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論文審査委員	主　査　教授　田中　晃二 教授　渡辺　芳人 助教授　岡本　祐幸 助教授　鈴木　敏泰 教授　清水　透（東北大学）

論文内容の要旨

Part I GENERAL INTRODUCTION

Myoglobin (Mb) functions as an oxygen storage and carrier protein in muscle. This protein has been one of the most intensively investigated hemoproteins as evident from the accumulated biochemical and spectroscopic data. It has protoporphyrin IX as a prosthetic group, and is the first protein structure determined to high resolution by X-ray crystallographic analyses. Thus, myoglobin has ever been serving as a model system for the study of structure-function relationships in heme proteins.

Part II Chapter 1. The Role of Val68 (E11) on Oxidation Activity and Enantioselectivity of Sperm Whale Myoglobin

To probe the role of the distal valine 68 (E11) in sperm whale myoglobin (Mb) on the oxidation activity, site-directed mutagenesis was performed. A series of Mb mutants, H64D/V68X Mbs, have been prepared by replacing Val-68 with Gly, Ala, Ser, Leu, Ile, and Phe in H64D Mb. All of the mutant proteins are stable enough to be purified except for the H64D/V68G mutant. The oxidation of the substrate thioanisole by H64D/V68X Mb-I besides H64D/V68S was monitored by stopped-flow spectrometer and the sulfoxidation rate constants increase in the order of Phe ≤ Val < Leu < Ala < Ile. The results suggest that the volume of hydrophobic residue at the 68 position influences the sulfoxidation activity. A similar pattern is observed for the catalytic sulfoxidation of thioanisole by H64D/V68X Mbs and H_2O_2. The dominant product in the catalytic sulfoxidation is the *R* isomer for the H64D/V68A and H64D/V68S mutants, with more than 84% enantiomeric excess (% ee). However, increasing the polarity of the distal pocket by substituting Ala-68 with Ser in H64D Mb decelerates the catalytic sulfoxidation rate by 2-fold. On the other hand, the H64D/V68I mutant affords dominantly the *S* isomer with the highest turnover rate up to 413 turnover/min. The substitution of Val-68 with Leu has little effect on enantioselectivity in the catalytic sulfoxidation but increases the reactivity with H_2O_2. Both the value of % ee and rate in the catalytic sulfoxidation decrease for H64D/V68F Mb in comparison with H64D/V68A Mb, implying a large benzyl side chain of phenylalanine at the 68 position inhibits the access of substrate to the heme pocket. Furthermore, the crystal structure of the mutant, H64D/V68A, has confirmed the previous report (*J. Am. Chem. Soc. 121*, 9952–9957, **1999**, Matsui *et al.*) on catalytic mechanism and the spectroscopic studies on H64D/V68X Mb phenylethylamine complexes which are prepared to mimic the transition sate of thioanisole sulfoxidation, have provided some information on enantioselectivity in the sulfoxidation.

Chapter 2. Conversion of Sperm Whale Myoglobin into a Catalase-like Enzyme

The sperm whale myoglobin active site mutants (F43H/H64A and F43H/H64N Mb) have been constructed to mimic the active site of catalase in which the distal histidine is suggested to facilitate compound I formation with H_2O_2. The F43H/H64A and F43H/H64N mutants exhibit 3.8- and 13-fold higher reactivity in the ABTS oxidation by H_2O_2 than the wild type, respectively. However such mutation does not increase the reactivity of the ferric state with H_2O_2 enough to accumulate compound I and even depress compound I accumulation with *m*CPBA. Some reasons

for the failure in the observation of compound I for the novel double mutants would be suggested when the crystal structural analysis of those mutants is completed.

Part III Chapter 1. Asymmetric Oxidation Catalyzed by Sperm Whale Myoglobin Mutants

The sperm whale myoglobin active site mutants (L29H/H64L and F43H/H64L Mb) have been shown to catalyze the asymmetric oxidation of sulfides and olefins. Thioanisole, ethyl phenyl sulfide, and *cis*-β-methylstyrene are oxidized by L29H/H64L Mb with more than 95% enantiomeric excess (% ee). On the other hand, the F43H/H64L mutant transforms *trans*-β-methylstyrene into *trans*-epoxide with 96% ee. The dominant sulfoxide product in the incubation of alkyl phenyl thioethers is the *R* isomer; however, the mutants afford dominantly the *S* isomer of aromatic bicyclic sulfoxides. The results help us for the rationalization of the difference in the preferred stereochemistry of the Mb mutants-catalyzed reactions. Furthermore, the Mb mutants exhibit the improvement of the oxidation rate up to 300-fold with respect to wild type.

