# Mechanisms of Sulfoxidation Catalyzed by High-Valent Intermediates of Heme Enzymes: Electron-Transfer vs Oxygen-Transfer Mechanism

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**Abstract:** Mechanisms of sulfoxidation catalyzed by high-valent intermediates of heme enzymes have been investigated by direct observation of sulfide-induced reduction of three different compound I species including HRP (horseradish peroxidase), the His64Ser myoglobin (Mb) mutant, and O=Fe$^{IV}$TMP$^{+•}$ (**1**) (TMP = 5,10,15,20-tetramesitylporphyrin dianion). The reaction of thioanisole and compound I of HRP (10 $\mu$M, pH 7.0, 298 K) gives the resting state of HRP with accumulation of compound II as an intermediate. The yield of sulfoxide by a stoichiometric reaction of HRP compound I with thioanisole was only 25% $\pm$ 5%. On the other hand, the same sulfoxidation by both **1** and His64Ser Mb compound I exclusively exhibited a two-electron process, resulting in quantitative formation of sulfoxide. When 1,5-dithiacyclooctane (DTCO) is employed as a substrate, the reaction of His64Ser Mb compound I with DTCO exhibits rapid formation of compound II, which decays to the ferric state due to the low oxidation potential of DTCO. The observed rate constants (log $k_{obs}$) of the reactions of **1** and compounds I of HRP and His64Ser Mb with a series of *p*-substituted thioanisoles correlate with the one-electron oxidation potentials ($E^0_{ox}$) of the sulfides. A comparison of these correlations with the established correlation between log $k_{obs}$ and $E^0_{ox}$ for the corresponding electron-transfer reactions of substituted *N,N*-dimethylanilines has revealed that the sulfoxidation reactions of compound I of HRP with the sulfides proceed via electron transfer while the sulfoxidations catalyzed by **1** and compound I of His64Ser Mb occur via direct oxygen transfer.

Cytochromes P450 utilize molecular oxygen to catalyze monooxygenation of organic compounds such as hydrocarbons, sulfides, and amines.[1,2] Many efforts have been made to elucidate molecular mechanisms of oxygen activation and oxidation reactions for decades. To clarify the reaction mechanisms, it is crucial to characterize the reactive intermediates in the multistep catalytic cycle of P450. In the case of peroxidases, compound I, an oxoferryl porphyrin $\pi$-cation radical, has been well characterized as a species equivalent to the proposed active intermediate of P450.[3] Horseradish peroxidase (HRP) was reported to convert thioanisole to the corresponding sulfoxide,[4] although peroxidases typically catalyze two sequential one-electron oxidations such as one-electron oxidation of phenol derivatives to phenoxyl radicals.[5] The sulfoxidation involves an oxygen-transfer process from an oxoferryl species

to sulfide, since 18-labeled oxygen in H$_2$$^{18}$O$_2$ has been shown to be incorporated into the product sulfoxide.[4,6] The oxygen transfer was suggested to proceed via electron transfer/oxygen coupling (overall two electron oxo transfer) in competition with typical sequential electron-transfer based on the kinetic study on the reaction of HRP compound I with *p*-methoxythioanisole.[7,8] Such an electron-transfer/oxygen-coupling mechanism was previously proposed for sulfoxidations catalyzed by P450.[9−12] Higher sulfoxidation activity has been achieved by using several HRP mutants that are designed to improve the access of sulfides to the oxo-ferryl group of compound I.[13−16]

An inability to observe the active intermediate of P450 due to a preceding rate-limiting step has precluded acquisition of a complete understanding of the monooxygenation mechanisms,[17−19]

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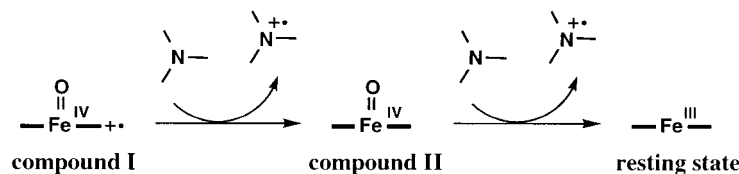(14) Newmyer, S. L.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1995**, *270*, 19430−19438.

