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Spectroscopic characterization of a manganese–lignin peroxidase hybrid isozyme produced by *Bjerkandera adusta* in the absence of manganese: evidence of a protein centred radical by hydrogen peroxide

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Abstract

Electronic absorption and electron paramagnetic resonance (EPR) spectra are reported for a novel manganese–lignin peroxidase (MnLiP) hybrid isozyme produced by *Bjerkandera adusta* in the absence of manganese at pH 5. The room temperature absorption and the low temperature (10 K) EPR spectra indicate that the same coordination and spin states are present at both temperatures: mainly six coordinate high spin containing low percentage six coordinate low spin ferric heme, the latter probably with a bis-imidazole coordination. A protein centred radical was detected in the presence of an excess of hydrogen peroxide and assumed to be a tryptophanyl radical. The catalytic significance of this site was addressed by specific chemical modification of the tryptophan residues that revealed a marked effect on the specific activity of the enzyme. It is proposed that substrate oxidation might proceed through a long range-electron transfer process.

Keywords: EPR; Spin state; Heme protein; Peroxidase; Protein radical

1. Introduction

The biodegradation of lignin, a major constituent of wood, is far from being fully elucidated. White rot fungi, with a complex enzymatic system, contribute in the recycling of this biopolymer in nature. Several extracellular enzymes from these ligninolytic fungi are involved in the degradation of lignin polymer. Among these enzymes, lignin peroxidase (LiP) and manganese peroxidase (MnP) from *Phanerochaete chrysosporium* have been extensively studied [1,2]. The catalytic cycle for both enzymes is similar to that of other heme peroxidases and begins with a two-electron oxidation of the heme prosthetic group by hydrogen peroxide yielding compound I. These two oxidizing equivalents are then sequentially transferred to substrate molecules. One of the oxidizing equivalents of compound I is located on the heme iron atom, leading to the formation of an oxyferryl iron(IV) centre. However, differences have been noted with

Abbreviations: LiP, lignin peroxidase; MnP, manganese peroxidase; MnLiP, manganese–lignin peroxidase hybrid; HS, high spin; LS, low spin; 6c-HS, hexacoordinated high spin; 5c-HS, pentacoordinated high spin; NBS, *N*-bromosuccinimide; BSA, bovine serum albumin; VA, veratryl alcohol

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respect to the second one. In all, peroxidases characterized up to date, the UV–VIS and resonance Raman (RR) spectroscopy have provided evidence that the second oxidizing equivalent is in fact a porphyrin π radical cation [3] except in cytochrome c peroxidase (CcP). In this case the radical resides on a tryptophan residue in the vicinity of the proximal histidine [4].

Although the exact mechanism of lignin degradation is not fully understood, it has been proposed that in vivo the process by which LiP and MnP attack the lignin polymer is different. LiP directly interacts with its substrates to form radical cations whereas MnP oxidizes Mn(II) to Mn(III), which in turn acts as a diffusable oxidizing intermediate [5].

Recently, two novel fungal peroxidases belonging to class II of the plant peroxidase superfamily [6] were detected in the white rot fungi *Bjerkandera adusta* and *Pleurotus eryngii* [7,8]. Both enzymes are able to catalyze manganese-dependent as well as manganese-independent reactions. The manganese-dependent activity proceeds optimally at pH 5, while the manganese-independent reactions require more acidic conditions, showing maximum rates at pH 3. The catalytic intermediates seem to be the same in these enzymes, as they oxidize Mn(II) and aromatic substrates with comparable values of V_{max} and K_m [8]. The biotechnological interest for the manganese–lignin peroxidase hybrid (MnLiP) from *B. adusta* lies in its ability to oxidize large substrate molecules in the absence of Mn(II).

Camarero et al. [9] suggested that MnLiP might be a structural hybrid, based on a comparison of a molecular model of the MnLiP structure from *P. eryngii* with the known structures of LiP and MnP from *P. chrysosporium*. This hybrid possesses a MnP-like Mn(II) binding site as well as a long range-electron transfer mechanism similar to that recently found for LiP [10]. Therefore, depending on the reaction conditions this enzyme would oxidize substrates unable to reach the heme pocket through the mediation of a diffusable, highly reactive intermediate or through an activated surface amino acid residue in the case of less accessible substrates [11].

