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Coordination Chemistry Reviews 226 (2002) 219–226

COORDINATION CHEMISTRY REVIEWS

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# Synthetic models for the active site of cytochrome P450

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Received 4 April 2001; accepted 21 September 2001

**Contents**



**Abstract**

As cytochrome P450 models, two kinds of iron porphyrins were synthesized, each of which has an alkanethiolate axial ligand and hydroxyl groups inside the molecular cavities. The stable dioxygen adducts were obtained by the reaction of the ferric complexes with $KO_2$ under an oxygen atmosphere. This study first demonstrated a hydrogen bond to bound dioxygen in an oxy form of a thiolate-coordinated heme model system. These results are discussed in relation to the process of dioxygen binding and activation in cytochrome P450. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Cytochrome P450; Porphyrin; Iron; Oxygen; Thiolate; Hydrogen bond

## 1. Introduction

Cytochrome P450 efficiently utilizes dioxygen to catalyze oxygenation in various biosyntheses of endogenous organic compounds and in detoxification of exogenous ones [1]. This enzyme as a hemoprotein is structurally distinguished from the large majority of hemoproteins by its unique thiolate group as an axial ligand to the heme [2]. The strong electron-donation from the cysteinyl thiolate ligand to the heme in P450 causes distinct spectroscopic features in addition to a critical structural factor for P450's unique reactivity.

In most reactions catalyzed by cytochrome P450s, one oxygen atom of a heme-bound dioxygen molecule is added to a substrate (monoxygenation) via heterolysis of the O–O bond, while the other oxygen atom is reduced to water with two electrons and two protons [3]. Reductive activation of dioxygen by cytochrome $P450_{cam}$, the most well-characterized P450, has been proposed to be assisted by the heme itself as well as by the peripheral amino acids such as Cys357 and Thr252, etc., which are conserved in most P450 isozymes and are considered to be critical for enzymatic oxygen activation. The thiolate group of Cys357 axially coordinates to the central iron atom to strongly donate electrons so that the electron density of the terminal oxygen atom of the bound dioxygen is increased. The hydroxyl group of Thr252 and several water molecules

*Abbreviations:* $TP_{piv}P$, *meso*-tetrakis(α,α,α,α,*o*-pivalamidophenyl)-porphyrin; TMP, *meso*-tetramesitylporphyrin; K222, Kryptofix®222: 4,7,13,16,21,24-Hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane.

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PII: S0010-8545(01)00444-1

constitute a hydrogen bonding network to relay protons to the terminal oxygen atom [4,5]. The cooperative functions (push–pull effect [6]) of these amino acids could enhance the heterolysis of the dioxygen, and depress the homolysis, resulting in the efficient generation of an active oxidizing species. However, the entire molecular mechanism of the catalytic cycle, especially the activation of dioxygen, has not been fully elucidated because all of the responsible intermediates have not yet been identified [7].

Both of these functional and structural distinctions of cytochrome P450 have induced not only extensive studies of the enzyme itself but also synthetic model studies. A variety of thiolate-coordinated heme models has been synthesized [8–13]. To enable coordination of the thiolate ligand to the iron center and to prevent oxidation of the thiolate ligand to the disulfide, either a large excess of the thiolate has been used [8] or a thiolate group has been fixed covalently to the porphyrin via one [9,12] or two [10] linkers. Most of the thiolate-coordinated P450 models without bulky protecting groups around the thiolate axial ligand were found to be fairly sensitive to light and air. It was rather difficult to synthesize a stable P450 model complex with a thiolate axial ligand. Thus, the steric protection of both the thiolate axial ligand and the $O_2$ binding site is indispensable for the model construction.

