed analysis of how the protein environment affects and modulates the electronic structure, stability, and reactivity of the species in the CYP were clarified by studying the functional and structural properties. It opens the door for further researches, which can be carried out on thermodynamical aspects, involvement of reductase protein and electron transport system in CYP.

論文審査結果の要旨

本研究は代謝酵素シトクロムP450（CYP）の代謝特性を構造および機能面から理論的に解析することを目的として行われた。本論文は「Computational Studies for Structural and Functional Analysis of Cytochrome P450 Enzyme (計算化学手法によるシトクロムP450の構造・機能解析)」と題し、以下の8章からなる。

第1章では、CYPおよび薬分子の生体内の動態に与えるその重要性について概説した後に、本論文で対象としたCYPの構造・機能の解析について述べている。CYPによる代謝反応の経路、代謝産物の予測の重要性に加えて、それらに与えるタンパク質構造の影響の重要性について具体的に説明し、本論文の目的を明確にしている。

第2章では、本論文で用いた手法について説明している。具体的には化学反応経路および代謝産物の解析に用いた密度汎関数法および分子動力学法について詳説し、さらにはタンパク質巨大分子における化学反応ダイナミクスを解析するための新規化学反応対応型分子動力学法、タンパク質のフレキシビリティ解析のための新規手法について紹介している。

第3章では、CYP3A4による(S)-N-[1-(3-morpholin-4-ylphenyl)ethyl]-3-phenylacrylamideの代謝反応について、密度汎関数法および分子動力学法を用いて調べている。密度汎関数法と分子動力学法の両方を統合的に活用することで、単独の手法では解明不可能であった反応経路および代謝産物の理論的解明に成功している。

第4章では、CYP3A4による(S)-N-[1-(3-morpholin-4ylphenyl)ethyl]-3-phenylacrylamideおよびその類似化合物(S)-N-[1-(4-fluoro-3-morpholin-4-ylphenyl)ethyl]-3-(4-fluorophenyl)acrylamideの代謝反応について、密度汎関数法および分子動力学法を用いて調べている。類似化合物に対する異なる代謝反応特性を解明し、第3章の結果と併せ、主代謝産物がアルコールであることを確認することに成功している。

第5章では、CYPによるMorpholineの代謝特性について密度汎関数法を用いて詳細に調べている。代謝反応におけるエネルギー障壁は、スピン状態により大きく影響されることを解明している。また、その反応において水分子の介在が重要であることを理論的に解明することに成功している。

第6章では、新規化学反応対応型分子動力学法を用いてP450camによるcamphorの代謝反応ダイナミクスについて調べている。巨大分子であるP450camの代謝反応の有限温度における動的特性の世界に先駆けた理論解明に成功している。

第7章では、CYPのフレキシビリティに与えるリガンドの相互作用の影響を調べている。最初に、新規フレキシビリティ解析手法の妥当性を実験的な指標との比較などにより調べている。続いて、CYP3A4とそのリガンドが相互作用した系へと手法を応用し、リガンドの相互作用に伴うCYP3A4の構造変化に関する示唆など、従来法では全く得られなかった有用な知見を得ることに成功している。

第8章は、本論文の総括である。

以上、本論文は、量子論や分子動力学法に加えて新規計算化学手法を活用することで、従来解明が不十分であったCYPの構造・機能に関する新規知見を得ることに成功している。

よって，本論文は博士(工学)の学位論文として合格と認める。