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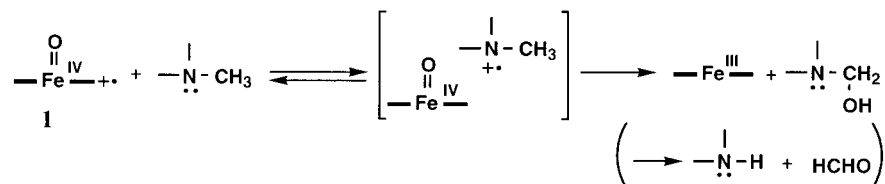
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**Scheme 1**



In this context we have recently reported reaction mechanisms of N-demethylation of *N,N*-dimethylaniline by compounds I on the basis of direct observation of the reactions with stopped-flow UV−vis spectroscopy.[20] In these experiments, we have used the relatively stable compound I: compound I of horseradish peroxidase (HRP) and a synthetic model, O=Fe$^{IV}$TMP$^{+•}$ (**1**; TMP, 5,10,15,20-tetramesitylporphyrin dianion),[21] which enabled us to monitor the reactions with substrates directly. In the HRP system, stepwise reduction of compound I to the ferric state via compound II (oxoferryl porphyrin) has been observed. A linear correlation of the observed rate constants (log $k_{obs}$) for each step with the oxidation potential of *N,N*-dimethylanilines (DMAs) and without observation of kinetic isotope effects are results consistent with the currently accepted sequential one electron oxidation mechanism by HRP.[22−24] A comparison of the log $k_{obs}$ values for the synthetic model (**1**) system with those of the HRP compound I system, combined with the kinetic and product isotope effects, has revealed that N-demethylation of DMAs by **1** also proceeds via a rate-determining electron-transfer step (Scheme 1).

This study reports the first kinetic data obtained by direct observation of the reactions of three different compound I species with a series of sulfides using stopped-flow UV−vis spectroscopy. The compound I species investigated are (1) compound I of HRP prepared by the addition of a stoichiometric amount of H$_2$O$_2$ to the ferric resting enzyme; (2) a synthetic model complex, O=Fe$^{IV}$TMP$^{+•}$, which is synthesized by the reaction of Fe$^{III}$TMP and *m*CPBA in CH$_2$Cl$_2$ at 233 K;[21] and (3) compound I of a sperm whale myoglobin His64Ser mutant formed in the reaction of a ferric form with *m*CPBA as reported previously.[25] These preparations provide us compounds I stable enough to ensure the direct observation of the reactions with

sulfides. A detailed comparison of the rate constants of sulfoxidation and those of *N,N*-dimethylaniline demethylation (Scheme 1) provides an excellent opportunity to evaluate the contribution of an electron-transfer pathway in sulfoxidations catalyzed by the high-valent intermediates of heme enzymes and a synthetic model compound.

## Experimental Section

**Materials.** All chemicals were purchased from Sigma-Aldrich, Wako, Nacalai Tesque, and Lancaster Co., Ltd. and used without further purification unless otherwise noted. *m*-Chloroperoxybenzoic acid (*m*CPBA) was purified by washing with phosphate buffer (pH 7.4) followed by water and then dried under reduced pressure. Dichloromethane was purchased from Wako Pure Chemical Ind. Ltd. and purified by distillation over CaH$_2$ prior to use. Acetonitrile (MeCN) was purchased from Wako Pure Chemical Ind. Ltd. and purified by successive distillation over CaH$_2$ and P$_2$O$_5$. Tetra-*n*-butylammonium hexafluorophosphate was purchased from Sigma Chemical Co., recrystallized from ethyl alcohol, and dried under vacuum at 40 °C for at least 1 week prior to use. HRP was purchased from Sigma-Aldrich Co., Ltd. The sperm whale myoglobin His64Ser mutant was constructed by cassette mutagenesis as described elsewhere.[25] Tetramesitylporphyrin (TMPH$_2$) was prepared as reported.[27] Iron was inserted in the porphyrin to form TMPFe$^{III}$(Cl) by a standard method.[28] TMPFe$^{III}$(OH) was prepared before use by passing TMPFe$^{III}$(Cl) through Al$_2$O$_3$ column (eluent, ethyl acetate), and the solvent was evaporated.[29] After being dried under reduced pressure, a solid TMPFe(OH) was dissolved in dichloromethane.