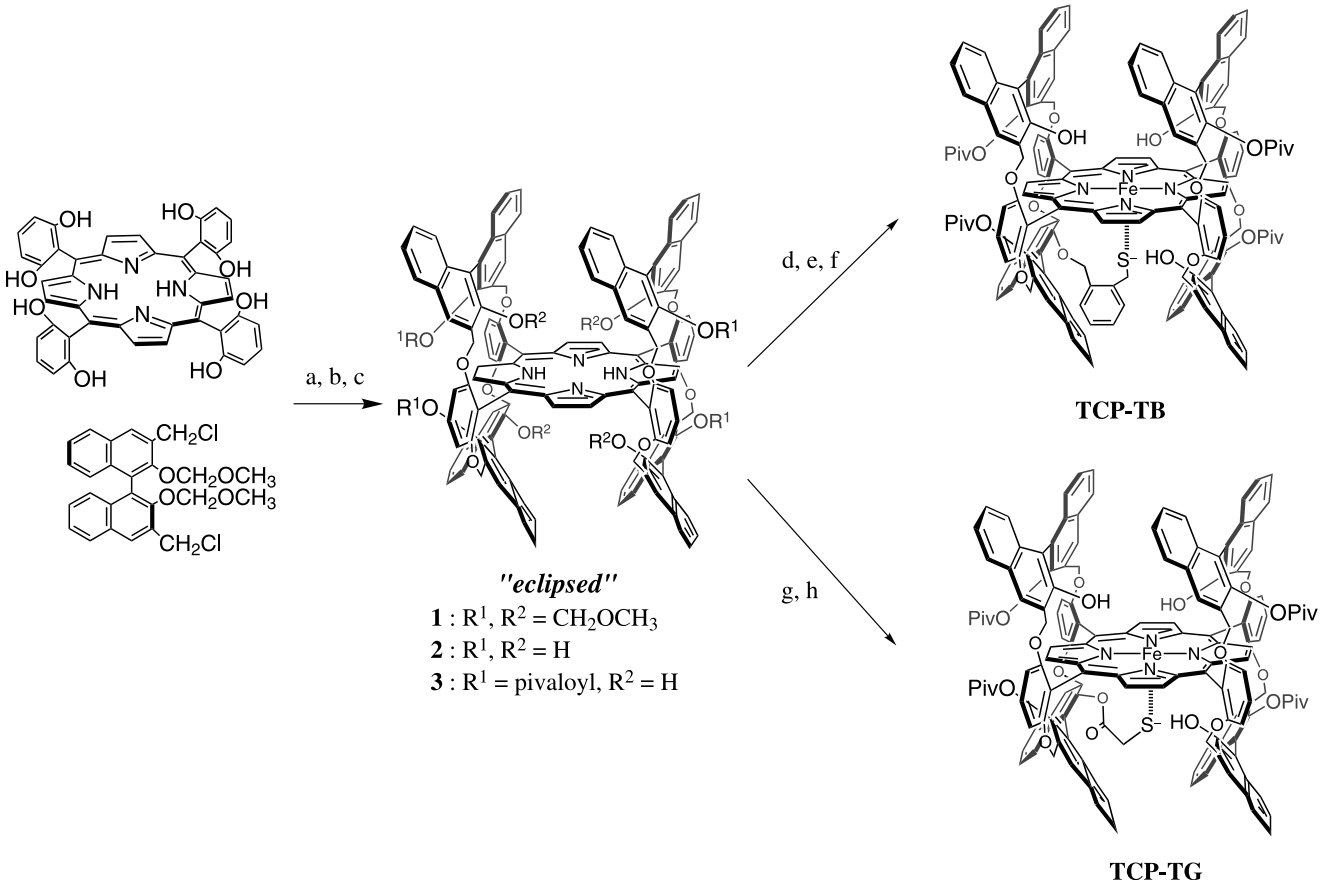
Higuchi and co-workers first prepared a remarkably stable alkanethiolate-coordinated heme model (Swan Resting; SR complex), in which the sulfur atom is sterically protected by bulky pivaloyl groups [12]. The SR complex retains its axial thiolate coordination during catalytic oxidation reactions and shows P450-like catalytic activity by the use of single oxygen donors, such as peroxy acids and hydroperoxides. However, it is unable to afford a stable dioxygen adduct due to the absence of an oxygen binding pocket on the distal side.

The dioxygen adduct of P450 is the key intermediate to be firmly identified in the catalytic oxygen activation process so far [5]. Preparation of an analogous oxy complex has also been attempted in model studies. However, examples of dioxygen adducts of thiolate-coordinated hemes have been severely limited because of their notorious instability [10e,13]. On the other hand, there are a lot of oxy-heme model systems with a nitrogen axial ligand which have been prepared as globin models [14]. Weiss and co-workers reported a sole example of the oxy complex as a P450 model and fully characterized by various spectroscopic analyses [13]. The thermal stability of the oxygen adduct, $Fe–O_2(TP_{piv}P)(C_6HF_4S^-)$, was remarkable; the concentrated solutions could be manipulated at room temperature and could be kept for days at 4 °C. This higher stability was attributed to the strongly electron-withdrawing substituents on the thiophenolate, which in regard to electronic property is greatly different from the electron-donating alkanethiolate ligand in natural P450. However, according to the X-ray crystal structure, the $S^-–Fe–O_2$ geometry of the model is very similar to that of oxy-P450 [5,13b]. In both cases, diatomic oxygen is bound to the heme iron in an end-on bent manner.

