Ligninolytic Peroxidase-Like Activity of a Synthetic Metalloporphine Immobilized onto Mercapto-Grafted Crosslinked PVA Inspired by the Active Site of Cytochrome P450

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Abstract: A synthetic metalloporphine was immobilized onto a PVA-based and mercapto-grafted solid support, emulating the active site of cytochrome P450. Its ligninolytic peroxidase-like catalytic activity was studied. The coordinated mercapto ligand significantly affected the catalytic features of the catalyst because the oxidation of lignin-model compounds was very slow by comparison with imidazole- and pyridine-coordinated immobilized metalloporphines. Conversely, the catalyst efficiently bleached several industrial dyes and thus demonstrated promising activity for this application. Based on this altered substrate specificity the oxygen-donor catalytic route seems to be more favorable than a single electron oxidation pathway.

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Recently, catalysts that promote oxidative delignification under mild conditions have attracted interest. Synthetic metalloporphines have proven to be promising catalysts for this reaction because they are able to oxidize lignin-related molecules [1] and other pollutant (and industrially relevant) substrates [2,3]. In particular, the structural and functional emulation of lignin peroxidase has been described [4] using imidazole as a coordinating group for metalloporphines. This catalyst showed promising catalytic features and worked well under very mild conditions. Based on these promising results, other coordinating groups (such as pyridine [5]) have been studied for the synthesis of immobilized metalloporphine-based catalysts. Herein, we report on the immobilization of a synthetic metalloporphine onto a mercapto-grafted solid support to emulate the structure of the active site of cytochrome P450. Cytochrome P450 is a large superfamily of hemoproteins that catalyze monooxygenase reactions [6,7] with O₂ as an oxidizing agent and NADPH as a co-reducing agent. In this enzyme the mercapto group of a cysteine residue is present in the active site as the proximal ligand of ferriheme [8]. The ability of metalloporphines to emulate P450 catalysis during monooxygenase reactions has been thoroughly studied [9–11]. In this paper, we describe the influence of the mercapto ligand

during the ligninolytic peroxidase-like catalysis of immobilized metalloporphine in the presence of H₂O₂. To our knowledge, no studies exist regarding similar characterizations of metalloporphines immobilized on mercapto-functionalized supports.

All reagents were of the best commercial grade available and were used without further purification. ARS was purchased from Fluka (cat. No. 05600), FeTFPP from Aldrich (cat. No. 252913), lignin peroxidase from Sigma-Aldrich (cat. No. 42603), and PVA from Aldrich (cat. No. 363138). Laccase from *P. sajor-caju* was purified by two chromatographic steps (details in [12]).

Spectrophotometric measurements were carried out with an UltroSpec 2100pro (Amersham Bioscience). The concentrations of the substrates were also measured by UV-HPLC and GC-MS using previously described methods [4,13]. Amino-grafted PVA (AP-PVA) was synthesized as previously described [5,14].

Each gram of the AP-PVA powder was suspended in 10 ml of a 0.1 mol/L aqueous solution of NaHCO₃ containing 0.5 g *N*-acetyl-DL-homocysteine thiolactone. The slurry was then stirred gently for 24 h at 25 °C. The obtained M-PVA was then consecutively washed with H₂O, 0.1 mol/L aqueous KH₂PO₄, H₂O again, 0.1 mol/L aqueous solution of

sodium dithionite, H₂O again, and 2-propanol.

The mercapto-grafted PVA was then carefully dried overnight in a vacuum oven at 50 °C. To determine the amount of aminopropyl moieties and the extent of their further derivatization with mercapto functions, a chromogenic reaction with ninhydrin and hydriindantin was applied [15].

One-gram aliquots of M-PVA were treated with 20 mg of FeTFPP solubilized in 10 ml of DMSO. The slurry was kept in the dark for 24 h while stirring and washed repeatedly with DMSO and 2-propanol. Finally, the adduct was dried at 50 °C. The proposed structure of the obtained catalyst is shown in Fig. 1. Bound metalloporphine was quantified by differences in the spectrophotometric measurements at 411 nm ($\epsilon_{411} = 115000 \text{ mol}/(\text{L}\cdot\text{cm})$) [5]).

Fig. 1. Proposed structure of the catalytic adduct M-PVA/FeTFPP.

