# L-arginine oxidation with Hydrogen peroxide catalyzed by synthetic heme-thiolate complex relevant to Nitric oxide synthase

Monalisa Mukherjee*

**Abstract**—This work describes the synthetic heme-thiolate (SR complex) catalyzed biomimetic oxidation of L-arginine with hydrogen peroxide. The result showed that the SR complex catalyst was efficient for the release of nitric oxide (NO·) and citrulline formation on the oxidation of L-arginine with hydrogen peroxide. The thiolate ligation of SR complex during oxidation reaction was confirmed by EPR spectrum after and before the reaction. SR complex which is a stable thiolate-ligated iron porphyrin, mediated the oxidation of L-arginine much more preferentially than iron porphyrins coordinated by imidazole or chloride.

**Index Terms**—L-arginine, Heme-thiolate, Hydrogen peroxide, Iron porphyrin, Catalyst, NO-Synthase, Oxidation

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## 1 INTRODUCTION

Cytochrome P450 and NO synthase (NOS) among heme enzymes have strong oxidizing ability and unusual structure, in that their heme irons have thiolate coordination [1-6]. Nitric oxide synthase, unusual members of the cytochrome P-450 family, catalyze the five-electron oxidation of L-arginine to nitric oxide (.NO) and citrulline at the expense of NADPH and $O_2$ [7,8]. Nitric oxide a messenger molecule, participates in important physiological processes, such as vasodilatation [9], neuronal signal transmission [10], cytotoxicity against pathogens and tumors [11], and cellular respiration activity [12]. Metalloporphyrins are generally involved to prepare artificial mimics of heme proteins. Attempts to model Nitric oxide synthase using iron (III) porphyrins [13,14] suffer from some lacunae, most of them ignored L-arginine as the starting substrate for their models. There is inadequate information concerning the direct oxidation of L-arginine with hydrogen peroxide in the presence of stable thilote-ligated iron porphyrin as catalyst. We have synthesized the synthetic heme thiolate (SR complexes) which retains thiolate coordination during catalytic oxidation of L-arginine (Scheme I). Organic peroxide, or more rarely environmental friendly oxidant $H_2O_2$ are known to be a substitute for NADPH and $O_2$ in this catalytic cycle [15]. The introduction of ferryl group from Fe+3 in this complex requires the treatment of catalyst with hydrogen peroxide. Therefore the idea of the shunt mechanism was opted by using peroxide, to carry out these oxidation reactions.

## 2 EXPERIMENTAL

### 2.1 Materials

All chemicals and reagents used in this study were of analytical grade and procured from Sigma Aldrich, Fluka and Spectrochem Pvt. Ltd. Organic solvents were freshly distilled before use. Deuterated solvents were purchased from Aldrich and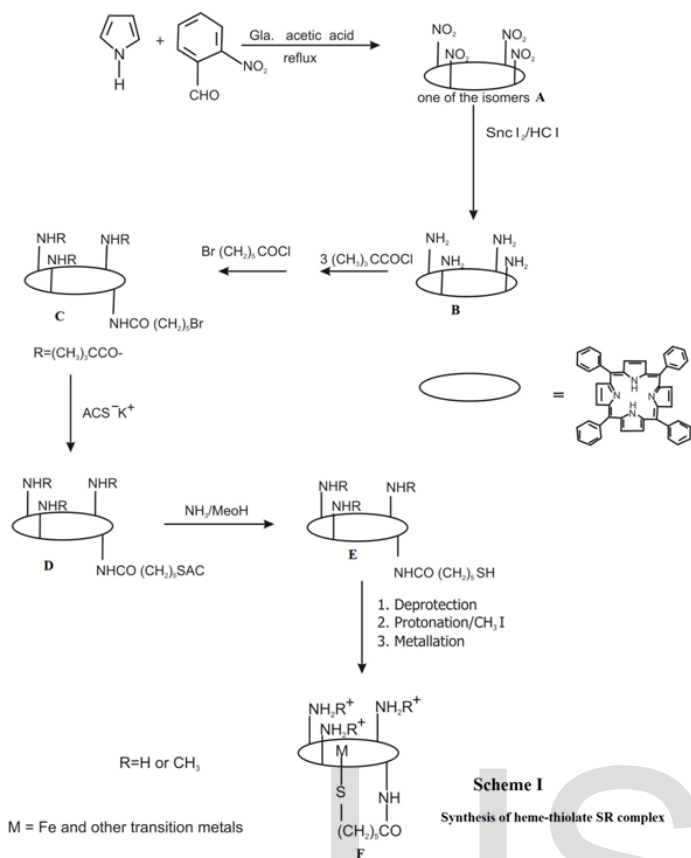 used without purification. Distilled water was obtained from Milli-Q water purification system (Millipore). Griess reagent used in the reactivity study was prepared freshly by mixing aliquots of 1% sulphanilamide in 4 N HCl and 0.2 ml of 0.1% N-(1-naphthyl) ethylenediamine in 0.4 N HCl and their absorption was measured at 543nm.

