Peroxidation of Pyrogallol by Antibody−Metalloporphyrin Complexes

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Antibody 03-1, which was prepared by immunization with *meso*-tetrakis(4-carboxyphenyl)porphyrin (TCPP) conjugate, has been found to bind strongly to Mn(III)−TCPP and Fe(III)−TCPP complexes with dissociation constants of 4.1×10^{-7} and 1.5×10^{-7} M, respectively, although other monoclonal antibodies raised against TCPP did not bind to these TCPP−metal complexes. The complexes of antibody 03-1 with Mn(III)−TCPP and Fe(III)−TCPP were found to catalyze oxidation of pyrogallol selectively. A Lineweaver-Burk plot for the oxidation of pyrogallol by the antibody−Fe−TCPP complex showed $K_m = 4.0$ mM and $k_{cat} = 50$ min^{-1}. Studies on the effect of the molar ratio of the antibody to metalloporphyrin on the catalytic activity showed that a 1:1 complex was the most effective for the reaction. The effect of salt (NaCl) on the reaction showed that electrostatic interaction between the antibody and the metalloporphyrin was important for the reaction. The antibody−metalloporphyrin complexes are stable enough to show catalytic activity in the presence of an excess amount of H_2O_2.

Introduction

Recently, much attention has been focused on catalytic antibodies.[1] Most catalytic antibodies have been prepared using "transition state analog compounds" as haptens. However, since antibodies are proteins composed of about 20 different amino acids, reactions promoted only by antibodies are limited.

On the other hand, a large number of metal complexes and organometallic compounds have been prepared and found to have catalytic activities,[2] and much effort has been devoted to improvement of their reactivities and selectivities. For development of highly reactive and selective catalysts, the environment of metal complexes must be manipulated. One of the most effective methods for changing the environment of metal complexes is to use the concept of the second sphere coordination.[3] Previously, we used cyclodextrins as second sphere coordinating ligands and found that they formed complexes with various organometallic compounds and had catalytic effects on metal-promoted reactions.[4] However, since cyclodextrins are ready-made hosts, the sizes and shapes of their cavities are limited.

Antibodies are unique in their specificities and diversities. We have focused our attention on monoclonal antibodies for metal complexes. One of the most important families of metal complexes is the metalloporphyrins, which function as oxygen carriers, redox enzymes, and photosynthetic mediators. Synthetic porphyrins and metalloporphyrins should function well when they are suitably incorporated into protein domains. One of the most convenient methods to incorporate porphyrins and metalloporphyrins into protein matrices is by preparation of monoclonal antibodies for porphyrins.

Previously, we prepared various monoclonal antibodies against *meso*-tetrakis(tetracarboxyphenyl)porphyrin[5] and showed that these monoclonal antibodies formed complexes with porphyrins and metalloporphyrins specifically.[6,7] We carried out spectroscopic studies on TCPP in the presence of the antibodies and found that one of the antibodies caused large shifts of the Soret bands and *Q* bands and induced Cotton effects. Later, Keinan et al. reported that antibodies against [*meso*-tetrakis(4-[2-carbonylvinyl]phenyl)porphinato]tin(IV) dihydroxide bind to corresponding porphyrins and cause some spectroscopic changes.[8]

Keinan et al. found that these antibody−metal complexes have catalytic effects on epoxidation reactions of styrenes.[9] Cochran and Schultz reported the oxidations of various substrates by a complex of antibody for *N*-methylmesoporphyrin with mesoporphyrin−metal complexes.[10] Catalysis of metalation of mesoporphyrin by the antibody has also been observed.[11] Moreover, metal-selective antibodies for metalloporphyrins have been reported.[12] In this study, we found that an antibody−metalloporphyrin complex had selective catalytic activity on the oxidation of pyrogallol and compared this with the catalytic activity of horseradish peroxidase.

Results and Discussion

Bindings of Metalloporphyrins to Monoclonal Antibodies. The bindings of metalloporphyrins to monoclonal antibodies were studied by enzyme-linked immunosorbent assay (ELISA). One (03-1) of four antibodies against TCPP strongly bound not

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Table 1. Dissociation Constants of Antibody 03-1 and Porphyrins[a]

	dissociation constants/M		
	TCPP	TCPP−Mn	TCPP−Fe
0.3-1	7.0×10^{-8}	4.1×10^{-7}	1.5×10^{-7}

[a] Estimated by fluorescence spectroscopy (excitation wavelength, 280 nm) assuming 1:1 complex formation.