**Spectral and Kinetic Measurements.** To a 0.6 mL HRP solution (10 $\mu$M) in a 50 mM sodium phosphate buffer (pH 7.0) was added a stoichiometric amount of H$_2$O$_2$ to generate HRP compound I at 298 K. The reactions of HRP compound I with thioanisoles were started by the addition of thioanisole (12.5−200 equiv), and the rates were monitored by the UV−vis spectral changes using a Shimadzu UV-2400 spectrophotometer equipped with a temperature controller. The rate constant for the decay of compound I was determined by fitting the change in absorbance at 411 nm (an isosbestic point of compound II and ferric state)[7] with a least-squares procedure using Igor Pro (WaveMetrics Inc.). The rate of the reaction of HRP compound I with DTCO was determined using a Hi-Tech SF-43 stopped-flow spectro-

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photometer with a sequential-mixing stopped-flow instrument at 298 K. HRP compound II was prepared by addition of a stoichiometric amount of $H_2O_2$ to a solution of ferric HRP followed by reduction with stoichiometric amount of potassium ferricyanide. UV−vis spectra on the reaction of HRP-compound II with DTCO were recorded with a Shimadzu UV-2400 spectrophotometer, and the rate constant of the reaction was determined by fitting the change in absorbance at 420 nm.

Kinetic experiments for the reduction of $O=Fe^{IV}TMP^{+•}$ (**1**) by sulfides were performed on a stopped-flow spectrophotometer. In a typical run, a $CH_2Cl_2$ solution of $Fe^{III}TMP(OH)$ (40 $\mu$M) was mixed with an equal volume of $CH_2Cl_2$ containing 4 equiv of *m*CPBA and 1 equiv of 3-chlorobenzoic acid at 223 K. The formation of **1** was completed in 3 min and confirmed by monitoring its UV−vis spectrum. Thioanisole (10−100 equiv) in $CH_2Cl_2$ was then mixed with **1** (final concentration of **1**, 10 $\mu$M), and the rates of reduction of **1** to $Fe^{III}TMP$ were determined by monitoring the absorbance changes at 413 nm.

His64Ser Mb compound I was prepared by mixing His64Ser Mb (10 $\mu$M) with a 1.5 equiv of *m*CPBA, followed by mixing with 10−100 equiv of sulfide at 277 K in 50 mM sodium acetate buffer (pH 5.0; final concentration of Mb, 2.5 $\mu$M). The rates of reduction of His64Ser Mb compound I by thioanisoles were determined by monitoring the change in absorbance at 409 nm. The rates for the reactions of His64Ser Mb compound I and compound II with DTCO were determined by fitting the changes in absorbance at 420 and 399 nm, respectively.

**Product Yields in Single-Turnover Reactions.** To 3 mL of 10 $\mu$M HRP compound I solution prepared by the addition of 1 equiv of $H_2O_2$ in a 50 mM sodium phosphate buffer, pH 7.0, in a UV-cuvette was added 30 $\mu$L of 100 mM thioanisole in methanol at 298 K. The UV−vis spectral change was monitored for 2 h to ensure the completion of the reaction. Then, benzophenone was added as an internal standard, and the products were extracted with hexane for HPLC analysis as reported elsewhere.[13] A standard curve prepared with the authentic sulfoxide was used to determine the product yield. In a separate run, the same reaction without HRP was carried out and a negligible amount of sulfoxide was obtained.

The single-turnover experiment of Mb compound I with thioanisole was also performed in a similar manner for that of HRP, except that His64Ser Mb compound I was prepared by the addition of 1.1 equiv of *m*CPBA to a 10 $\mu$M Mb solution in a 50 mM sodium acetate buffer, pH 5.0, at 277 K.

The single-turnover sulfoxidation by **1** was performed as follows: **1** was prepared by a stoichiometric reaction of *m*CPBA and $Fe^{III}TMP(OH)$ (0.1 mM in $CH_2Cl_2$) at 223 K, and then a $CH_2Cl_2$ solution of thioanisole (10 equiv) was added to the resulting green solution. Immediate completion of the reaction was confirmed by the solution color change from green to brown. The product solution was then submitted to GC-MS for determing the yield of the sulfoxide in comparison with the authentic sample (internal standard, benzophenone).