Weiss' complex is a good model for the study on the static $O_2$ binding and the physical properties of the oxy complex. However, it is unsatisfactory for the study of $O_2$ activation for the following two reasons: (1) It uses an electron-deficient arenethiolate group, which is different from the alkanethiolate as in the enzyme and would not be suitable for the strong electron demand at the stage of the O–O bond cleavage. (2) There is no direct evidence for a hydrogen bond to the bound oxygen, although a hydrogen bond to the bound oxygen at the distal site is essential in respect of the stabilization of the oxy-intermediate and $O_2$ activation by protonation to the terminal oxygen atom through the proton relay system. Therefore, a model in the next generation should have a structure to satisfy the requisites described above; an alkanethiolate ligand should be sterically protected from undesirable oxidation, and in the distal side, there should be an oxygen-binding cavity with protic residues in an appropriate position. Thus, we designed and synthesized two kinds of alkanethiolate-coordinated model hemes that fulfill the above requisites [15]. The carbon monoxide and dioxygen adducts were formed directly from the ferric complexes and characterized by UV–vis and resonance Raman (RR) spectroscopies. Further, direct evidence for hydrogen bonding to the bound dioxygen was obtained.

## 2. Design and synthesis of model complexes

The following are the most important structural features of **TCP-TB** and **TCP-TG** (Twin Coronet Porphyrins [16] with ThioBenzyloxy and ThioGlycolate groups) (Scheme 1): (1) On both sides of the porphyrin plane, bulky binaphthyl moieties can form hydrophobic molecular cavities, which are suitable for providing an oxygen binding site to prevent possible irreversible autoxidation of the iron center. (2) In one of the cavities, an alkanethiolate group is covalently fixed to coordinate axially to the central iron and is sterically protected from undesirable disulfide formation and other oxidative decomposition. (3) In the opposite cavity, two naphtholic hydroxyl groups are oriented toward the center above the porphyrin with the aim of forming a hydrogen bond to bound dioxygen. (4) To prepare hemes having an alkanethiolate ligand of different steric and electronic characters, two kinds of alkanethiolates were selected. The thiolate part of **TCP-TG** is more compact and less electron-donating due to its




Scheme 1. Reagents and conditions: (a) $K_2CO_3$, THF, NMP, 110 °C, 19%. (b) Trimethylsilyl bromide, $CH_2Cl_2$, −40 °C, 96%. (c) Pivaloyl chloride, pyridine, $CH_2Cl_2$, 97%. (d) 2-(Iodomethyl)benzyl thiobenzoate, $K_2CO_3$, NMP, 100 °C, 62%. (e) $Fe(CO)_5$, $I_2$, toluene, 50 °C, 78%. (f) $BuNH_2$, $CH_3CN$, 48%. (g) Ethyldisulfanylacetic acid, 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride, 4-dimethylamino-pyridine, $CH_2Cl_2$, 36%. (h) $Fe(CO)_5$, $I_2$, toluene, 50 °C, 41%.

electron-withdrawing carbonyl group, compared with that of **TCP-TB**.

The major framework, twin-coronet porphyrin (**TCP**), is synthesized by forming bridges between the *ortho* positions of the adjacent *meso* aryl rings with the binaphthyl derivatives through ethereal linkages (Scheme 1). Next, a protected thiolate moiety is covalently fixed into one of the cavities. Finally, metal insertion and the deprotection of the thiolate group are achieved to afford **TCP-TB** or **TCP-TG**.

## 3. Spectroscopic characterization of the thiolate-coordinated model complexes

EPR spectroscopy has been employed as a useful method to provide various important information on hemoproteins and their models, such as electronic states, magnetic properties, axial ligands and porphyrin ring distortion etc. [17]. EPR spectra of ferric **TCP-TB** and **TCP-TG** in THF at 77 K (Table 1) clearly showed near-axial rhombic low-spin signals. The two crystal field parameters, tetragonality $|\mu/\lambda|$ and rhombicity $|R/\mu|$, both of which are calculated from $g$ values, have been used to characterize rhombic types of low-spin ferric hemoproteins and their model complexes [19]. By Bohan's method [20], two crystal field parameters, $|\mu/\lambda|$ and $|R/\mu|$, were calculated from the three observed $g$ values to be 8.230, 0.414 (**TCP-TB**) and 8.512, 0.370 (**TCP-TG**), respectively. According to the classification of axial donor ligands based on the crystal field parameters [21], these values indicate six-coordinate low-spin ferric hemes with a thiolate axial ligand and an oxygen-donor ligand (THF). Compared with cy-