The present results show that MnLiP from *B. adusta* exhibits a prevalent 6c-high spin ferric form with a low percentage of 6c-low spin probably due to a bis-imidazole co-ordination at both room and low temperature. A protein centred radical was detected at room temperature in the presence of hydrogen peroxide, supporting the hypothesis of a long range-electron transfer mechanism for the oxidation of large molecules.

2. Experimental

Lignin peroxidase (LiP) and manganese peroxidase (MnP) from *P. chrysosporium* were obtained from Tienzyme, Inc. (Salt Lake City, UT). The partially purified hybrid peroxidase (MnLiP) from *B. adusta* UAMH 7308 (R_z $(A_{403}/A_{280}) = 1.3$) was kindly provided by Prof. M.A. Pickard, from the University of Alberta, Canada. Protein concentration was determined with BioRad reagent using BSA as standard. The molecular weight of the protein was estimated from a Coomasie-stained 10% SDS–PAGE gel and it is equal to 45 kDa. The single band in the gel shows that there is no contamination from other proteins in the preparation for this work (data not shown). 2-Methyl-2-nitroso propane (MNP) was purchased from Sigma (St. Louis, MO) and H_2O_2 from Fluka (Germany). All reagents were used without further purification.

EPR and UV–VIS measurements were performed on the native enzyme and the enzyme modified with *N*-bromo succinimide (NBS) [12]. EPR solutions of the native enzyme were prepared with a final concentration of 0.21 mM MnLiP and 2 mM H_2O_2 at pH 5. The chemical modification of Trp residues was done using NBS recrystallized from hot water just before use. To follow the effect on activity of the chemical modification of Trp, 0.2 ml of a 0.24 mM MnLiP solution were mixed with different molar excesses of NBS dissolved in water. After 2 min, the reaction was stopped by adding a 600-fold molar excess of Trp in water. To follow the decrease of Trp fluorescence at 315 nm, 2 ml of a 0.0015 mM MnLiP solution were mixed with increasing amounts of NBS dissolved in water, and after 1 min the emission spectra were recorded. For the EPR measurements, 1 ml of a 0.17 mM MnLiP solution was mixed with a 60-fold molar excess of NBS dissolved in water. The reaction was allowed to proceed for 2 min and stopped by adding 0.1 g tryptophan dissolved in water. The reaction mixtures were extensively washed

with 100 mM acetate buffer pH 4.5 in an ultrafiltration cell using a 10 kDa cutoff membrane, in order to remove substances that could interfere with the EPR and enzymatic assays.

Absorption spectra of a 0.005 mM solution of MnP, LiP and MnLiP were measured in 60 mM phosphate buffer pH 5. The Mn(II)-independent activity at different pH was estimated using hydroquinone as substrate. Reactions were performed in 60 mM phosphate buffer, pH 3 or 5, in the presence of 1 mM of hydroquinone and 0.1 mM H_2O_2. The reaction was started by adding H_2O_2 and followed by measuring the changes in absorbance at 247 nm ($\epsilon = 21\,mM^{-1}\,cm^{-1}$). LiP and MnP activity were measured as reported elsewhere [13,14]. For LiP activity, the reaction mixture contained 40 mM succinate buffer pH 4, 4 mM veratryl alcohol (VA) and 0.4 mM hydrogen peroxide. The production of veratryl aldehyde was followed at 310 nm ($\epsilon = 9.3\,mM^{-1}\,cm^{-1}$). For MnP activity, the reaction consisted of 50 mM malonate buffer pH 4.5, 0.1 mM manganese sulfate and 0.1 mM hydrogen peroxide. The production of the malonate–Mn^{3+} complex was monitored at 270 nm ($\epsilon = 11.59\,mM^{-1}\,cm^{-1}$). For all reactions, the enzyme concentration was in the pH range.