**Cyclic Voltammetry.** The one-electron oxidation potentials ($E^0_{ox}$) of dimethylaniline derivatives (DMAs) were previously reported.[20] $E^0_{ox}$ values of sulfides measured in MeCN containing 0.1 M $Bu_4NPF_6$ as supporting electrolyte were determined at room temperature by cyclic voltammetry under deaerated conditions using a three electrode system and a BAS 100B electrochemical analyzer. The working and counter electrodes were platinum, while $Ag/AgNO_3$ (0.01 M) was used as the reference electrode. All potentials are reported as *V* vs SCE. The $E_{1/2}$ value of ferrocene used as a standard is 0.37 V vs SCE in MeCN under our solution conditions.

## Results and Discussion

**Electron-Transfer Pathway for HRP Compound I.** The HRP resting state is converted to compound I (oxoferryl porphyrin radical cation) by the reaction with a stoichiometric amount of $H_2O_2$ in 50 mM phosphate buffer (pH 7.0) at 298 K. Addition of *p*-methylthioanisole to HRP compound I (10 $\mu$M) gave a mixture of compound II (oxoferryl), as evidenced by a small accumulation of absorption around 420 nm, and the resting
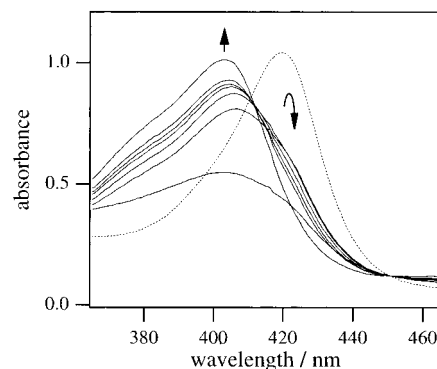


**Figure 1.** UV−Vis spectral changes of the reaction of HRP−compound I with *p*-methyl thioanisole in 50 mM sodium phosphate buffer, pH 7.0, at 298 K: [HRP] = 10 $\mu$M, [*p*-methylthioanisole] = 1.0 mM. Spectra were recorded 0, 20, 40, 60, 80, 100, and 200 min after mixing. The dotted line represents the spectrum of HRP−compound II.

ferric state as shown in Figure 1. The initial conversion of compound I to compound II is followed by the further reduction to the resting state, consistent with the results by Pérez and Dunford.[7,8] On the other hand, incorporation of 18-labeled oxygen into sulfoxides from the oxidant $H_2^{18}O_2$ as well as the moderate enantioselectivity indicates the occurrence of an oxygen atom transfer from HRP compound I to sulfides.[4,6] These results are well explained if one assumes an electron transfer from sulfide to HRP compound I in the protein cage followed by two competitive processes, (i) oxygen rebound to afford the sulfoxide and (ii) diffusion of a sulfenium radical from the protein cage to allow the observation of HRP compound II as shown in Scheme 2.

To estimate the efficiency of the oxygen-rebound step over the diffusion in Scheme 2, we have determined the yield of sulfoxide by the stoichiometric reaction of HRP compound I with thioanisole to be only 25% ± 5%.

Rates of decrease of HRP compound I in the reaction with a series of thioanisoles were determined from the absorbance change at 411 nm (see Experimental Section). The rates in the presence of large excess of thioanisole (12.5−100 equiv) obeyed pseudo-first-order kinetics, and the pseudo-first-order rate constants increased linearly with the thioanisole concentration (see Supporting Information).[30] The second-order rate constants ($k_{obs}$) are determined from the slopes of the linear correlations for the reduction of HRP compound I by a series of *p*-substituted thioanisoles in phosphate buffer (pH 7.0, 50 mM) at 298 K, and the $k_{obs}$ values are listed in Table 1 together with the one-electron reduction potentials of thioanisoles.