Table 1
EPR spectroscopic data of thiolate-coordinated hemes

| Heme | EPR $g$ value | | | Crystal field parameter | |
|---|---|---|---|---|---|
| | $g_1$ | $g_2$ | $g_3$ | $\|\mu/\lambda\|$ | $\|R/\mu\|$ |
| **TCP-TB** [a] | 2.334 | 2.210 | 1.959 | 8.230 | 0.414 |
| **TCP-TG** [a] | 2.313 | 2.209 | 1.966 | 8.512 | 0.370 |
| $P450_{cam}$ [b] | 2.45 | 2.26 | 1.91 | 5.998 | 0.469 |

[a] The sixth ligand is THF. Taken from Ref. [15b].
[b] Substrate-free state. Taken from Refs. [18,21].

Table 2
Redox potentials of **TCP-TB**, **TCP-TG**, FeTMPCl [a], and $P450_{cam}$

| Heme | Redox potentials/V, Fe(III)/Fe(II) | |
|---|---|---|
| | vs. $Fc^+/Fc$ [b] | vs. NHE |
| **TCP-TB** [c]; $E_{p/2}$ | −1.35 | (−0.95) |
| **TCP-TG** [c]; $E_{1/2}$ | −1.12 | (−0.72) |
| FeTMPCl [c]; $E_{1/2}$ | −0.73 | (−0.33) |
| $P450_{cam}$ [d]; $E_{1/2}$ | – | −0.33 |

[a] 0.1 M $Bu_4NBF_4/CH_3CN$ with Pt working/counter electrodes, [Fe-por] = 0.5 mM, at scanning rate 50 mV $s^{-1}$.
[b] $Fc^+/Fc$ = ferrocenium/ferrocene.
[c] Ref. [15b].
[d] Ref. [3a].

tochrome $P450_{cam}$, **TCP-TB** and **TCP-TG** showed higher tetragonality and lower rhombicity values, implying relatively high axial symmetry and strong electron donation from the thiolate ligands. Previous alkanethiolate-coordinated model complexes having an oxygen-donor ligand as the sixth ligand have showed very similar EPR spectra [8c,12a].

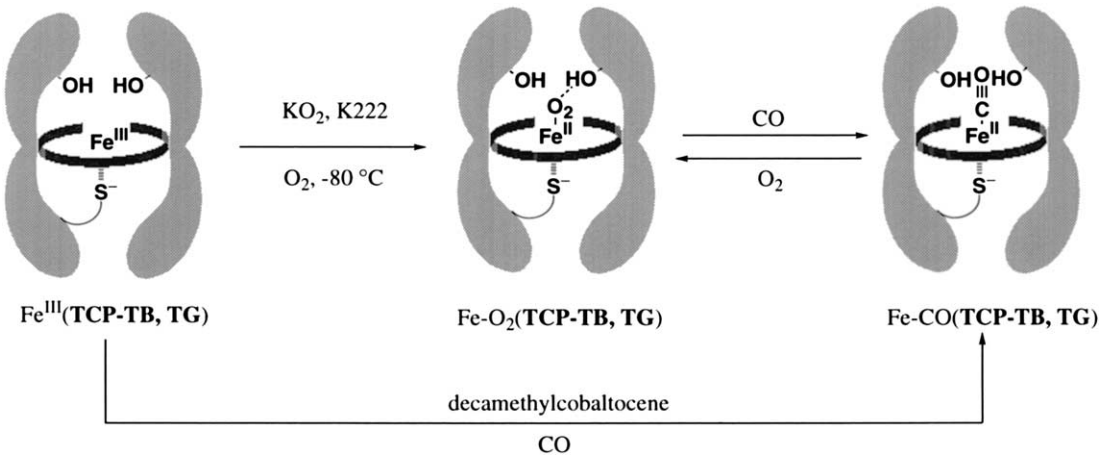
The redox potentials of **TCP-TB** and **TCP-TG** were determined by cyclic voltammetry (Table 2). A quasi-reversible $Fe^{III}/Fe^{II}$ couple was observed in the voltammogram of **TCP-TB**, while **TCP-TG** showed a reversible couple. The slightly positive-shifted potential of **TCP-TG** is due to the coordination of the less electron-donating thiolate ligand relative to that of **TCP-TB**. The more negative potentials of both **TCP-TB** and **TCP-TG** compared with FeTMPCl and cytochrome $P450_{cam}$, indicate the strong electron donation mainly from the alkanethiolate ligands to the iron atoms.