UV–VIS measurements were performed on a Hewlett-Packard 8453 spectrophotometer. Fluorescence measurements were performed on a Perkin Elmer LS 50 Luminescence spectrometer, with an excitation wavelength of 285 nm, excitation slit = 8 nm and emission slit = 5 nm. EPR spectra were recorded on a Bruker 200D SRC instrument equipped with a microwave frequency counter XL (Jagmar, Krakow, Poland). The spectrometer was interfaced with a PS/2 technical instrument hardware computer and the data acquired using the EPR data system CS–EPR produced by Stelar Inc. (Mede, Italy). An Oxford instrument ESR 900 cryostat was used to obtain low temperatures. The spectra were recorded under non-saturating conditions at 10 K, 9.466 GHz microwave frequency, 10 mW microwave power and 1 mT modulation amplitude. Spectra were also recorded at 20 K and 80 mW. All samples were frozen rapidly by immersion of the EPR tube into liquid nitrogen. The spectrum at 120 K was recorded using an ER4111VT Bruker variable temperature unit, 9.565 GHz microwave frequency and 63 mW microwave power.

3. Results

Fig. 1 shows the electronic absorption spectrum at room temperature and pH 5 of the hybrid MnLiP from *B. adusta* compared with those of LiP and MnP from *P. chrysosporium*. The spectrum shows a Soret band at 409 nm, α and β bands at 579 (shoulder) and 541 nm, and two charge transfer bands at 501 nm (CT2) and 632 nm (CT1).

All the electronic absorption spectra reported in Fig. 1 show a CT1 band at 632 nm. The wavelength of the band in the 600–650 nm region is sensitive to the heme pocket environment [15]. This band, observed only in the high spin (HS) heme proteins, was assigned to a charge transfer transition (CT1) from the porphyrin to the iron [$a'_{2u}(\pi) \rightarrow e_g(d_\pi)$]. For the hexacoordinate (6c) proteins containing an imidazole as the fifth ligand, the CT1 ranges from 600–637 nm [16]. It has been suggested [15,17–19] that the room temperature electronic absorption spectra of MnP and LiP indicate that the heme iron is predominantly in the hexacoordinated HS state (6c-HS) with a water molecule bound at the sixth coordination position of the Fe atom. The close similarity of the absorption spectrum from MnLiP with those from MnP and LiP suggests that the major heme state of MnLiP is also 6c-HS. Furthermore, the presence of a low percentage of 6c-LS heme in MnLiP of bis-imidazole coordination is revealed by the pronounced β band at 541 nm and the α band at 579 nm, the latter being absent in the spectra of LiP and MnP.

Fig. 2a shows the X-band EPR spectrum of MnLiP at pH 5, recorded under non-saturating conditions (temperature 10 K, microwave power 10 mW). The spectrum is characterized by two distinct signals which indicate the coexistence in MnLiP of a HS species ($g = 6.00, 2.00$) and a small amount of LS species ($g = 3.15, 2.07$) at liquid helium temperature. The third value of the latter species is too weak to be observed. The feature at $g = 4.3$ corresponds to a non-heme iron impurity often seen in protein samples [20].

The LS signal was optimized at a temperature of 20 K and a microwave power of 80 mW as shown in Fig. 2b. Particular attention should be given to the signal at $g = 3.15$. Many model compounds and heme proteins which have bis-imidazole coordination have g values close to 3.0 or slightly lower [21,22].