### 2.2 Physical Measurements

IR spectra were recorded on (5-Dx) Nicolet FTIR instrument with KBr pellets. NMR spectral characterization was carried out with a Bruker DPX 300 MHz instrument with tetramethylsilane as internal standard for chemical shift measurements. UV-Vis spectral measurements were taken with a Perkin-Elmer $\lambda_{2S}$ spectrophotometer interfaced with a 486 digital computer. The cell holder of the spectrophotometer was connected to a Julabo F-30 temperature regulator. Absorbances against time were recorded by using PECSS software developed by Perkin Elmer. X-ray diffraction patterns of powdered samples were obtained, using Bruker D8 advanced diffractometer equipped with a rotating anode and Cu Kα radiation. HPLC measurements were done by reverse phase HPLC, of o-phthalaldehyde (OPA) derivatives with SF 970 fluorescence detector equipped with a deuterium lamp. The OPA derivatives were detected with a monochromator set at around 330nm(excitation wavelength) and around 418nm(emission wavelength) cut-off filter. Column used was Ultrasphere ODS 25cm X 4.6mm I.D., 5μm particle size.

### 2.3 Catalyst Preparation

Condensation of pyrrole and four equivalents of o-nitrobenzaldehyde in acetic acid gave meso-tetra(o-nitrophenyl)porphyrin which was reduced by stannous chloride to the meso-tetra(o-aminophenyl)porphyrin (Scheme I). The four atropisomers were separated by chromatography, the slowest moving of which was the desired isomer A shown in the scheme I.

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**Scheme I**

**Synthesis of heme-thiolate SR complex**

porphyrins FeTPAP and FeTMPyP were synthesized with modified literature procedure [17-21]. These ionic porphyrin complexes were loaded on counter ionic dowex resin. Imidazole covalently bound to iron porphyrin are synthesized and investigated by literature procedures [22-23].



**Scheme II**

**Variartions in peripheral postions were as follows**

Interconversion of the atropisomers was sufficiently slow at room temperature to afford clean separation. Refluxing the unwanted products in toluene for 20 min effected reequilibration to the statistical mixture allowing further isolation of the desired isomer. Reaction of the amino groups with pivaloyl chloride( trimethylacetyl chloride) gave the "picket-fence" porphyrin in which the configuration is frozen by the bulky substituents. Reaction with omega bromo acetoyl chloride gives the compound C, which on treatment with potassium salt of thio-acetic acid gives the compound D. D further reacts with ammonia in presence methanol to give compound E. Finally compound E undergoes de-protection, followed by protonation in presence of methyl iodide and metallation with iron bromide to give desired SR complex F.  Since the S-protected pentanoic acid derivatives were more difficult to obtain, the bromoalkyl chain was first attached to the porphyrin to give C and the thio group was introduced by treatment with ACS-K+. Deacetylation (MeOH/NH₃) gave the free thiol. The reaction of compound B with excess isonicotinic chloride hydrochloride yielded the tetra-isonicotinamide TPP as a statistical mixture of atropisomers which could be separated by chromatography. The isonicotinamide groups were then methylated with methyl iodide, followed by anion exchange, to yield the water soluble tetrakis-(N-methylisonicotinamidophenyl) porphyrin-tetracation. This compound can undergo remaining steps to yield water soluble heme-thiolate complex as shown in Scheme I. SR-Imidazole complex was prepared as per literature procedure [16].