We have previously reported that the reduction of HRP compound I by a series of para-substituted *N*,*N*-dimethylanilines (DMAs) proceeds via electron transfer from DMAs to compound I and that there is a linear correlation between the rate constants (log $k_{obs}$) and the one-electron oxidation potentials of DMAs ($E^0_{ox}$).[20] The electron-transfer pathway in Scheme 1 has been confirmed by the absence of kinetic isotope effects for the reactions of deuterated compounds DMAs(-$CD_3$)$_2$.[20] When the plot of log $k_{obs}$ for the reduction of HRP compound I by a series of para-substituted thioanisoles vs $E^0_{ox}$ of thioanisoles (Figure

(30) Linear relationships were observed between the pseudo-first-order rate constants and the sulfide concentrations for the reactions with *p*-methylthioanisole and thioanisole. In the case of the reaction with *p*-methoxythioanisole, however, the pseudo-first-order rate constant exhibits a saturation behavior at the high concentrations. Thus the rate at [*p*-methoxythioanisole] = 125 $\mu$M was used for the determination of a bimolecular rate constant for this reaction.
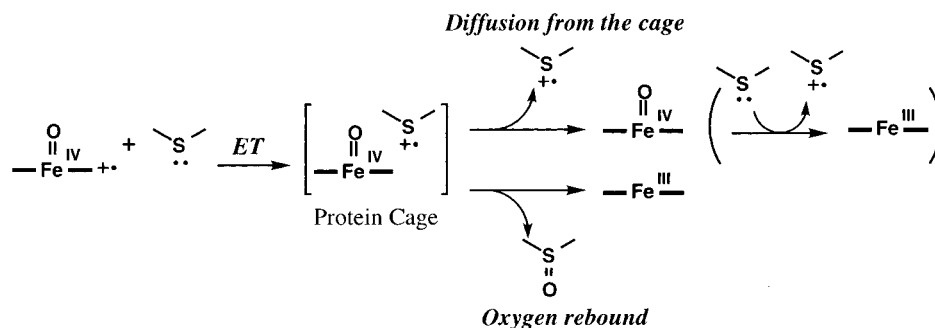
**Scheme 2**



**Table 1.** Bimolecular Rate Constants of the Reactions of HRP−I, O=Fe$^{IV}$TMP$^{•+}$, and His64Ser Mb Compound I with a Series of *p*-Substituted Thioanisoles and DTCO

| sulfide | $E^0_{ox}$ vs SCE [V] | HRP $k_{HRP}$ [M$^{-1}$ s$^{-1}$] | TMPFe $k_{TMP}$ [M$^{-1}$ s$^{-1}$] | Mb His64Ser $k_{Mb}$ [M$^{-1}$ s$^{-1}$] |
|---|---|---|---|---|
| thioanisoles | | | | |
| *p*-OMe | 1.13 | $(1.6 \pm 0.1) \times 10^2$ | $(1.7 \pm 0.1) \times 10^4$ | $(1.2 \pm 0.1) \times 10^6$ |
| *p*-Me | 1.24 | $5.5 \pm 0.1$ | $(5.8 \pm 0.2) \times 10^3$ | $(6.2 \pm 0.2) \times 10^5$ |
| *p*-H | 1.34 | $1.0 \pm 0.1$ | $(3.0 \pm 0.1) \times 10^3$ | $(5.2 \pm 0.2) \times 10^5$ |
| *p*-Cl | 1.37 | nd$^a$ | nd$^a$ | $(7.2 \pm 0.4) \times 10^5$ |
| *p*-Br | 1.41 | nd$^a$ | $(2.4 \pm 0.1) \times 10^3$ | $(5.2 \pm 0.1) \times 10^5$ |
| *p*-CN | 1.61 | nd$^a$ | $(7.8 \pm 0.1) \times 10^2$ | $(8.4 \pm 0.1) \times 10^4$ |
| *p*-NO$_2$ | 1.70 | nd$^a$ | $(5.1 \pm 0.1) \times 10^2$ | nd$^a$ |
| DTCO$^b$ | 0.72 | $k_2$: $(1.2 \pm 0.1) \times 10^3$ | $(8.6 \pm 0.8) \times 10^3$ | $k_2$: $(4.3 \pm 0.1) \times 10^5$ |
| | | $k_3$: $(3.4 \pm 0.3) \times 10$ $^b$ | | $k_3$: $(1.2 \pm 0.1) \times 10^{3\,b}$ |