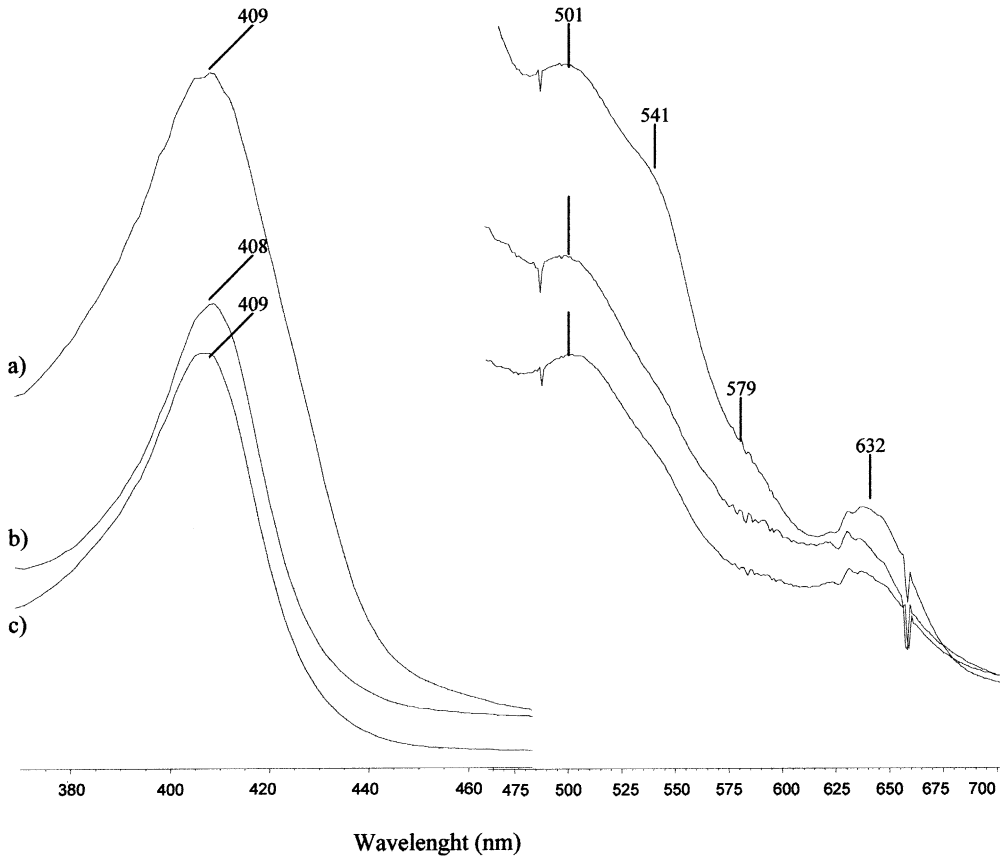

Fig. 1. Electronic absorption spectra of ferric (a) MnLiP, (b) LiP, (c) MnP obtained at room temperature in 60 mM phosphate buffer solution at pH 5. The region between 475–700 nm has been expanded.

Furthermore, a wide variety of membrane bound cytochromes b, which have bis-imidazole coordination, have $g > 3.0$. These large g values have been shown to result from a non-parallel orientation of the imidazole ligands [23–25]. The LS signal at $g = 3.15$ observed in our case supports the assignment of the LS species noted in the absorption spectra to a bis-imidazole complex from an endogenous ligand. Thus, the low temperature EPR and absorption measurements indicate that MnLiP has a similar heme environment at both temperatures, in which HS and LS species coexist in similar proportions. Consequently, it seems that the protein does not undergo a conformational change upon lowering the temperature, which would lead to a significant modification of the heme spin state proportions.

The addition of H_2O_2 to the enzyme solution shows the presence of an enzyme intermediate at both EPR liquid helium temperature and UV–VIS room temperature region (data not shown). The UV–VIS spectrum of this intermediate is similar to those reported for compound I from other peroxidases [26,27]. The signal of this intermediate is stable for at least 4 min, after which the spectrum spontaneously begins to slowly revert to that of the native enzyme. The room temperature EPR spectrum of a protein centred radical with a $g_{iso} = 2.005$ is reported in Fig. 3a. Although the signal is clearly evident, it is characterized by a very rapid decrease in intensity, indicative of a short lifetime species. The spectrum reveals superhyperfine structure that becomes more evident in the second derivative display, as evidenced