## 4. Formation and characterization of carbon monoxide adducts

CO also binds to cytochrome P450 to inhibit the catalytic activity. The visible spectrum of the CO adduct exhibits a hyperporphyrin spectrum with a unique feature: the Soret band splits to 380 and 450 nm (the origin of the name). This spectroscopic feature has been employed as a criterion for the axial coordination of a thiolate group to a ferrous heme. The split Soret band is due to the strong electron donation of the thiolate ligand [10b,22]. Some of the carbon monoxide adducts of P450 models also show very similar electronic spectra with those of the proteins; for example, $Fe–CO(TP_{piv}P)(CH_3S^-)$ and $Fe–CO(TP_{piv}P)(C_6HF_4S^-)$ exhibited a Soret band at 449 and 451 nm, respectively [8b,13a].

A CO adduct of a heme is usually prepared stepwise via reduction of a ferric form to the ferrous one under aqueous conditions and subsequent addition of CO gas. However, during the reduction of the ferric **TCP-TB** and **TCP-TG** in a protic media, such as an aqueous dithionite solution, the thiolate groups tend to be easily protonated to turn into the corresponding thiol forms with the neutralization of the unstable negative charge [23]. Thus, we applied an aprotic method to obtain the CO adducts directly from the ferric complexes with non-aqueous reductant under CO atmosphere. A THF solution of the ferric **TCP-TB**, **TG** was cooled to − 80 °C under a CO atmosphere and then a small excess of decamethylcobaltocene [24] solution (1.5 molar equiv. to the amount of the heme) as a reductant was added (Scheme 2). A hyperporphyrin spectrum appeared with split Soret bands at 373 and 456 nm for **TCP-TB** and 364 and 452 nm for **TCP-TG** (Fig. 1), indicating the axial coordination of the thiolate group also at the ferrous oxidation state [25].

Resonance Raman (RR) spectroscopy has been a very powerful technique to elucidate the detailed structure of hemoproteins and their model complexes [26]. The CO adduct of **TCP-TB** was further characterized by RR spectroscopy, because **TCP-TB**, which has the




Scheme 2.

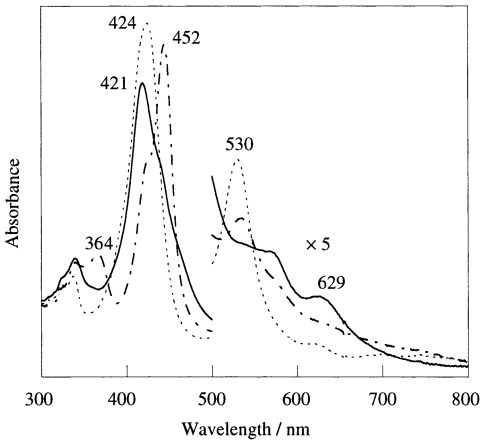



Fig. 1. Electronic spectra of $O_2$, CO and ferric complexes of **TCP-TG** in THF at −80 °C: solid line, $O_2$ complex; dash–dot line, CO complex; broken line, ferric complex.

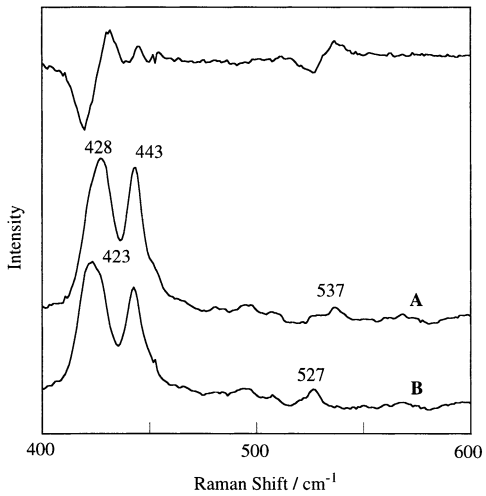



Fig. 2. Low-frequency region of resonance Raman spectra of Fe–CO(**TCP-TB**). THF, −80 °C, 457.8 nm excitation, 20 mW: trace **A**, $^{12}CO$; trace **B**, $^{13}CO$; top, difference spectrum **A**–**B**.