Chapter 2. Characterization of I107H/H64L Myoglobin Mutant

Since the alignment of the distal histidine is important for the reactivity with hydrogen peroxide as well as the prolonged life time of Mb-I [Ozaki et al. (1997), *J. Am. Chem. Soc.*, 119, 6666], I107H/H64L Mb was constructed with an objective of obtaining highly active Mb in the reaction with H_2O_2. However, the increased activities in peroxidation and peroxygenation are not observed for the I107H/H64L mutant in comparison with H64L and WT Mbs. The finding indicates that not only the distance of distal histidine to the heme iron but also its conformation might be crucial in the activation of H_2O_2 for Mb. In addition, attempts are made to define the mechanism of influence of the distal histidine on regioselectivity in the coupled oxidation of several distal histidine relocation Mbs including I107H/H64L. HPLC analysis of biliverdin isomers shows that relocation of the distal histidine at the 107 position (I107H/H64L Mb) affords the amount of γ-isomer to 22%, while L29H/H64L Mb almost exclusively gives γ-isomer compared with H64L and WT Mbs which mainly afford α-isomer.

Part IV SUMMARY AND CONCLUSION

In the present thesis, the author aimed to clarify enantioselectivity of oxidation reactions catalyzed by hemeproteins. Sperm whale myoglobin (Mb) is employed as a model hemoprotein for the purpose, and some Mb mutants have been prepared by site-directed mutagenesis.

On the one hand, a series of H64D/V68X Mb mutants have been prepared to investigate the function of residues at the position 68 on the oxidation activity. The results presented here demonstrate that the size and polarity of residues 68 in Mb play absolutely important roles on the oxidation activity and enantioselectivity in peroxidation and peroxygenation. The changes in the oxidation activity would be rationalized by different reactivity of compound I for H64D/V68X Mbs. It has been clearly that polarity of the active site instead of a general acid-base catalyst of residue Asp-64 is involved in catalytic mechanism. Analysis of 1-phenylethylamine-Mb-complex crystal structure is under way in order to elucidate the precise substrate binding mode. This type of information would provide a basis for the development of myoglobin mutants designed to

catalyze enantioselective oxidation of interest, an important goal in synthetic organic chemistry.

On the other, substrate specificity is also important in controlling enatioselectivity of enzymatic oxidation. Previously, L29H/H64L and F43H/H64L Mb were reported to exhibit catalytic turnover with high stereospecificity for the sulfoxidation of thioanisole and the epoxidation of styrene. To determine how substrate structures influence on the enantioselectivity oxidations by Mb, the scope of asymmetric oxygenation by the use of various sulfides and styrene were explored for L29H/H64L and F43H/H64L Mb. On the basis of changes in stereoselectivity, the substrate binding mode for Mb mutant-catalyzed reactions was rationalized.

論文の審査結果の要旨

楊慧君さんの提出論文の題名は Stereochemistry in Catalytic Oxidation by Heme Enzymes (ヘム酵素による触媒的な酸化反応における立体化学)である。本論文は4章よりなる。第1章はヘムたんぱく質が触媒する酸化反応の概略とマッコウクジラノのミオグロビンのヒスチジン64をアスパラギン酸に変えたH64Dでは過酸化水素と反応させることで、効率よく酸化反応の活性種であるCompound I が効率よく蓄積されることを述べている。第2章では Compound I の反応性を明らかにする目的で、H64Dとヘム近傍のバリン68を Gly, Ala, Ser. Leu, Ile と Phe に置き換えた一連の修飾ミオグロビン(H64D/V68X Mb)を合成している。さらに、H64D/V68X Mb を用いて各種の酸化反応行っている。たとえば、チオアニソールの酸化の反応速度は Phe<<Val<Leu<Ala<Ile であることから、68番目のタンパクの疎水空間の大きさに酸化反応の速度が依存していることを明らかにしている。また、主生成物として光学純度が84%以上で R-体が得られている。一方、H64D/V68I Mb を用いた反応ではS－体が主生成物となることを明らかにしている。3 章ではロイシン29をヒスチジンにヒスチジン64をロイシン置換した L29H/H64L とフェニルアラニン43をヒスチジンに置換した F43H/H64L Mb を合成を記載している。L29H/H64L はシスーβーメチルスチレンを95%以上の光学純度で酸化し、F43H/H64L ではトランスーβーメチルスチレンが96%の光学純度でトランスーエポキシドになることを見出した。この事実はヘムポケット内で基質の配向を制御することが可能であることを示した。以上の研究を通じて、蛋白質の改変によって希望する機能を有するヘム酵素を自在に創成するための基本的な原理と要件を確立したことの意義は大きいと考える. 特に、金属錯体などを触媒として用いている様々な化学合成プロセスを、人工酵素による環境調和型プロセスへと置き換えることが強く望まれており、本研究の成果がそうした研究に対して一つの指針を与えている点は高く評価される成果といえる。これらの成果の主要部分は既に2編の学術論文に報告されており、審査委員全員一致して審査に合格したものと判定した。

楊　慧君　君の博士論文に対する口述試験は1月22日の午後に実施した。4章からなる博士論文に記載された具体的な研究成果について，1時間の発表が行われた。その後審査委員による種々の角度からの質疑がなされた。その結果、論文内容と同君の専門的な学力は十分であると認められた。

語学力については、論文が英語で書かれており、また、その大部分は既に楊君は筆頭著者とする2報の原著論文として公表されており、水準に達していると結論した。1月29日の公開発表会においても、研究内容が簡潔にまとめられ、質疑に対しても的確な対応がなされた。よって、審査委員全員一致で合格と判定した。