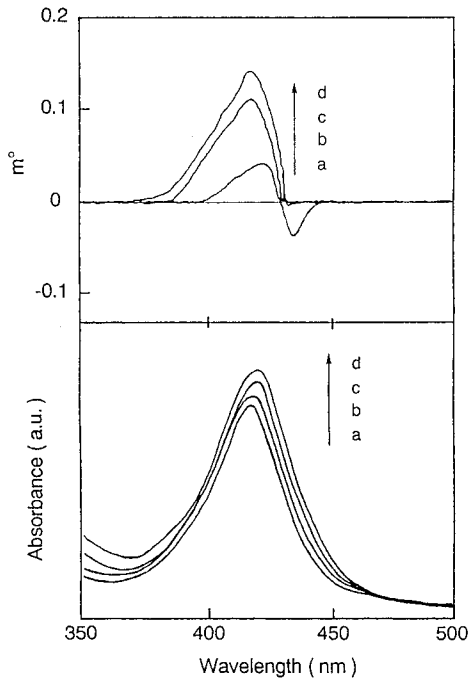
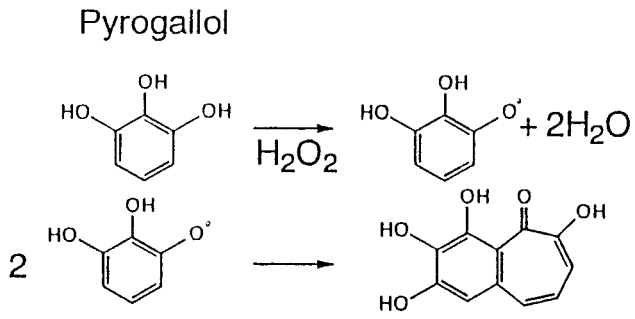


Figure 1. Absorption spectra and circular dichroism spectra of Fe(III)−TCPP in the presence of antibody 03-1(Fab) in phosphate borate buffer (PBB) at pH 9.0. [Fe(III)−TCPP] = 1.8 μM; [antibody 03-1(Fab)] = (a) 0, (b) 0.49, (c) 1.5, and (d) 2.9 μM.

only to TCPP but also to Mn(III)−TCPP and Fe(III)−TCPP, whereas the other three antibodies against TCPP did not form complexes with Mn−TCPP and Fe−TCPP. Table 1 shows the dissociation constants of the complexes of TCPP and its metal complexes with monoclonal antibody 03-1 determined by emission quenching studies. Antibody 03-1 bound strongly to TCPP with a dissociation constant of 7.0×10^{-8} M and to Mn−TCPP and Fe−TCPP with dissociation constants of 4.1×10^{-7} and 1.5×10^{-7} M, respectively. Under the conditions used for catalytic reactions, most of the metal complexes were incorporated into the binding site of the antibody. Figure 1 shows the UV−vis and circular dichroism spectra of Fe(III)−TCPP in the presence of antibody 03-1. The UV−vis spectra showed some hyperchroism in the region of the Soret band, indicating that the metalloporphyrin is placed in the low-polar environment. The circular dichroism spectra showed induced Cotton effects on Fe(III)−TCPP, indicating that the metalloporphyrin is incorporated into the chiral environment of the binding site of the antibody.

Catalytic Effects on Peroxidation by Antibody−Metalloporphyrin (TCPP) Complexes. We studied the catalytic effects of complexes of Mn(III)−TCPP and Fe(III)−TCPP with antibody 03-1 on the oxidations of various substrates. For this, Fab fragments (antigen binding fragments, 50 kDa) were used instead of whole antibody 03-1 (IgG_{2b}) because Fab is more soluble in aqueous buffer than whole antibody. We tested various substrates for peroxidase, such as hydroquinone, catechol, resorcinol, pyrogallol, guaiacol, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) 2-ammonate (ABTS), and *o*-dianisidine. Results showed that the complexes of metal−