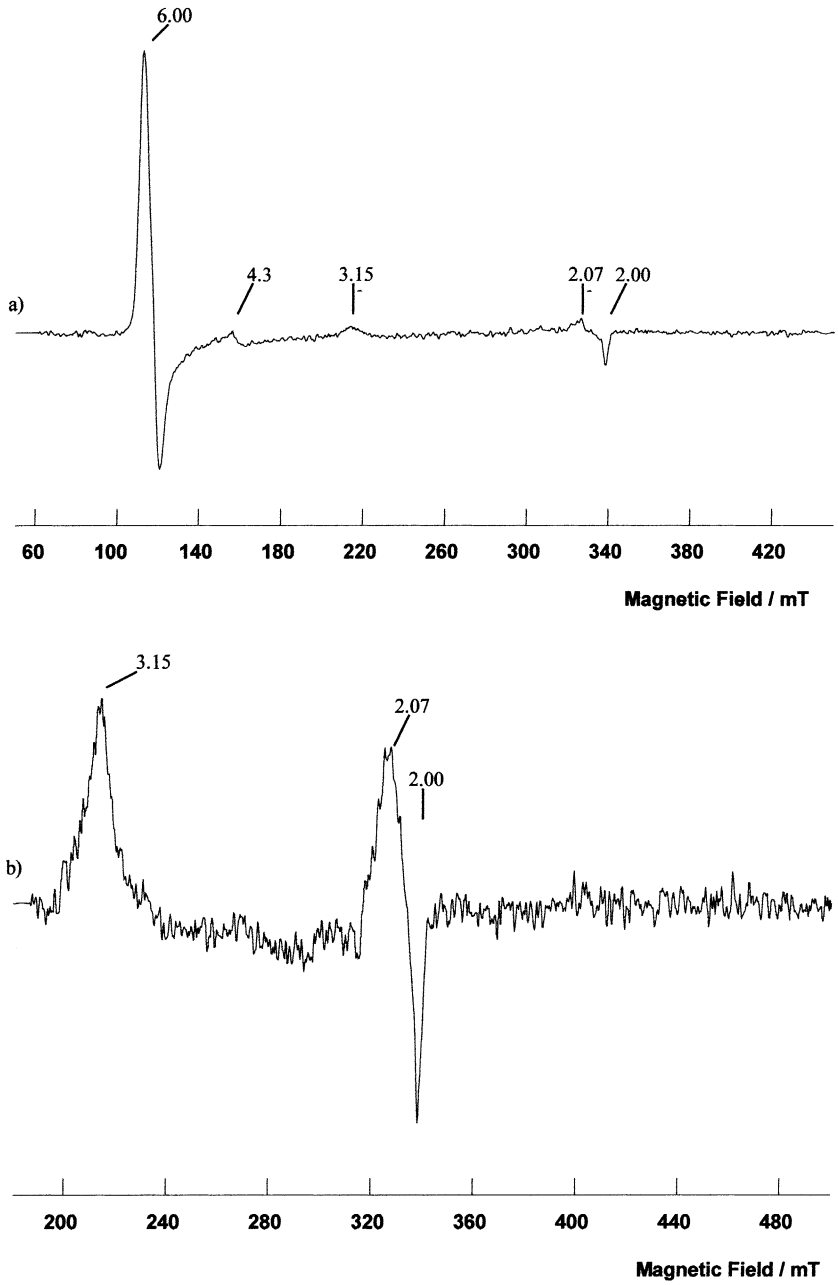

Fig. 2. X-band EPR spectra of the ferric iron MnLiP in 60 mM phosphate buffer solution at pH 5 (a) recorded at 10 K with 1 mT modulation amplitude, 10 mW power and $\nu = 9.466$; (b) recorded at 20 K with 1 mT modulation amplitude, 80 mW power and $\nu = 9.464$.

previously by others [28]. In Fig. 3b the same radical at nitrogen temperature is reported. The reaction was performed in the absence of Mn(II) ions at acidic pH.

To investigate the role of a Trp residue in the protein centred radical, a chemical modification of the Trp residues of MnLiP was performed. Table 1 shows the specific activity of MnLiP with hydroquinone at two