$^a$ Not determined. $^b$ $k_2$ and $k_3$ correspond to the rate constant from compound I to compound II and from compound II to ferric, respectively.
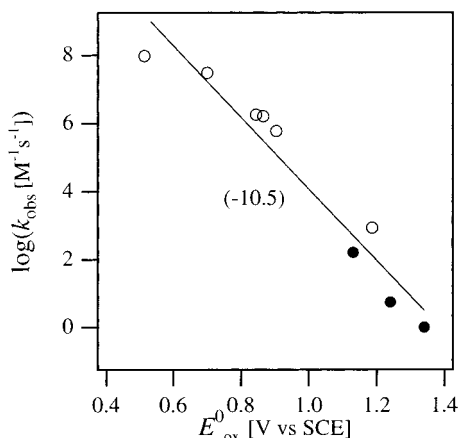


**Figure 2.** Plots of bimolecular rate constants of the reaction of HRP compound I with DMAs (○) and thioanisoles (●) against oxidation potential of DMAs and thioanisoles ($E^0_{ox}$). The slope of the line is shown in parenthesis.



**Figure 3.** UV−Vis spectral changes on the reaction of O=Fe$^{IV}$TMP$^{+•}$ (5.0 $\mu$M) with thioanisole (400 $\mu$M) in CH$_2$Cl$_2$ at 223 K. The spectra were recorded for 880 ms (every 80 ms) after mixing.

2, ●) is incorporated in the linear correlation between log $k_{obs}$ for the reduction of HRP compound I by DMAs and $E^0_{ox}$ of DMAs (Figure 2, ○), there is a single linear correlation between log $k_{obs}$ and $E^0_{ox}$ as shown in Figure 2. Thus, the rate constants for reduction of HRP compound I by DMAs and thioanisoles are both expressed by a single, common relationship (eq 1). Such a unified correlation together with the minor yield of

$$\log k_{obs} = -10.5 E^0_{ox} + 14.6 \qquad (1)$$

sulfoxide clearly indicates that the reduction of HRP compound I by thioanisoles proceeds via electron transfer rather than direct oxygen transfer.

**Direct Oxygen Transfer.** Although the active form of P450 is believed to be compound I or its equivalent, any high valent intermediates of P450 have yet to be well characterized.[18,19] Thus, we have employed a synthetic model of compound I, O=Fe$^{IV}$TMP$^{•+}$ (**1**), since **1** is well characterized and can mimic a
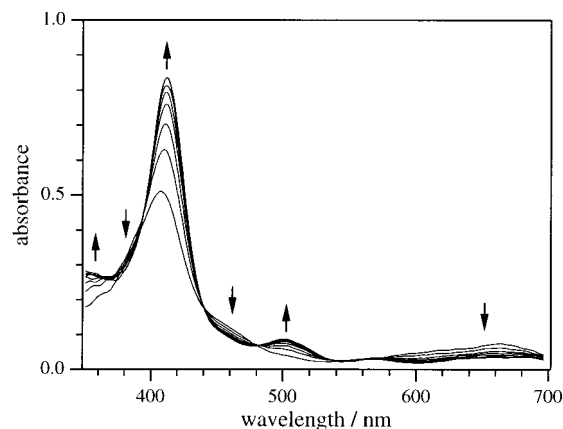
number of P450-type reactions.[21] The synthetic model **1** was prepared by the reaction of Fe$^{III}$TMP(OH) with *m*CPBA in CH$_2$Cl$_2$ at 223 K (see Experimental Section).[21] The reaction of **1** with *p*-substituted thioanisoles in CH$_2$Cl$_2$ at 223 K monitored directly by a stopped-flow technique afforded Fe$^{III}$TMP without accumulating any intermediates as shown in Figure 3.[7] The yield of methylphenyl sulfoxide was quantitative, indicating that the UV−vis spectral change corresponds to the oxygen-transfer process. The rates of formation of Fe$^{III}$TMP in CH$_2$Cl$_2$ at 223 K were determined by monitoring the absorbance changes at 413 nm due to Fe$^{III}$TMP. The rates in the presence of a large excess of thioanisole obeyed pseudo-first-order kinetics, and the pseudo-first-order rate constants increased proportionally with the thioanisole concentration (see Supporting Information). The second-order rate constants ($k_{obs}$) thus determined for the reduction of **1** by a series of *p*-substituted thioanisoles are also listed in Table 1.