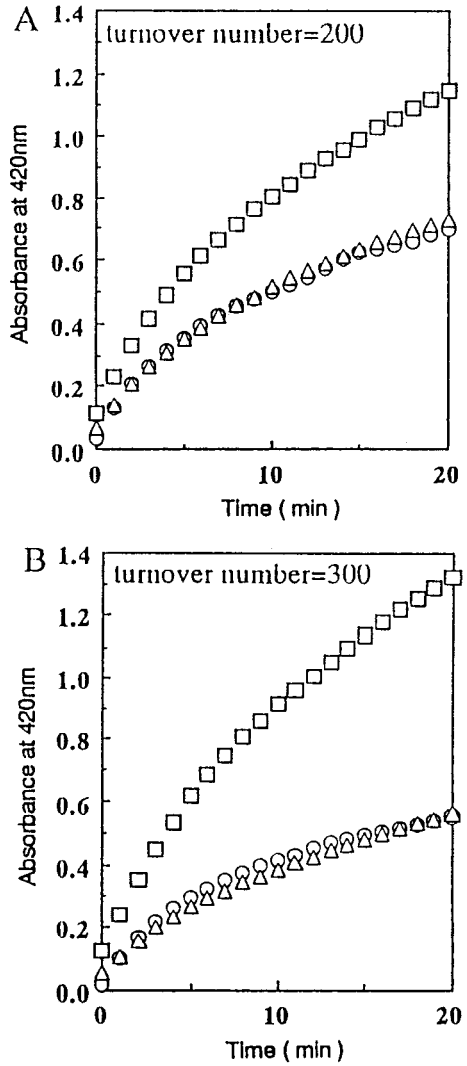


Figure 2. Peroxidation of pyrogallol in the presence of Fab−porphyrin complex (□), Fc−porphyrin (○), and TCPP−metal complex (△). (A) Mn(III)−TCPP and (B) Fe(III)−TCPP. Reaction mixtures contained 5 mM H_2O_2, 1.2 mM pyrogallol, 0.5 μM Mn(III)−TCPP or Fe(III)−TCPP, 0.8 μM Fab or Fc, 90 mM Tris−acetate buffer, and 4% (v/v) DMSO.

TCPP with antibody 03-1 had specific catalytic effects only on pyrogallol. This result is in contrast to the oxidations of various substrates by mesoporphyrin−iron complexes with antibody against *N*-methylmesoporphyrin reported by Cochran and Schultz.[10] They found that these complexes catalyzed not only the oxidation of pyrogallol but also those of other peroxidase substrates, such as hydroquinone, resorcinol, catechol, and ABTS. Since we have not designed the substrate binding site of the antibody, the substrate specificities shown by the antibody−metalloporphyrin are fortuitous.

Figure 2 shows the time dependencies of oxidation of pyrogallol by metal−TCPP complexes in the presence and

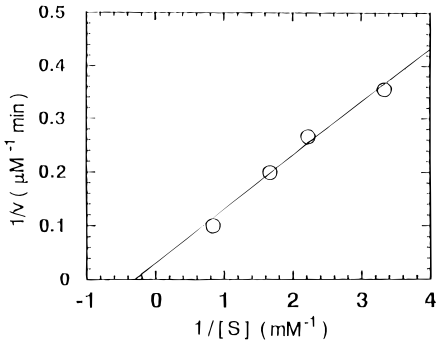


Figure 3. Lineweaver-Burk plot for the oxidation of pyrogallol as a function of [pyrogallol] for the Fe(III)−TCPP−antibody 03-1 complex. Whole antibody was used for kinetic analysis. Initial rates of oxidation were determined by measuring the absorbance increase at 420 nm. Reaction mixtures contained 5 mM H_2O_2, 0.5 μM Fe(III)−TCPP, 0.4 μM antibody ([Fab] = 0.8 μM), 90 mM Tris−acetate buffer, and 4% (v/v) DMSO.