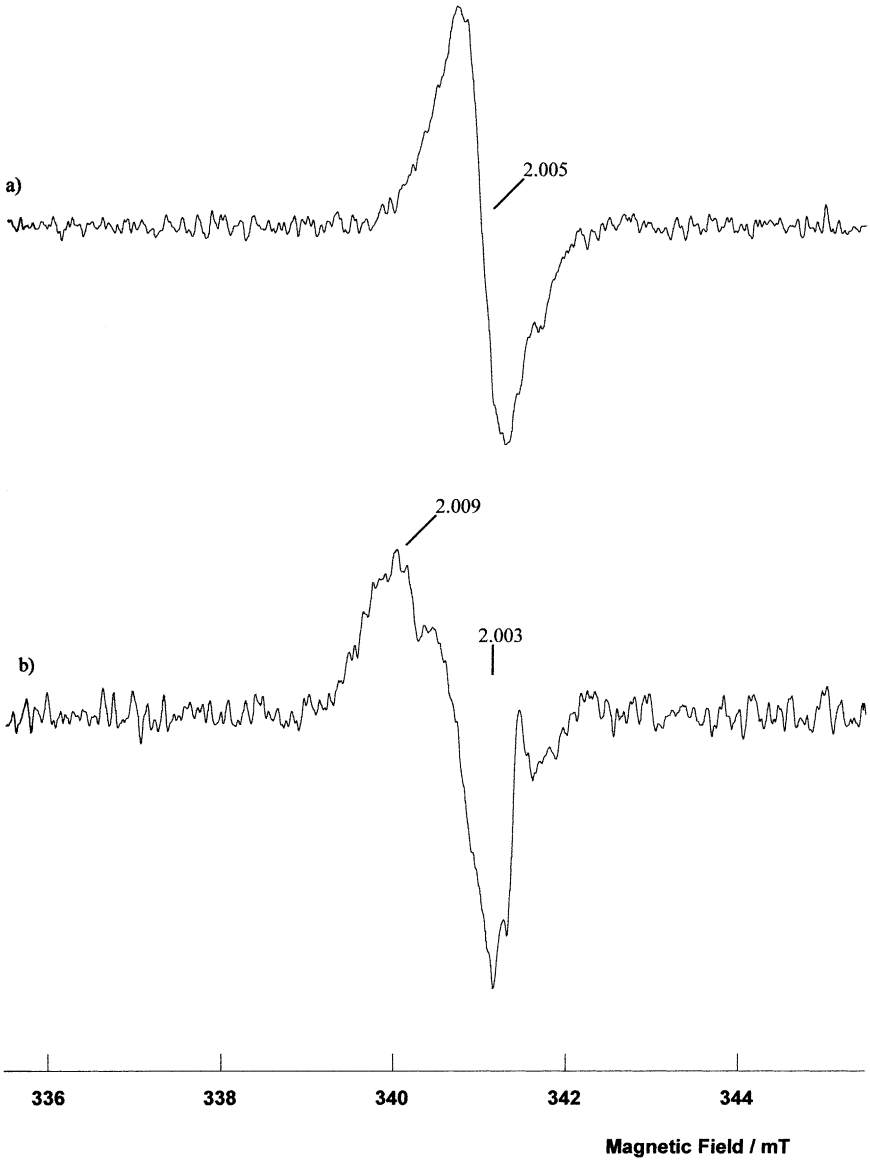

Fig. 3. X-band EPR spectra of the protein centred radical of MnLiP in 60 mM buffer solution at pH 5 recorded at (a) 298 K, $\nu = 9.5716$ and (b) 120 K, $\nu = 9.5652$. The spectra were recorded with 0.02 mT modulation amplitude and 63 mW power.

Table 1
Enzymatic activity of the hybrid peroxidase from *B. adusta*

Substrate	Specific activity (min^{-1})
Hydroquinone, pH 3	16.4 (±0.8)
Hydroquinone, pH 5	4.5 (±0.2)
Veratryl alcohol	263 (±13)
Manganese(II)	2847 (±142)

different pH. As expected, the Mn(II)-independent activity is higher at low pH values. The MnP-like activity and the LiP-like activity are reported as well. Although the enzyme catalyzes both Mn(II)-dependent and Mn(II)-independent reactions, it is more efficient when Mn(II) is used as substrate. Fig. 4 shows the changes in fluorescence at 315 nm and the decrease in