Table 3
RR frequencies for carbon monoxide adducts of hemes

| Heme | $\nu$(Fe–CO) [a] ($cm^{-1}$) | $\delta$(Fe–C–O) [a] ($cm^{-1}$) | Ref. |
|---|---|---|---|
| Fe–CO (**TCP-TB**) | 428 (423) | 537 (527) | [25] |
| Fe–CO ($P450_{cam}$) | 464 (–) | 556 (–) | [27] |
| Fe–CO ($TP_{piv}P$)-($C_6HF_4S^-$) | 479 (474) | – | [28] |
| Fe–CO (hemoglobin) | 507 (503) | 578 (563) | [29] |
| Fe–CO (myoglobin) | 512 (509) | 577 (563) | [29] |

[a] Values in parentheses are for $^{13}CO$ adducts.

stronger donating ligand, can more clearly exaggerate the effect of an axial ligand on the vibrational frequencies of a FeCO unit.

The RR spectrum of Fe–CO(**TCP-TB**) exhibited $\nu$(Fe–CO) band at 428 $cm^{-1}$ (Fig. 2), which shifted to 423 $cm^{-1}$ by replacement of $^{12}CO$ with $^{13}CO$. The observed isotopic shift upon $^{13}CO$ substitution is in good agreement with the value (5 $cm^{-1}$) calculated from the harmonic oscillator approximation of Fe–CO stretching vibration. Compared with the reported $\nu$(Fe–CO) frequencies of substrate-unbound CO-cytochrome P450 [27] and the CO adduct of modeling thiolate heme [28], the observed value is much lower (Table 3). A strong σ-donor *trans* to CO increases the population of Fe $dz^2$ orbital, which is not favorable for Fe–CO σ bonding, indicated by the decrease of $\nu$(Fe–CO). In contrast, a good π-donor, which enhances Fe–CO π-back bonding, increases $\nu$(Fe–CO). Hence, the present anomalously low frequency of $\nu$(Fe–CO) could be due to the extremely strong σ-donation from the thiolate axial ligand to the iron atom, as already shown by the electrochemical data.

The other weak CO sensitive band assignable to $\delta$(Fe–C–O) was also observed at 537 $cm^{-1}$ (Fig. 2) and shifted to 527 $cm^{-1}$ on $^{13}CO$ substitution. This band showed the two similarities with $\delta$(Fe–C–O) of the CO complexes of P450 [27], hemoglobin [29] and myoglobin [29] (Table 3); (1) higher in frequency and weaker in intensity than the corresponding $\nu$(Fe–CO), (2) a larger $^{12}CO/^{13}CO$ shift ($\Delta = 10$–$15$ $cm^{-1}$) than that of $\nu$(Fe–CO). The simultaneous enhancement of $\nu$(Fe–CO) and $\delta$(Fe–C–O) was observed in the CO adduct of cytochrome P450 [27]. It has been known that the activation of $\delta$(Fe–C–O) mode in RR spectrum requires symmetry lowering [30]. The observation of $\delta$(Fe–C–O) mode in Fe–CO(**TCP-TB**), which has no symmetry, is consistent with this argument. In contrast, $\delta$(Fe–C–O) mode was not detected in the previous thiolate-coordinated model of high symmetry [28].

## 5. Formation and characterization of dioxygen adducts

As already mentioned in the formation of the CO adducts, it is necessary to avoid the protonation of the thiolate groups in the ferrous forms of **TCP-TB** and **TCP-TG**. We have obtained dioxygen adducts directly from the ferric forms of **TCP-TB** and **TCP-TG** by addition of an equimolar amount of $KO_2$ [31] under an oxygen atmosphere at −80 °C (Scheme 2). In the reaction, the intensity of the Soret band slightly decreased and the Q-band around 530 nm significantly diminished (Fig. 1). When the atmosphere was replaced with CO, the dioxygen adducts were converted to the corresponding CO adducts, which exhibit typical hyperporphyrin spectra with split Soret bands. The reversibility between the $O_2$ and CO adducts indicates that the

ferrous oxidation state and the axial coordination of the thiolate remain under these conditions without any autoxidation or other oxidative decomposition.