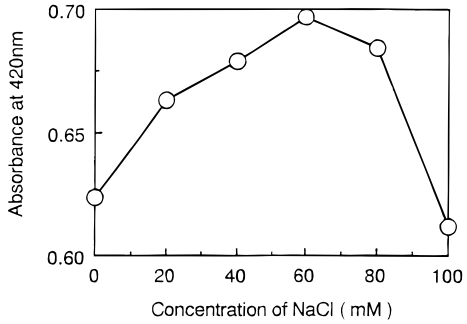


Figure 4. Effect of salt concentrations on the oxidation of pyrogallol by the antibody 03-1(Fab)−Mn(III)−TCPP complex.

absence of antibody 03-1(Fab). The antibody 03-1(Fab)−metal−TCPP complexes had higher catalytic effects than metal−TCPP alone. Metal−TCPP and the Fc fraction (which does not bind antigen), which has no binding site for porphyrin, had no effect on the reaction. The turnover number was 200 with the antibody−Mn−TCPP complex and 300 with the antibody−Fe−TCPP complex. Further addition of substrates caused a further catalytic reaction, indicating that the catalysts were still active. Fe−TCPP was more active than Mn−TCPP in the presence of antibody 03-1(Fab).

Figure 3 shows a Lineweaver-Burk plot of the oxidation of pyrogallol by the antibody 03-1−Fe(III)−TCPP complex. From the plot, the K_m and k_{cat} values were calculated. The K_m value in the presence of antibody 03-1−Fe−TCPP complex is 4.0 mM. The k_{cat} is 50 min^{-1}, higher than that in the absence of the antibody (8.7 min^{-1}).

Figure 4 shows the effects of NaCl on the catalytic effect of the antibody−metalloporphyrin complexes. The effect increased with an increase in the concentration of salt, indicating that electrostatic interactions between metal−TCPP and the antibody combining site are important in formation of the complex. NaCl at 60 mM was the most effective on the reactions, higher concentrations of NaCl possibly having a salting-out effect, causing dissociation of the antibody−metalloporphyrin complex. This kind of dissociation was observed by comparison of the absorption spectra of metal−TCPP complexes in the presence and in the absence of NaCl.[7b]

Figure 5 shows the effect on the reaction of the molar ratio of antibody binding site to the metal−TCPP complex. The plot shows a maximum at a molar ratio of 1:1, indicating that a 1:1 complex was the most effective. These results indicate that excess antibody inhibited the reactions. This suggests the formation of a 2:1 complex (two binding sites per porphyrin),

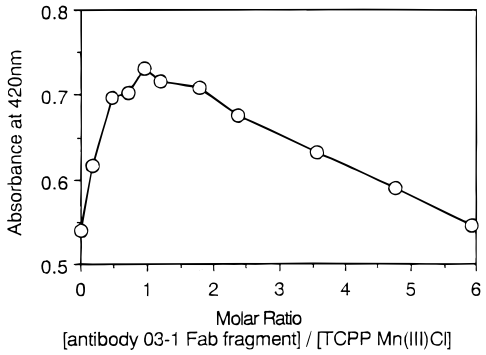


Figure 5. Effects of the molar ratio of the Fab fragment of antibody 03-1−Mn^{III}Cl−TCPP on oxidation of pyrogallol.

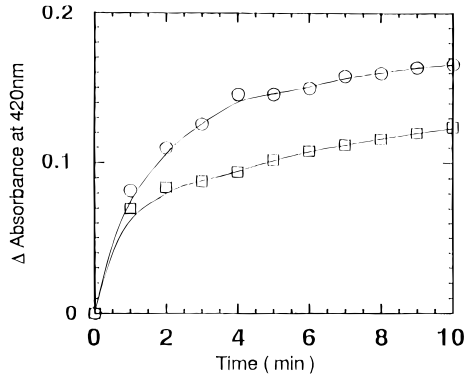


Figure 6. Absorbance versus time curve for the oxidation of pyrogallol in the presence of horseradish peroxidase and Fe(III)−TCPP (○) and that in the presence of antibody 03-1(Fab)−Fe(III)−TCPP complex (□). Reaction mixtures contained 800 mM H_2O_2, 1.2 mM pyrogallol, 0.5 μM Fe(III)−TCPP, 0.8 μM horseradish peroxidase or Fab, PBB (pH 9.0), and 4% (v/v) DMSO.

as indicated by studies on the binding properties of antibody 03-1.[7b]

In Tris buffer (at pH 8.3), the reactions proceeded rather fast without metalloporphyrins, making comparison of the rates in the presence and in the absence of antibody difficult. So, we used phosphate borate buffer (PBB buffer, pH 9.0) for these oxidation reactions.