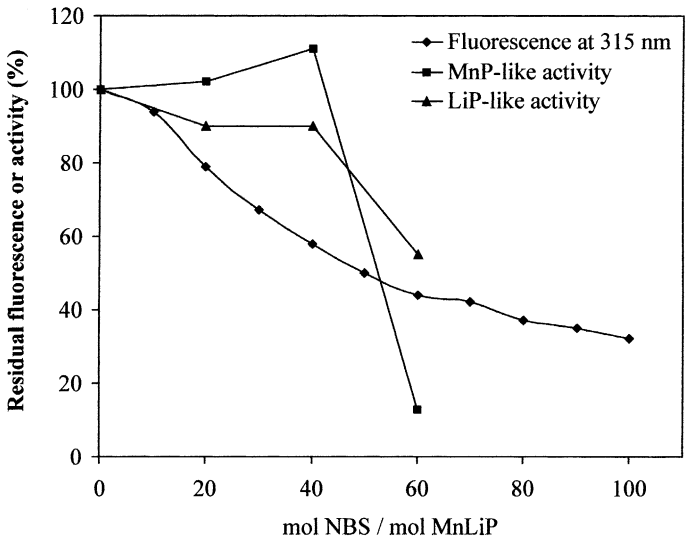

Fig. 4. Effect of the enzymatic activity and fluorescence reduction of the NBS modified Trp residues in MnLiP.

enzymatic activity as the molar excess of NBS over MnLiP increases. Although the chemical modification of Trp lowers both the MnP-like and the LiP-like activities, a more pronounced effect is observed on the Mn(II)-independent activity. This indirect evidence also supports the hypothesis of a tryptophanyl radical involved in the catalytic cycle of this enzyme.

4. Discussion

The crystal structures and the available sequence alignments show that the heme active sites of peroxidases share many common features. The conserved functional residues on the distal (His, Arg, Asn) and proximal (His, Asp) sides are linked by a hydrogen bond network, mediated by polar residues and water molecules, connecting the proximal and the distal sides of the heme. Nevertheless, small structural variations from one type of peroxidase to another which modulate the strength of the axial histidine hydrogen bond could easily account for the considerable range of midpoint potentials found for this class of enzymes [29]. Normally the heme iron is pentacoordinate at room temperature, an exception being LiP and its various isozymes which have a water molecule located close to the heme iron, giving rise to a 6c-HS heme [15,18,19].

The percentage of a 6c-LS form in MnLiP distinguishes this enzyme with respect to LiP and MnP. The presence of a 6c-LS species is confirmed by EPR at liquid helium temperature (Fig. 2a and b), thus revealing the same coordination at both temperatures. The signal at $g = 3.15$ indicates that this LS species might be assigned to a bis-imidazole complex. Such large g values have been shown to arise from a non-parallel orientation of the imidazole ligands [23,30]. Crystal field and thermodynamic analysis, based upon reasonable estimates of the tetragonal and rhombic splittings of the d orbitals of low spin Fe(III) porphyrins, suggest that perpendicular alignment of planar axial ligands could lead to a positive shift in redox potential of about 50 mV over that observed for parallel alignment, all other structural and environmental factors being equal [30].

The ability of MnLiP to oxidize large substrates prompted the hypothesis that different sites in the protein were involved in the oxidation of the substrate. In a recent work by Doyle et al. [10] two distinct substrate interaction sites in LiP were postulated. One was a heme-edge site typical of those encountered in other peroxidases. The second one was a novel site centred around Trp 171 which is required for the oxidation of VA even if, until now, this latter protein radical has not been directly detected. Considering the structural homology of MnLiP from *Pleurotus*

eryngii with LiP and MnP from *P. chrysosporium* for which crystal structure data are available [18,19,31], the substrate access channel and heme appear relatively inaccessible suggesting that the classical heme-edge site may not be the only site for substrate interaction [9]. The presence of a tryptophan residue exposed to the solvent and in close proximity to the heme might be a probable site for the oxidation of substrates.

A long-range electron transfer pathway in the presence of H_2O_2 was envisaged based on the structural homology with LiP, where Trp 171 participates in the oxidation of VA [8]. Our results show that at room temperature a radical is formed ($g = 2.005$) in the presence of excess hydrogen peroxide (Fig. 3a). The signal intensity decreases very rapidly, indicative of a short lifetime. The spectrum has been obtained at room temperature performing the reaction with an excess of hydrogen peroxide, even if the formation of the radical is evident also for an equivalent quantity of hydrogen peroxide with respect to the protein. Our attempts to highlight the protein radical adduct with the 2-methyl-2-nitroso propane (MNP) spin trap were prevented [11,29,32]. The protein radical might be a Trp with a similar role to that of Trp 171 in LiP. The C3 of the indolyl radical cation is generally believed to be the prime target of a spin trap like MNP or dioxygen, since this is the site of the highest spin density in tryptophan radicals. In LiP the positions of the pyrrole moiety of the Trp 171 indole ring are hardly accessible by the solvent in contrast with the benzene ring that becomes more accessible site giving an adduct that promptly becomes diamagnetic [11]. The structural model of MnLiP from *P. eryngii* was examined and two Trp residues (Trp 250 and Trp 170) were identified. Both tryptophans are on the proximal side of the protein, the Trp 250 is buried inside while the Trp 170 is solvent exposed via the benzene ring, in close proximity of the heme and Ser 174 that is bound to the proximal His 175.