Routine measurements of the catalytic activity of immobilized metalloporphine were performed by spectrophotometric assays. A suspension of 5 mg of catalyst in 1 ml of 25 mmol/L buffer solution containing 12 mmol/L VA and 8.8 mmol/L hydrogen peroxide was stirred at 25 °C in the dark. After 30 min, the increase in absorbance at 310 nm was measured to detect the formed veratraldehyde ($\epsilon_{310} = 9300 \text{ mol}/(\text{L}\cdot\text{cm})$) [16]). In the case of ARS the reducing substrate concentration was 1.2 mmol/L and the absorbance decrease at 520 nm was recorded ($\epsilon_{520} = 7200 \text{ mol}/(\text{L}\cdot\text{cm})$) [17]).

In some experiments the well-known hydroxyl radical scavenger 0.1 mol/L mannitol was also added. When used, redox mediators (OH-TEMPO, NPH, NHS, and NHT) were

present in the reaction medium at a final concentration of 1 mmol/L.

To test the catalytic performance at various pH levels, different McIlvaine buffers were used at pH = 3, 4, 5, 6, 7, and 8.

Other compounds were also tested as substrates, including 4-methoxybenzyl alcohol, 4-hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol), 3-hydroxy-4-methoxybenzyl alcohol (isovanillyl alcohol), 3,4,5-trimethoxybenzyl alcohol, 3,4-dimethoxybenzaldehyde (veratraldehyde), 3,4-dimethoxybenzoic acid (veratric acid), 1,2-dimethoxybenzene (veratrole), 1,2,3-trimethoxybenzene, and 4-hydroxy-3-methoxycinnamic acid (ferulic acid).

For the MnP-like assays all the experiments were repeated in aqueous media with 1 mmol/L MnSO₄ and 50 mmol/L malonic acid. For the multicyclic runs, the catalyst was regenerated between consecutive cycles by repeated washing with water and 2-propanol and by drying at 50 °C.

Inexpensive and hydrophilic PVA was efficiently grafted with mercapto functional groups (280 $\mu\text{mol}/\text{g}$ of support) using the well-known reaction between amines and *N*-acetyl-DL-homocysteine thiolactone. This reaction also allowed for the insertion of a long and flexible molecular spacer arm between the support and the catalyst.

After being grafted the support was immediately treated with metalloporphine to avoid the possible oxidation of the -SH group. FeTFPP was chosen for this study because of its notable catalytic efficiency and chemical stability compared with its physiological counterpart ferriprotoporphyrin IX [18]. The metalloporphine bound to the support gave a typical saturation curve (not shown) but the highest specific catalytic activity was observed when 20 mg of FeTFPP per g of M-PVA was added (leading to an effective binding of 8.8 mg or 8.3 μmol). Accordingly, this FeTFPP concentration was chosen for further study.

Significantly, in the presence of O₂ and NADPH, M-PVA/FeTFPP was unable to act as a monooxygenase for a variety of possible oxygen acceptors.

However, when H₂O₂ was present in the reaction mixture, M-PVA/FeTFPP catalyzed the desired peroxidase-like reactions of both VA and ARS.

For VA oxidation the observed catalytic activity was quite poor (1 h provided 17% conversion of VA) by comparison with similar metalloporphines immobilized with different ligands [4,5]. The product distribution remained similar: veratraldehyde was only obtained with 21% selectivity along with byproducts that were unidentifiable by UV-HPLC and GC-MS (only very small, non-quantifiable amounts of 2-hydroxymethyl-5-methoxy-1,4-benzoquinone and veratric acid were observed).

For ARS oxidation, however, a significantly higher conversion was observed (over 80% in 1 h). This behavior

could be rationalized by considering that VA can only be oxidized by a single electron transfer pathway whereas ARS (or other ARS partial oxidation-derived species) can act as a substrate for monooxygenase-like reactions [3].

The ability of metalloporphines to act as both monoelectronic oxidants and as oxygen donors has previously been described [3]. Our results suggest that the mercapto coordinating group is able to make the latter route more favorable in accordance with the attempted structural emulation. In fact, ligninolytic peroxidases that present imidazoles (of a specific histidine residue) as proximal coordinating groups are only able to achieve monoelectronic oxidation. Thus, supported metalloporphines that resemble their active sites are capable of catalyzing the nearly complete conversion of substrates such as VA. Mercapto coordinating groups, however, can change the activity of the metalloporphine oxygen-donor ability as in cytochrome P450.

The effects of Mn^{2+} on the catalytic process were also studied. In its absence, the M-PVA/FeTFPP adduct showed a pH optimum of 3 for both VA and ARS (Fig. 2). In the presence of Mn^{2+} , however, VA oxidation values increased whereas ARS oxidation seemed to be inhibited by Mn^{2+} .