Two anionic porphyrins FeTPPS and FeTCP and two cationic
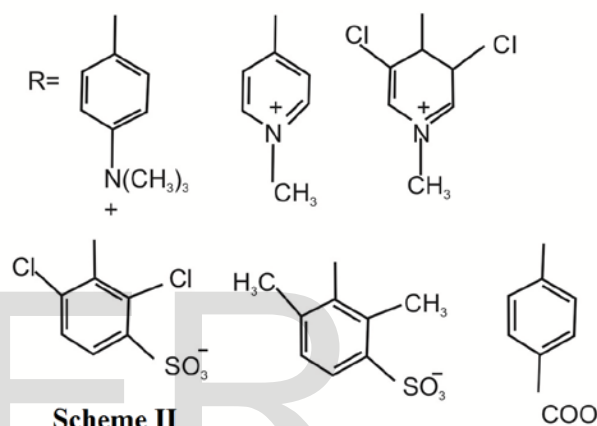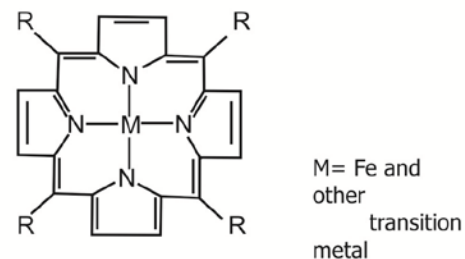
## 2.4 Catalysis

The The nonthiolated and thiolated porphyrins (20 mg) was taken in a cuvette and 1 ml of L-arginine (50 mM) was added with a pipette. The reaction was initiated by adding 0.5 ml of $H_2O_2$ (100 mM) and continued for 20min at 25°C. $NO_2^-$ formation was determined by an automated procedure based on Griess reaction. The contents were shaken for a minute and the absorbance at 543 nm was noted. Nitrite standard reference curve was prepared in water in high concentration range as well as in low concentration range. The average absorbance value of each concentration of nitrite standard was plotted against nitrite concentration. The amount of ($NO_2^-$) released was determined from the nitrite standard reference curve.

Amino acid product was identified using two different techniques.

RP-HPLC of OPA derivative. The derivatization procedure was followed as described by Umagat et al. [24]. The product was analyzed with good selectivity and sensitivity by reversed-phase high-performance liquid chromatography. 10μl of the derivatized sample was injected onto the column. The mobile phase was linear step gradient with a ternary solvent mixture, THF/ 0.05mol/L sodium acetate, pH 6.6/ methanol. The flow rate for this method was 1.5ml/min. Amino acid standards were used to quantify samples. 2-aminoethanol was

used as an internal standard. Detection was generally possible in picomole range.

The formation of amino acid was also identified using NMR probe. NMR spectra of L-arginine and L-citrulline procured from the market were recorded in $D_2O$ in presence of SR complex. The spectra are shown in fig.1a &1b. 20mg of axial thiolated water-soluble porphyrin was used for these experiments because of the limitation in NMR tube. 5mg of L-arginine dissolved in 0.6 ml $D_2O$ was added. Finally 6.4 μl of aqueous 30% $H_2O_2$ was added to initiate the reaction. The contents of the tube were shaken for taking the NMR spectrum.
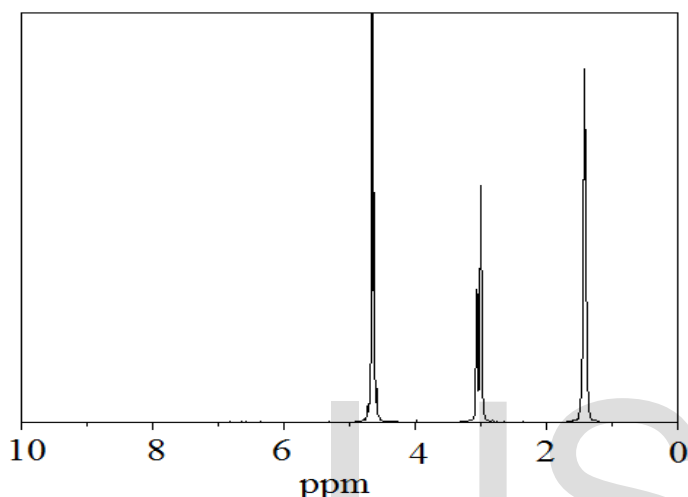


Fig.1a. NMR spectra of L-arginine(5mg) + heme-thiolate complex (20mg) + $D_2O$(0.6ml)