If the oxidation reactions proceed in the same way as with natural enzymes, such as peroxidases and catalase, an imidazole group in the histidine residue should be important as an axial ligand for metals in the porphyrins. So, we studied the effects of amines on the reactions. Addition of various amines, such as imidazole and pyridine, did not have marked effects on the oxidation reactions by the antibody−porphyrin complexes. These results suggest that an imidazole group in the antibody serves as a coordinating ligand for metals in porphyrins. Preliminary results on the sequence analyses showed that there are a few histidine residues around the binding site of the antibody. There is a possibility that one of the histidine residue is involved in the association with a metal−TCPP complex. However, there is also a possibility that the ligands have no binding capacity for the antibody.

Comparison of Catalytic Activities of Antibody−Metalloporphyrin Complexes with Those of Peroxidase. Figure 6 shows the changes of absorbance with time curve in the oxidation of pyrogallol in the presence of naturally occurring horseradish peroxidase and Fe−TCPP and in the presence of the antibody 03-1(Fab)−Fe−TCPP complex. The results indicated that the activity of the antibody−Fe−TCPP complex was high under these conditions, although the metalloporphyrins show some effects on the reaction. The antibody−porphyrin

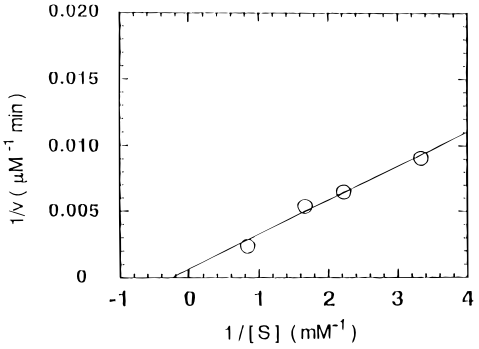


Figure 7. Lineweaver-Burk plot for the oxidation of pyrogallol in the presence of peroxidase. Reaction conditions were the same as those given in Figure 3.

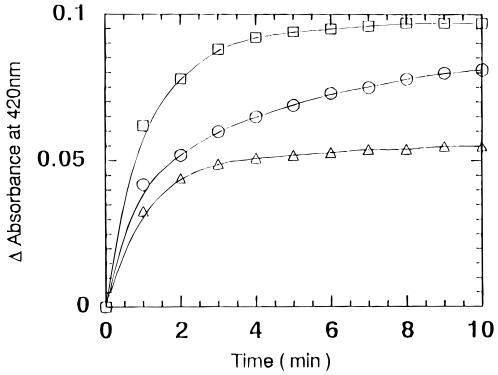


Figure 8. Absorbance versus time curve for the oxidation of pyrogallol in the presence of Fab−porphyrin (□), heavy chains−porphyrin (○), and light chains−porphyrin (△). Reaction mixtures contained 800 mM H_2O_2, 3.3 mM pyrogallol, 0.8 μM Mn(III)−TCPP, 1.2 μM Fab, heavy chains, or light chains, 90 mM Tris−acetate buffer, and 4% (v/v) DMSO.

complexes are stable enough to show catalytic activity in the presence of an excess amount of H_2O_2. Horseradish peroxidase showed some loss of activity under the same conditions.

Figure 7 shows a Lineweaver-Burk plot for the oxidation of pyrogallol in the presence of horseradish peroxidase. From the plot, the K_m and k_{cat} values for horseradish peroxidase were calculated to be 4.6 mM and 2.5×10^3 min^{-1}, respectively. The k_2/K_m value was 9.1×10^3 M^{-1} s^{-1}. This value is 50 times that of the antibody−Fe−TCPP complex. The antibody−Fe−TCPP complex was highly reactive. The antibody−Fe−TCPP complex was also highly selective, catalyzing the oxidation of pyrogallol specifically.

Naturally occurring catalyst horseradish peroxidase catalyzes the oxidations of various substrates, not only pyrogallol but also hydroquinone, catechol, resorcinol, guaiacol, ABTS, and *o*-dianisidine. The reactions promoted by peroxidases are nonspecific. In contrast, the antibody 03-1−metal−TCPP complex was selective for pyrogallol, although we did not design a substrate binding site for pyrogallol. This selectivity seems to be accidental, but the high susceptibility of pyrogallol to oxidation was probably because pyrogallol has the lowest redox potential of the substrates tested, and the antibody−metalloporphyrin complex selected such a compound as a substrate. We determined the dissociation constant of the complex between antibody 03-1 and pyrogallol to be 10^{-3} M. The antibody has the ability to bind pyrogallol, and the metalloporphyrins selected such a compound as a substrate.