The catalyst was also characterized kinetically, showing a typical Michaelis-Menten saturation for both substrates. A relatively high K_m value (7.6 ± 2.5 mmol/L) confirmed the low specificity of M-PVA/FeTFPP for VA while the K_m for ARS (1.12 ± 0.02 mmol/L) was similar to those observed in

the presence of ligands other than the mercapto group [3,5].

The poor LiP-like performances of M-PVA/FeTFPP were confirmed during oxidation screenings of various lignin-model compounds (Table 1). The conversions were very low for both phenolic and non-phenolic substrates, especially upon comparison with similar experiments containing pyridine as the FeTFPP ligand [5].

Table 1 Substrate specificity of the M-PVA/FeTFPP adduct with various lignin model compounds

Substrate	Conversion (%)
Veratryl alcohol	18.0
Veratraldehyde	9.9
Veratric acid	2.7
Isovanillyl alcohol	29.6
Vanillyl alcohol	46.2
1,2,3-Trimethoxybenzene	0
Ferulic acid	86.2
Veratrole	18.2
4-Methoxybenzyl alcohol	10.6
3,4,5-Trimethoxybenzyl alcohol	1.8

Catalytic activity was detected in the presence of 5 mg of catalyst, 8.8 mmol/L hydrogen peroxide, 1 mmol/L substrate, DME 10%, 1 mmol/L MnSO_4 , 50 mmol/L malonic acid, and 25 mmol/L buffer (pH = 4, final volume 2 mL).

A homolytic cleavage of the O–O bond was excluded as a possible pathway [19] because mannitol (a well-known OH• scavenger) did not affect the catalytic properties of the adduct.

Additional redox mediators were also tested: OH-TEMPO, NPH, NHS, and NHT. ARS bleaching was not greatly affected by their presence and the rate of VA oxidation was tripled in the presence of OH-TEMPO and NHS.

According to all the data reported above, M-PVA/FeTFPP is not a good LiP emulator. This finding was further corroborated by a multicycle analysis. For VA oxidation, only 7% catalytic activity was obtained in the second cycle and for subsequent cycles no activity was observed. More promising results, however, were obtained for ARS since about 20% of the catalytic activity was obtained after 6 cycles.

In addition to ARS, the ability of M-PVA/FeTFPP to bleach a complete series of dyes belonging to different chemical classes was also tested (Table 2). The conversion rates over 1 h were quite high for almost all the dyes tested and ranged from 50% to 90% (only XO conversion was low at 23%). However, in all cases the adduct was catalytically active confirming the wide substrate specificity of the immobilized metalloporphine, particularly when pathways apart from single electron oxidation are feasible.

The substrate specificity with industrial dyes was even broader than that of ligninolytic enzymes. For example,

Fig. 2. Relative activity of the M-PVA/FeTFPP adduct toward VA (a) and ARS (b) at various pH values.

Table 2 M-PVA/FeTFPP adduct bleaching activity with several dyes

Dye	Conversion (%)
ARS	83
0.3 mmol/L PNS	59
2 mmol/L XO	23
0.15 mmol/L MB	50
1.5 mmol/L MG	47
1.2 mmol/L MO	97

Catalytic activity was detected in the presence of 5 mg of catalyst, 8.8 mmol/L hydrogen peroxide and 25 mmol/L buffer (pH = 4, final volume 2 ml). The dyes were present at a concentration of 0.3 mmol/L PNS, 2 mmol/L XO, 0.15 mmol/L methylene blue, 1.5 mmol/L methyl green, and 1.2 mmol/L methyl orange.

laccase and lignin peroxidases under similar operational conditions gave lower conversion rates [20] and were completely unable to oxidize some of the dyes such as PNS.

In conclusion, the use of a mercapto group to immobilize metalloporphines did not allow for an efficient ligninolytic peroxidase-like emulsion with lignin-model compounds (contrary to what has been observed with imidazole and pyridine as ligands of similar metalloporphines). However, the M-PVA/FeTFPP adduct showed interesting features in the treatment of several industrial dyes that were efficiently bleached under very mild operational conditions. These results are very promising, particularly from an industrial application point of view. Furthermore, the collected data suggests that the mercapto group can change the catalytic mechanism of FeTFPP since its oxygen-donor behavior becomes more favorable than its single electron transfer behavior.

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