### 2.5 Formation of nitrite ($NO_2^-$) as a function of hydrogen peroxide concentration in the oxidation of L-arginine with SR complex

The nonthiolated and thiolated porphyrins oxidation reactions of L-arginine were carried out at 25°C by varying the $H_2O_2$ concentration using heme-thiolated (SR complex). L-arginine (35 μg, 200 μM) in 1ml deionized water and SR-complex (20 mg, 0.2752 μmol axial thiolated porphyrin, SR complex) were taken in a vial pre-occupied with a magnetic stir-bar. In each experiment after the addition of aqueous 30% $H_2O_2$, the reaction mixture was stirred for 5-6 min. Finally the reaction mixture was transferred to the cuvette containing Griess reagent. The cuvette was shaken for a minute and the absorbance was measured at 543 nm. From the calibration plot of nitrite, the concentration of nitrite corresponding to measured absorbance value was obtained.
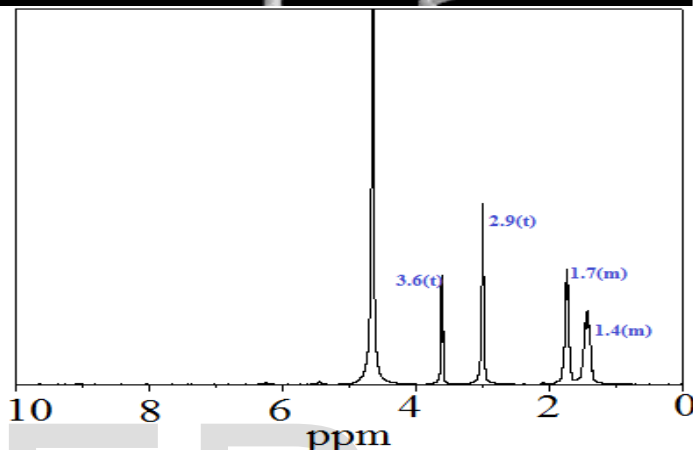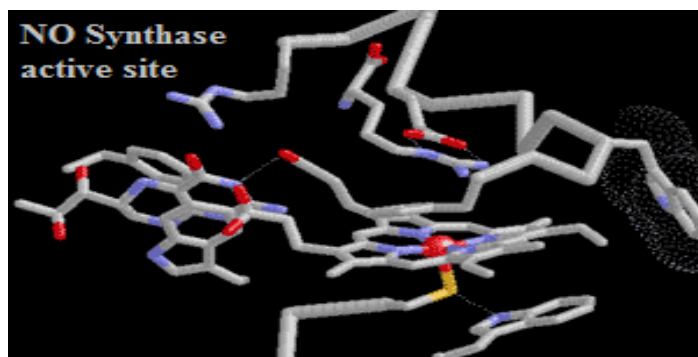


Fig.1b. NMR spectra of L-arginine(5mg) + 30% aqueous $H_2O_2$(6.4μl) + $D_2O$(0.6ml) + Heme-thiolate complex (20mg)

### 2.6 Stoichimetry of L-arginine consumption and product formation

The reaction mixture contains: 38.7 mM $H_2O_2$, 200 μM L-arginine in 1ml deionized water and heme-thiolate SR complex (20-50mg). After addition of aqueous 30% $H_2O_2$, the reaction was continued for 20 min at 25°C. Finally 2 ml of Griess reagent (freshly prepared) was added to it. The contents were shaken for a minute and the absorbance at 543 nm was noted. Nitrite standard reference curve was prepared in water in high concentration range as well as in low concentration range. The average absorbance value of each concentration of nitrite standard was plotted against nitrite concentration. The amount of ($NO_2^-$) released was determined from the nitrite standard reference curve. Amino acid product was analyzed as described in section **2.4.** Concentration of L-arginine and Citrulline were determined from their standard curves (concentration vs. peak area) using HPLC methodology.