Despite the coordination of the strongly electron-donating alkanethiolate anions, these dioxygen adducts exhibited high thermal stability, especially in the case of **TCP-TG**. The UV–vis spectra of the adducts were stable and unchanged at elevated temperatures up to −20 °C for **TCP-TB** and 0 °C for **TCP-TG**. The difference in thermal stability between the two complexes could be due to the difference in electron density at the iron resulting from the strength of the electron donation from the axial thiolate ligand.

We have applied RR spectroscopy to the dioxygen adducts of **TCP-TB** and **TCP-TG** in order to confirm the existence of the Fe–$O_2$ moiety. The RR spectra of the dioxygen adducts obtained with Soret excitation (413.1 or 441.6 nm) exhibited a strong line at 1138 $cm^{-1}$ for **TCP-TB** and 1137 $cm^{-1}$ for **TCP-TG** (Fig. 3). By replacement of $K^{16}O_2$ and $^{16}O_2$ with $K^{18}O_2$ and $^{18}O_2$, these bands shifted to 1074 and 1073 $cm^{-1}$, respectively. The observed isotopic shifts upon $^{18}O_2$ substitution are in good accordance with the values calculated from the harmonic oscillator approximation of the O–O stretching vibration ($\Delta_{obs}(^{16}O_2/^{18}O_2) = 64$ $cm^{-1}$; $\Delta_{calc} = 65$ $cm^{-1}$). These bands are therefore assigned to the $\nu$(O–O) mode. The observed frequencies of the $\nu$(O–O) modes are very close to those of oxy-cytochrome $P450_{cam}$ [32,33] and oxy-model complexes [28,32] (Table 4). A Raman active $\nu$(O–O) mode has been known to be characteristic of the dioxygen adduct of a thiolate-coordinated heme. In contrast, the $\nu$(O–O) mode of an oxy-heme with an axial ligand of a nitrogen-base has been found to be Raman inactive [34]. RR enhancement of $\nu$(Fe–$O_2$) and $\nu$(O–O) was observed in a thiolate-coordinated oxy heme and explained as follows [35]; a thiolate ligand increases the electron density of porphyrin π* through $p_\pi$ and $d_\pi$ to promote the charge-transfer transition from the π* to the empty Fe–O σ* orbital of the oxy form, resulting in the enhancement of the $\nu$(O–O) band. Therefore, the present RR results show successful formation of the dioxygen adducts of the thiolate-coordinated hemes.

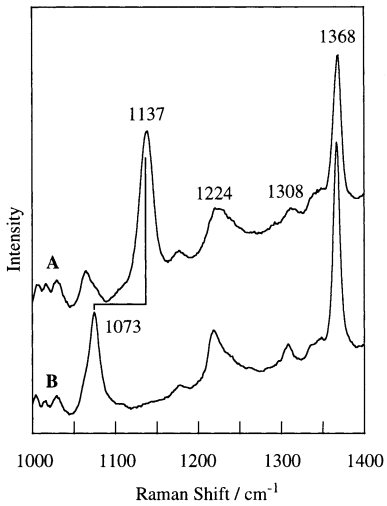



Fig. 3. High-frequency region of resonance Raman spectra of Fe–$O_2$(**TCP-TG**). THF/$CH_3CN$, −60 °C, 413.1 nm excitation, 20 mW: trace **A**, $K^{16}O_2 + {}^{16}O_2$; trace **B**, $K^{18}O_2 + {}^{18}O_2$.

Table 4
RR $\nu$(O–O) frequencies for dioxygen adducts of thiolate-coordinated hemes

| Heme | $\nu$(O–O) [a] ($cm^{-1}$) | Ref. |
|---|---|---|
| Fe–$O_2$ (**TCP-TB**) | 1138 (1074) | [15] |
| Fe–$O_2$ (**TCP-TG**) | 1137 (1073) | [15] |
| Fe–$O_2$ ($P450_{cam}$)/camphor | 1140 (1074) | [32,33] |
| Fe–$O_2$ ($P450_{cam}$)/adamantanone | 1147 (1080) | [32] |
| Fe–$O_2$ ($TP_{piv}P$)($C_6HF_4S^-$) | 1140 (1080) | [28,32] |
| Fe–$O_2$ ($TP_{piv}P$)($C_6F_5S^-$) | 1147 (1084) | [32] |

[a] Values in parentheses are for $^{18}O_2$ adducts.

