ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 21 (2003) 291–297

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Electrochemical characterization of lignin peroxidase from the white-rot basidiomycete *Phanerochaete chrysosporium*

Masafumi Oyadomari [a], Hiroaki Shinohara [b], Toru Johjima [a,1], Hiroyuki Wariishi [a,*], Hiroo Tanaka [a]

[a] *Faculty of Agriculture, Kyushu University, 6-10-1, Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan*
[b] *Faculty of Engineering, Okayama University, 3-1-1, Tsushima, Okayama 700-8531, Japan*

Received 17 September 2002; received in revised form 22 October 2002; accepted 22 October 2002

Abstract

Electrochemical analysis of lignin peroxidase (LiP) was performed using a pyrolytic graphite electrode coated with peroxidase-embedded tributylmethyl phosphonium chloride membrane. The formal redox potential of ferric/ferrous couples of LiP was −126 mV (versus SHE), which was comparable with that of manganese peroxidase (MnP) and horseradish peroxidase (HRP). Yet, only LiP is capable of oxidizing non-phenolic substrates with a high redox potential. Since with decreasing pH, the redox potential increased, an incredibly low pH optimum of LiP as peroxidase at 3.0 or lower was proposed as the clue to explain LiP mechanisms. A low pH might be the key for LiP to possess a high redox potential. The pK_a values for the distal His in peroxidases were calculated using redox data and the Nernst equation, to be 5.8 for LiP, 4.7 for MnP, and 3.8 for HRP. A high pK_a value of the distal His might be crucial for LiP compound II to uptake a proton from the solvent. As a result, LiP is able to complete its catalytic cycle during the oxidation of non-proton-donating substrates. In compensation, LiP has diminished its reactivity toward hydrogen peroxide.

Keywords: Aryl cation radical; Basidiomycete; Electrochemical analysis; Lignin peroxidase; Redox potential

1. Introduction

Lignin is a heterogeneous, phenylpropanoid polymer that constitutes 20–30% of woody plant cell walls [1]. White-rot basidiomycetous fungi are primarily responsible for initiating the depolymerization of lignin, which is a key step in the earth's carbon cycle [2–4]. One of the well-studied lignin-degrading fungi, *Phanerochaete chrysosporium*, secretes two types of extracellular heme peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), which are the major extracellular components of its lignin degradative system [3–5]. Although LiP shares many structural and mechanistic features with other peroxidases [3,6–11], it still shows several unique characteristics, such as an apparently high redox potential [4,12], a low pH optimum [13,14], and an oxidation of macromolecules [15–18]. Recent spectroscopic, kinetic, and crystallographic studies proposed that LiP oxidizes reducing substrates at the protein surface [16–19]. Furthermore, mutagenesis studies demonstrated that the Trp171 residue located on the LiP

* Corresponding author. Tel.: +81-92-642-2993; fax: +81-92-642-2993.
E-mail address: hirowari@agr.kyushu-u.ac.jp (H. Wariishi).
[1] Present address: International Cooperative Research Project, Japan Science and Technology Corporation (JST-ICORP).

1381-1177/02/$ – see front matter © 2002 Published by Elsevier Science B.V.
PII: S1381-1177(02)00256-4

protein surface is the substrate interaction site [19,20]. These observations may explain why LiP is capable of oxidizing a variety of reducing substrates including polymeric substrates. However, the mechanistic features involved in the higher redox potential of LiP are still unclear. Since the redox characteristics seem to be very sensitive to the microenvironment of the heme pocket, a site-directed mutagenesis may not be an effective strategy. A mutation introduced to the residue located in the heme pocket often caused the complete loss of the catalytic activity [21,22].

In this study, we utilized a voltammetric technique to measure the redox potential between ferric LiP and ferrous LiP. Among methods examined, a rapid electron transfer was observed, for the first time, when LiP was entrapped in a polymeric membrane coating on the pyrolytic graphite (PG) electrode. To better characterize the redox features of LiP, the electrochemical measurements were performed on not only LiP but also MnP and horseradish peroxidase (HRP). Based on the comparison of their characteristics, the role of the distal His and the importance of a low pH optimum of LiP in its catalytic mechanism were discussed.

2. Materials and methods

2.1. Proteins

LiP (H8) and MnP (H4) were purified from the extracellular culture media of *P. chrysosporium* (ATCC 34541) as previously described [18,23,24]. HRP (type C) was obtained from TOYOBO. LiP, MnP, and HRP were electrophoretically homogeneous showing RZ ($A_{Soret/280}$) values of 4.8, 4.9, and 3.0, respectively.

2.2. Preparation of electrode

Tributylmethyl phosphonium chloride polymer (TBMPC) bound anionic exchange resin (polystyrene cross-linked with 1% divinyl benzene) was purchased from Fluka. All the reagents were of analytical grade. Enzyme-embedded PG electrodes were prepared as previously described with a slight modification [25,26]. Twenty-five milliliters of 0.2 mM enzyme solution was mixed with 5 ml of 0.035% (w/v) TBMPC in DMSO. The mixture (2 μl) was deposited onto the PG electrode surface (3 mm diameter), previously polished using sandpaper followed by extensive sonication in deionized water. The modified PG electrode was dried under reduced pressure before use.