## 3 RESULT AND DISCUSSION

The most successful of the heme protein active site models is the "picket-fence" porphyrin. Steric encumbrance about the metal site of these substituted TPP( tetra phenyl porphyrin) molecules depends on two factors:

(i) due to steric repulsion, the TPP will adopt a conformation in which the four meso- phenyl rings are essentially perpendicular to the porphyrin ring; substituents at the orr/io-positions of the phenyl rings will lie above and below the por-

phyrin plane, and

(ii) for TPP molecules containing mono-ortho-substituted phenyl rings, separation and, depending on the bulk of the subsituent, interconversion of the four possible atropisomers may be achieved. The synthesis of a substituted iron(II) tetraphenylporphyrin having four pivalamido groups located on the same side of the porphyrin ring would give a "protected pocket". Ligands e.g. imidazole, could bind to the metal on the open face but could not penetrate the pocket, thereby ensuring five-coordination even in the presence of excess ligand. The formation of amino acid (citrulline) and inorganic product ($NO_2^-$) from L-arginine and $H_2O_2$ was determined as a function of the amount of the SR catalyst. Each sample was analyzed separately for citrulline and nitrite. Amount of NO released from L-arginine with $H_2O_2$ using the ctalysts were monitored spectrophotometrically at 543 nm with time. Griess reagent reacts with $NO_2^-$ to produce a chromophore which absorbs at 543nm. The formation of $NO_2^-$ produced from L-arginine and $H_2O_2$ was observed with time at 25°C for the thiolated and nonthiolated catalyst (data not shown). The result showed that the release of nitric oxide was instantaneous, when thiolated iron porphyrin was used. The reaction with iron porphyrins coordinated with chloride or imidazole is very slow process compared to the thiolated one. Most likely, hydrogen peroxide reacts with the catalyst to form high valent iron oxo intermediate, which contains ferryl group $[Fe=O]+3$, responsible for the oxidation of L-arginine. The dependence of formation of $NO_2^-$ on the concentration of hydrogen peroxide was determined for the heme-thiolate SR complex catalyzed oxidation of L-arginine at 25°C. All reactions were continued for six minutes in order to collect data for each concentration. The product formation was linear with time over this interval and after that deviates from linearity. The results of these reactions are shown in fig.2. The data presented were average of duplicate set of experiments. This study was done to determine the kinetics (or kinetic parameters) of $H_2O_2$ dependent oxidation of L-arginine.
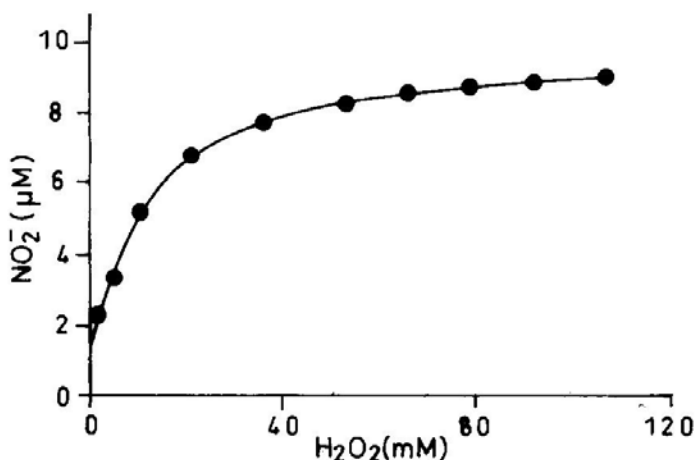


Fig.2. Formation of nitrite ($NO_2^-$) as a function of hydrogen peroxide concentration in the oxidation of L-arginine with heme-thiolate (SR complex)

The formation of citrulline and $NO_2^-$ were stoichiometric to the amount of L-arginine consumed. Within experimental error, for every mol of arginine consumed, one mol each of citrulline and $NO_2^-$ were formed shown in Table 1. The amino acid product of the reaction was shown to be L-citrulline. L-arginine and L-citrulline were eluted at 23.7 min and 21.4 minutes respectively. 2-aminoethanol (internal standard) was eluted at 32 minutes. No evidence was suggestive of the formation of any other amino acid product. Chromatographic identification of citrulline was made by reverse-phase HPCL of OPA derivative and agreed with the retention time (fig.3) of identically treated citrulline standard. Unequivocal evidence of citrulline formation was also obtained from NMR spectroscopy. Identification of citrulline can be readily made by monitoring its peak at 3.6 (t), 2.9(t), 1.74-1.66(m) and 1.4 – 1.3(m), which clearly distinguishes its presence even in presence of L-arginine.
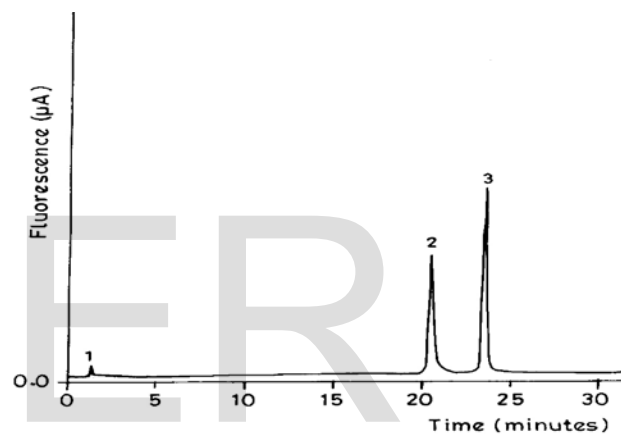