Figure 4 shows a correlation between log $k_{obs}$ for the sulfoxidation of thioanisoles by **1** and $E^0_{ox}$ of thioanisoles in
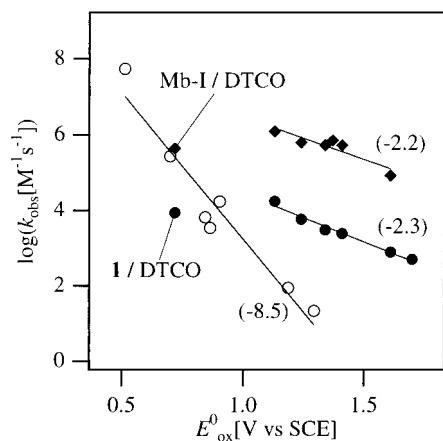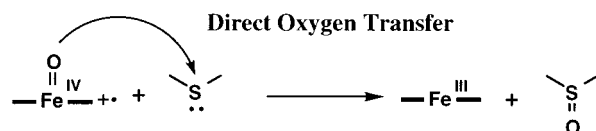
**Figure 4.** Plots of bimolecular rate constants of the reaction of compounds I against oxidation potential of DMAs and sulfides ($E^0_{ox}$): **1** with DMAs (○) and sulfides (●), Mb H64S compound I with sulfides (◆). The slopes of each lines are shown in parentheses.

**Scheme 3**



comparison with a linear correlation between log $k_{obs}$ for the electron transfer from DMAs to **1** and $E^0_{ox}$ of DMAs.[20] In contrast with the case of HRP compound I in Figure 2, the $k_{obs}$ values of thioanisoles in Figure 4 are at least two-orders of magnitude larger than the $k_{obs}$ values of electron transfer from DMAs to **1** when they are compared with the same $E^0_{ox}$ values. In addition, the $k_{obs}$ values of thioanisoles are much less sensitive to the $E^0_{ox}$ values of thioanisoles as compared to the large slope observed in the linear correlation between log $k_{obs}$ for the electron transfer from DMAs to **1** and $E^0_{ox}$. This indicates that the reduction of **1** by thioanisoles proceeds via direct oxygen transfer as shown in Scheme 3 rather than the electron transfer/oxygen rebound pathway in Scheme 2.

We have also employed the His64Ser Mb mutant[25] as a P450 model[26] to compare the reactivity of His64Ser Mb compound I toward thioanisoles with that of the synthetic model (**1**), since His64Ser Mb compound I can be readily produced by the reaction with *m*CPBA (see Experimental Section).[25] The reduction of His64Ser Mb compound I by thioanisoles was examined, and the spectral changes show direct isosbestic reduction of compound I by thioanisole to the ferric state as shown in Figure 5.[7] It was confirmed that the conversion of compound I to the ferric state was accompanied by quantitative formation of methylphenyl sulfoxide (see Experimental Section). The observed second-order rate constants ($k_{obs}$) for the reduction of His64Ser Mb compound I by thioanisoles were determined in phosphate buffer (pH 5.0) at 277 K. The results are compared with those of **1** in Figure 4, where the log $k_{obs}$ values are plotted against the $E^0_{ox}$ values of the thioanisoles. There is a parallel relationship between the plots of **1** and His64Ser Mb compound I, both of which are far above the electron-transfer correlation for the reduction of HRP compound I by DMAs and thioanisoles. This indicates that the reduction of His64Ser Mb compound I also proceeds via direct oxygen transfer in Scheme 3 rather than the electron transfer/oxygen rebound pathway.

**Electron Transfer vs Direct Oxygen Transfer.** A comparison of the linear correlation between log $k_{obs}$ and $E^0_{ox}$ for the direct oxygen transfer from **1** to sulfides with that for the