## 6. Evidence for hydrogen bonding to bound dioxygen

The thermal stability of these dioxygen adducts was also confirmed by RR spectroscopy. The $\nu$(O–O) bands were successfully observed at −20 °C (**TCP-TB**) and 0 °C (**TCP-TG**), respectively. We postulated that an intramolecular hydrogen bonding between the bound oxygen and the hydroxyl groups of the binaphthalene moieties was mainly responsible for this higher stability. To verify this prediction, the frequencies of the O–O stretching modes of the dioxygen adduct upon deuterium substitution of the exchangeable protons were investigated. Before the reaction with $KO_2$, ferric **TCP-TB** and **TCP-TG** were kept in $CH_3OH/CH_3CN$ or $CD_3OD/CH_3CN$ for several hours and dried in vacuo overnight. In the IR spectra of ferric **TCP-TB** and **TCP-TG**, $\nu$(O–H) signals were replaced with lower $\nu$(O–D) bands upon H/D exchange treatment and no other bands were shifted. In the RR spectra of both oxy-**TCP-TB** and **TCP-TG**, only the $\nu$(O–O) modes were up-shifted by 2 $cm^{-1}$ upon exchange of $CH_3OH$ by $CD_3OD$ (Fig. 4). No other vibrations exhibited frequency shifts. These results indicate that there is no perturbation in the porphyrin skeleton upon H/D exchange. We therefore conclude that the bound oxygen in **TCP-TB** and **TCP-TG** interacts with the adjacent exchangeable protons and that the dioxygen adducts of these complexes are stabilized by the hydrogen bonds between the dioxygen and the inner hydroxyl groups, as expected in the molecular design.

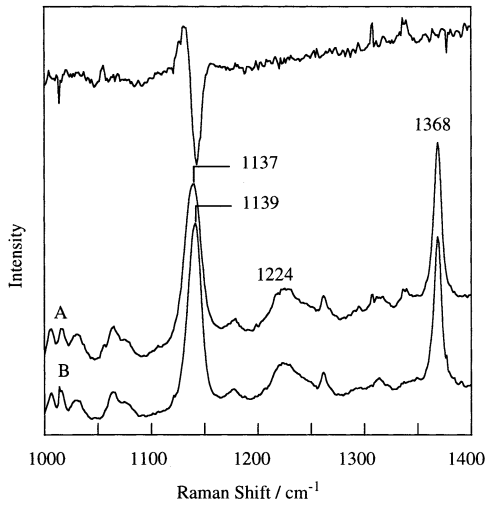



Fig. 4. Selected region of resonance Raman spectra of Fe–$O_2$(**TCP-TG**) on H/D exchange experiment. THF/$CH_3CN$, −80 °C, 413.1 nm excitation, 20 mW: trace **A**, $CH_3OH$; trace **B**, $CD_3OD$; top, difference spectrum **A**–**B**.

## 7. Conclusion

Synthesis and characterization of novel iron porphyrins, designated **TCP-TB** and **TCP-TG**, with an alkanethiolate axial ligand were carried out as models for the active center of cytochrome P450. The stable dioxygen adducts of **TCP-TB** and **TG** were obtained by reaction of the ferric complexes with an equimolar amount of $KO_2$ under an oxygen atmosphere. The frequencies of $\nu$(O–O) bands in the RR spectra were determined to be 1138 $cm^{-1}$ for **TCP-TB** and 1137 $cm^{-1}$ for **TCP-TG**. Finally, direct evidence for hydrogen bonding of the bound dioxygen to the distal hydroxyl groups were obtained also by RR spectroscopy.

It has been long recognized that the hydrogen bonding to bound dioxygen can stabilize the oxy-complexes of globins and their nitrogen-base coordinated models [14,36]. On the other hand, an analogous hydrogen bond in oxy-cytochrome P450 has been very recently shown by cryocrystallographic methods [5]. To the best of our knowledge, the present study also shows the first clear evidence for a hydrogen bonding to dioxygen in a thiolate-coordinated model. The results obtained have significance in studies of the process of binding and activation of dioxygen by cytochrome P450. Based on these dioxygen adducts, our model complexes open the way to further studies on the identification of the corresponding peroxy- and high-valent oxo-heme.

## Acknowledgements

This research was financially supported by Grant-in-Aids for COE Research (#08CE2005 to Y.N.), for Scientific Research on Priority Areas (#09235225 and 11228207 to Y.N.), and for Encouragement of Young Scientists (#08740500 to F.T.) from the Ministry of Education, Science and Culture, Japan and by Research Grants to Y.N. from the Sumitomo Foundation and to F.T. from the Takeda Science Foundation and Otsuka Chemical Co. Ltd.

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