Fig.3. Elution profile of OPA-derivatized amino acid product from oxidation of L-arginine of $H_2O_2$ dependent reaction catalyzed by heme-thiolate SR complex.

No evidence was to support for the formation Nδ-hydroxy-L-arginine (L-NHA) in this oxidation reaction. This may be due to the instability of L-NHA in presence of excess peroxide in the reaction mixture. The activity of the catalyst was indicated by the degree of L-arginine conversion. SR complex showed better performance than the iron porphyrin coordinated by chloride or imidazole. The yields of L-citrulline and $NO_2^-$ were relative to the substrate L-arginine used. $NO_2^-$ and citrulline formation in the 2nd did not vary much from first run. The filtrates were used for determination of iron leaching. The thiolate ligation of SR during the oxidation reaction was confirmed by measuring the EPR spectrum before and after the reaction at 2788. There was no change in the FT–IR spectra and XRD pattern of the recovered catalyst (data not shown).

TABLE 1

Stoichiometry of citrulline to $NO_2^-$ formation from L-arginine using heme-thiolate SR complex

| Experiment | heme-thiolate Complex(mg) | [citrulline][b] formation (μM) | [NO$_2^-$][d] formation (μM) | [arginine][c] consumption (μM) | [citrulline]: [NO$_2^-$] |
|---|---|---|---|---|---|
| 1 | 20[e] | 7.6 | 7.2 | 7.7 | 1.06 |
| 2 | 30[f] | 9.7 | 9.6 | 10.0 | 1.01 |
| 3 | 40[g] | 12.4 | 12.3 | 12.6 | 1.01 |
| 4 | 50[h] | 15.2 | 15.5 | 15.8 | 0.98 |

[a] All values are the average of duplicate measurements.
[b] citrulline formation and [c] arginine consumption were determined by HPLC methodology as described in the experimental section
[d] [NO$_2^-$] was determined by quantitation of NO$_2^-$ with Griess reagent as described in the experimental section
[e] 0.275 μmol of heme-thiolate complex was loaded
[f] 0.412 μmol of heme-thiolate was loaded
[g] 0.550 μmol of heme-thiolate was loaded
[h] 0.688 μmol of heme-thiolate was loaded

# 4  CONCLUSION

In summary a stable model for nitric oxide synthase activity using shunt mechanism has been developed, which showed the formation of nitrite and citrulline. The model consists of water-soluble heme-thiolate SR complex. This system is stable under the treatment of peroxide devoid of the degradation of the porphyrin moiety (active site). SR complex can be stored at room temperature under air for several months. L-arginine, instead of N$^\delta$-hydroxy-L-arginine (L-NHA) was taken as the substrate for oxidation. It is highly probable that thiolate axial ligation alters the reactivity of the active intermediate of heme so as to make L-arginine oxidation much more favorable than chloride or imidazole axial ligation. The acceleration of the catalytic reaction by thiolate ligation is undoubtedly due to the enhancement of O-O bond scission because the concentration of peroxides is high so O-O bond cleavage step is the rate determining. The cyclic voltammogram of SR complex in DMF showed much more negative reversible reduction couple than iron porphyrin coordinated to chloride. The negativity of redox potential of SR is probably due electron donation between thiolate to the iron atom. Further investigation to characterize the structure of active intermediate of heme–thiolate complex spectroscopically is in progress in our laboratory.

# 5  ACKNOWLEDGMENTS

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