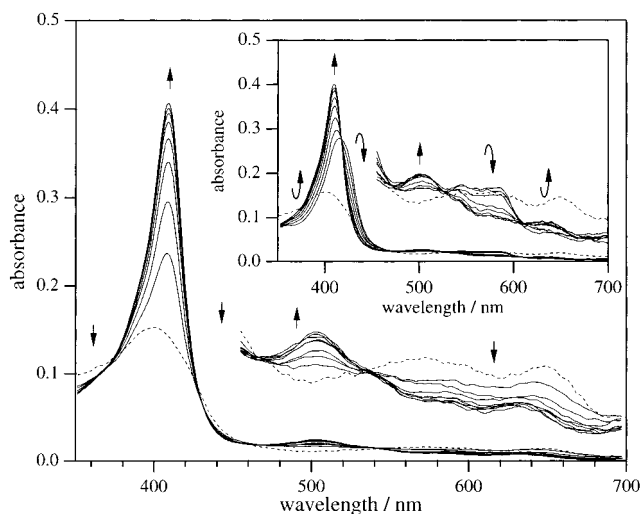**Figure 5.** UV−Vis spectral changes in the reaction of H64S Mb compound I (2.5 $\mu$M) with thioanisole (100 $\mu$M) in 50 mM sodium acetate buffer, pH 5.0, at 277 K. The spectra were recorded 0−84 ms after the mixing. (Inset) UV−Vis spectral change in the reaction of H64S Mb compound I (2.5 $\mu$M) with DTCO (25 $\mu$M) in 50 mM sodium acetate buffer, pH 5.0, at 277 K. The spectra were recorded 0−12.8 s (every 1.6 s) after the mixing. The dotted line is the spectrum before the reaction (His64Ser Mb compound I).

electron transfer from **1** to DMAs in Figure 4 suggests that the electron transfer from sulfides to **1** could become a preferable process over the oxygen transfer when sulfides having lower $E^0_{ox}$ values are employed for the reaction with **1**. This implies an alteration of the reaction mechanism from direct oxygen transfer to electron transfer, depending on the $E^0_{ox}$ value. This supposition was further examined by using 1,5-dithiacyclooctane (DTCO), the $E^0_{ox}$ value of which is much lower than those of thioanisoles. The $k_{obs}$ value for the reduction of **1** by DTCO becomes even smaller than the value expected from the electron-transfer correlation between log $k_{obs}$ and $E^0_{ox}$ in Figure 4. Such a small $k_{obs}$ value of DTCO as compared with the $k_{obs}$ value of DMAs at the same $E^0_{ox}$ value is consistent with the larger reorganization energy ($\lambda$) expected for the electron-transfer oxidation to produce a $\sigma$ radical cation (DTCO$^{\bullet+}$) than the $\lambda$ values of $\pi$ radical cations (DMAs$^{\bullet+}$).[31] Thus, the reduction of **1** by DTCO may proceed via electron transfer from DTCO to **1** rather than direct oxygen transfer. An alteration of the reaction mechanism from direct oxygen transfer to electron transfer becomes more evident in the reduction of His64Ser Mb compound I by DTCO, since rapid formation of compound II is clearly observed, followed by the slow conversion of compound II to the ferric state as shown in Figure 5 (inset). The electron-transfer rate constant from DTCO to His64Ser Mb compound I was determined as $(4.3 \pm 0.1) \times 10^5$ M$^{-1}$ s$^{-1}$. The subsequent slower electron transfer from DTCO to His64Ser compound II was also determined as $(1.2 \pm 0.1) \times 10^3$ M$^{-1}$ s$^{-1}$.

In conclusion, the reduction of HRP compound I by thioanisoles proceeds via electron transfer, while the sulfoxidation of thioanisoles with **1** and His64Ser Mb compound I occurs via direct oxygen transfer. When thioanisoles are replaced by a much stronger reductant (DTCO), however, the reactions of both **1** and His64Ser Mb compound I proceed via electron transfer. One question that must be addressed here is why the reactions of three different compound I systems proceed via different

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mechanisms: electron transfer or direct oxygen atom transfer, depending on the type of compound I and the oxidation potentials of substrates. The heme group of Mb has been shown to be located close to the protein surface and thereby more accessible for substrates as compared to the heme group of HRP.[32−37] It is therefore not surprising that the reactions of His64Ser Mb compound I and **1** proceed via direct oxygen transfer, which requires strong interaction between compound I and substrates rather than an electron transfer, which can occur at a longer distance for the reactions of HRP compound I. In

any case, the use of a strong electron donor such as DTCO as a substrate favors the electron-transfer pathway of compound I.

**Supporting Information Available:** Dependence of pseudo-first-order rate constant on the concentration of substrate in thioanisole sulfoxidation by HRP-compound I, **1**, and His64Ser Mb compound I, UV−vis spectral changes in the reduction of HRP-compound I and **1** by DTCO (Figures S1−S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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