To probe the localization of the radical on MnLiP tryptophan residues a chemical modification of the peroxidase from *B. adusta* was undertaken using the tryptophan-specific agent NBS. For LiP, it was reported [12] that the activity decreased when from 10- to 25-fold molar excess of NBS was added to reach a constant value of ∼15% residual activity at ∼30-fold excess of reagent. The change in activity was accompanied by a reduction of tryptophan fluorescence because oxindoles are non-fluorescent. The loss of about one-third of the initial fluorescence intensity was achieved with five equivalents of NBS while the activity was slightly affected. This could be attributed to the modification of a surface tryptophan residue (Trp 170). The second stage of the modification required higher amounts of NBS, and resulted in the loss of most of the activity and roughly another third of the initial fluorescence. The remaining emission was attributed to a tryptophan buried inside the protein (Trp 250) [12].

With MnLiP from *B. adusta* the fluorescence at 315 nm diminished as higher amounts of NBS were added (Fig. 4). This is indicative of chemical modification of the solvent exposed Trp residues in the protein. The enzymatic activity decreases when a 60-fold molar excess of NBS reacts with the protein. The Mn(II)-independent activity is drastically reduced and consequently the enzyme retains only 15% of its original activity. On the other hand, the Mn(II)-dependent activity is reduced to 55% of its original value. No EPR radical signals were detected with this chemically modified sample, showing that solvent exposed tryptophan residues are important for the formation of the protein radical.

These findings strongly suggest a mechanism in which a protein radical is formed on a Trp residue of MnLiP in the presence of H_2O_2 and in the absence of Mn(II). When Trp residues are chemically oxidized, no electrons can be extracted from the Trp residue and thus the radical cannot be formed. Consequently, the oxidation of the substrate at this site cannot be accomplished. Nevertheless, the oxidation pathway at the Mn(II)-binding site is still available.

The EPR low temperature (120 K) spectrum of the MnLiP radical (Fig. 3b) differs from that at room temperature (Fig. 3a) displaying a slightly axial symmetry with an apparent $g_{\parallel} > g_{\perp}$, which is similar to that obtained for the Trp 191 free radical species of wild-type CcP [28]. The radical signal is difficult to saturate even at 10 K, which is again similar to the behaviour of the Trp 191 radical of wild-type CcP, and thus it is presumably coupled to the $S = 1$ oxyferryl heme iron centre [33].

In conclusion, the UV–VIS absorption and EPR spectra show the presence of a percentage of 6c-LS species for MnLiP that might result from a

bis-imidazole complex, very likely due to the distal histidine becoming bound to the sixth coordination site of the iron atom. MnLiP from *B. adusta* described here is truly a hybrid peroxidase, as it possesses two oxidation pathways. One pathway resembles that of MnP and it proceeds through the oxidation of Mn(II) to the highly reactive Mn(III). A second different pathway resembles that of LiP and it involves the formation of a solvent exposed protein radical. To our knowledge, it is the first time that a protein centred radical has been directly detected by EPR in the presence of hydrogen peroxide at room temperature for a ligninolytic peroxidase, demonstrating the key role played by this site in the oxidation of substrates by a long range-electron transfer. The importance of Trp residues in both peroxidase activity and protein radical formation has been demonstrated by the specific chemical modification of Trp residues, even if further characterization of the free radical site is under way.

This is the fourth enzyme, together with CcP, DNA photolyase [34] and LiP [11], in a group of unique enzymes that display an active Trp required for the transformation of natural substrates.

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