TCPP had no catalytic effects, and the antibody 03-1−TCPP complex without metal also had no effect on the oxidation of pyrogallol. Antibody 03-1 and a mixture of TCPP and Fe−TCPP (1:1 molar ratio) had intermediary effects on the oxidation of pyrogallol. These results indicate that all these species, antibody, TCPP, and metal, are required for the catalytic reaction.

Catalytic Effects of Heavy and Light Chains. We compared the effects of the "heavy chains" (longer peptide chains, 50 kDa) and "light chains" (shorter peptide chains, 25 kDa) of antibody 03-1 in the presence of TCPP−metal complexes on the oxidation of pyrogallol. The heavy chains and light chains of antibody 03-1 were separated by the reduction of the disulfide bonds with cysteine. Figure 8 shows the time−absorbance curve for the oxidation of pyrogallol in the presence of Mn−TCPP and the heavy chain of antibody 03-1. Heavy chain−TCPP−metal complexes show effects comparable to those of the Fab and TCPP−metal complexes. The light chain had lower reactivity, comparable to that of the metal−TCPP complex alone. These results indicate that the heavy chain plays an important role in catalytic effects on the oxidation of pyrogallol.

In conclusion, we could obtain a monoclonal antibody that binds manganese(III) and iron(II) *meso*-tetrakis(4-carboxyphenyl)porphyrin (TCPP) strongly and shows catalytic activity toward the oxidation of pyrogallol selectively. Detailed studies on the structures and functions of the antibody are now under way.

Experimental Section

Material. *meso*-Tetrakis(4-carboxyphenyl)porphyrin (TCPP) was prepared by condensation of *p*-carboxybenzaldehyde with pyrrole in propionic acid according to the procedure of Longo et al.[13] The Mn(III)−TCPP complex was prepared by treatment of TCPP with excess $MnCl_2$ in acetic acid and acetic anhydride refluxing for 2 h. After evaporation of the solvents, the residue was recrystallized from methylene chloride. $Fe^{III}Cl$−TCPP was prepared by treatment of TCPP with excess $FeCl_3$ in glacial acetic acid with sodium acetate. After the solution was refluxed for 1 h and water added, the resulting crystals were filtered off, washed with water, and recrystallized from 0.5 N NaOH−EtOH. Monoclonal antibodies for TCPP were prepared as described previously.[5] Fab fractions of antibody 03-1 were obtained by digestion of the antibody with papain (Wako Chemical Ltd.) as described below.

Preparation of Fab Fragment. Antibody (25 mg) in 10 mL of PBB buffer (pH 8) was mixed with 0.1 M L-cysteine solution (1.11 mL) and then 25 mL of papain solution (Sigma). The mixture was incubated at 37 °C for 4 h. The reaction was terminated by the addition of iodoacetamide at a final concentration of 30 mM. The reaction mixture was passed through a prepacked Protein A column (Pierce immunopure kit). The Fab (antigen binding fragment, 50 kDa) eluted was passed through a Sephadex G100 column. The purity was checked by SDS−PAGE.

Method. (1) Spectroscopy. UV−vis spectra were recorded on a Shimadzu UV-2100 UV−visible spectrophotometer. Circular dichroism spectra were recorded in a JASCO J-40A spectrophotometer with a JASCO ORD/CD data processor using a 10 mm quartz cell. Proton NMR spectra were recorded at 270 MHz in $CDCl_3$ with a JEOL JNM GSX-270 NMR spectrometer. Chemical shifts were determined with reference to TMS. Emission spectra were recorded with a Shimadzu RF-502A spectrofluorophotometer using an excitation wavelength of 280 nm.

(2) Kinetics. Kinetics were conducted under the following conditions. The Fab fraction of antibody 03-1 and TCPP−metal complex was dissolved in Tris buffer (pH 8.0) or PBB buffer (0.1 M, pH 9.0) and incubated for 2 days. Hydrogen peroxide was then added (5.0 mM), followed by a substrate (e.g., pyrogallol). The starting time was set at this point. The reactions were followed by measuring the change of absorbance at 420 nm.

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