2.3. Electrochemical measurement

A BAS 100 B/W workstation (Bioanalytical Systems, USA) was used with a standard three-electrode configuration containing a platinum wire counter electrode and an Ag/AgCl (saturated KCl) reference electrode. Electrochemical experiments were performed at room temperature in 100 mM succinate, malonate, phosphate or Tris–HCl buffers, depending on the pH. All the buffers were purged with argon before use and a head space was flushed with argon during the measurements.

3. Results and discussion

LiP is the only known enzyme to effectively catalyze the one-electron oxidation of non-phenolic aromatic compounds with high redox potentials via the formation of a substrate cation radical [12,27,28]. The difference in the redox potentials of LiP and other peroxidases such as MnP and HRP is thought to be reflected from the difference in heme pocket environment. Although the crystal structures of these enzymes were elucidated [8–10], no reasonable mechanism to explain the relationship between redox potentials and geometrical differences of the heme environment was reported. In this report, we established an analytical method using an electrode to measure the redox potential between ferric and ferrous LiP. Utilizing this technique, the pH dependency of ferric/ferrous redox potentials of LiP, MnP, and HRP was extensively investigated.

3.1. Redox potential of ferric and ferrous LiP

Preliminary voltammetric measurements showed that neither LiP nor MnP solutions produce voltammetric signal at a naked electrode. To facilitate the electron transfer between peroxidase and electrode, a surface-modified glassy carbon electrode and electrodes coated with peroxidase-entrapped membrane (TBMPC/PG electrode and polypyrrole/platinum electrode) were utilized [25,26,29]. Among methods

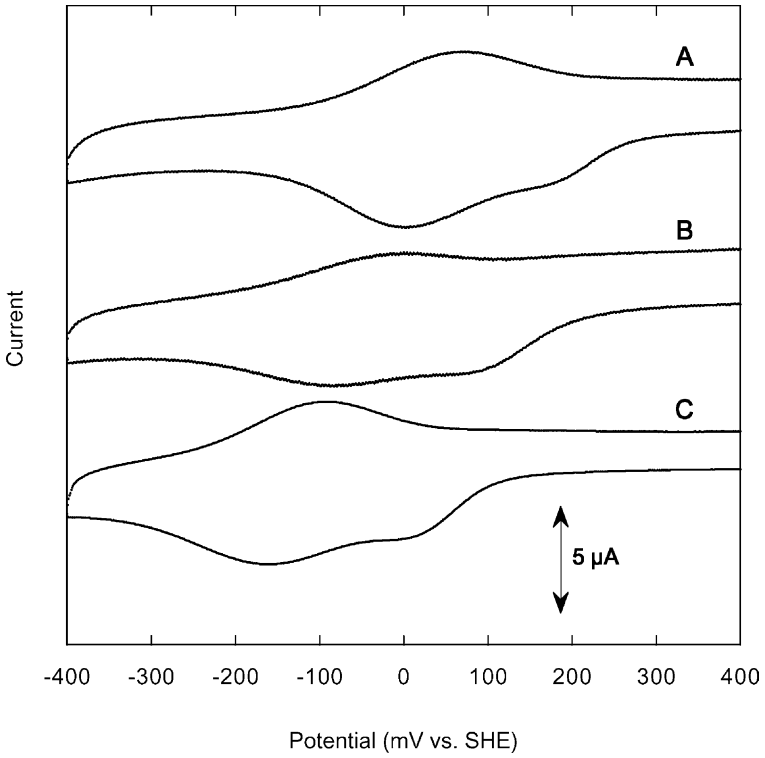

Fig. 1. Cyclic voltammograms of TBMPC-entrapped LiP at a PG electrode in the potential range between 400 and −400 mV vs. SHE at a scan rate of 80 mV s^{-1}. Cyclic voltammograms were run at a room temperature in: (A) 100 mM succinate buffer, pH 3.5; (B) 100 mM malonate buffer, pH 5.0; (C) 100 mM phosphate buffer, pH 7.0. Potential was corrected vs. SHE from vs. Ag/AgCl.

tested, a rapid electron transfer was observed when peroxidase was embedded in a TBMPC membrane coating the PG electrode. Recently, this TBMPC system was successfully applied to measure the redox potential of ferric/ferrous couples for HRP and MnP [25,26,30]. Quasi-reversible cyclic voltammograms were observed over the course of several cycles at various pH and were unchanged as shown in Fig. 1. Under the conditions utilized in this study, the voltammetric peak current was found to depend on the square root of the scan rate, as expected for a diffusion-controlled redox process. The formal redox potential for LiP (ferric/ferrous) was found to be $E = -126$ mV versus SHE at pH 7.0, which showed an agreement with that obtained by a potentiometric titration experiment ($E = -137$ mV versus SHE) [31]. The formal redox potentials for MnP ($E = -122$ mV versus SHE) and for HRP ($E = -136$ mV versus SHE) at pH 7.0 obtained in this study were similar with the previous results [25,26,31].

3.2. pH dependency of redox potential

To probe the role of a heme-linked ionizable amino acid residue, the pH dependency of the redox potential was investigated. The distorted voltammogram was observed with LiP at pH >10 (data not shown). This may be caused by an alkali transition occurring at pH 9.0, which was shown via concomitant spectroscopic changes [32]. Over the pH range from 3 to 8.5, well-defined cyclic voltammograms were observed upon scan rates ranging between 20 and 100 mV s^{-1}. Fig. 2 shows the effect of pH on the formal redox potential between ferric and ferrous forms of LiP, MnP, and HRP, clearly indicating that redox potentials and pH dependencies of these enzymes are comparable.

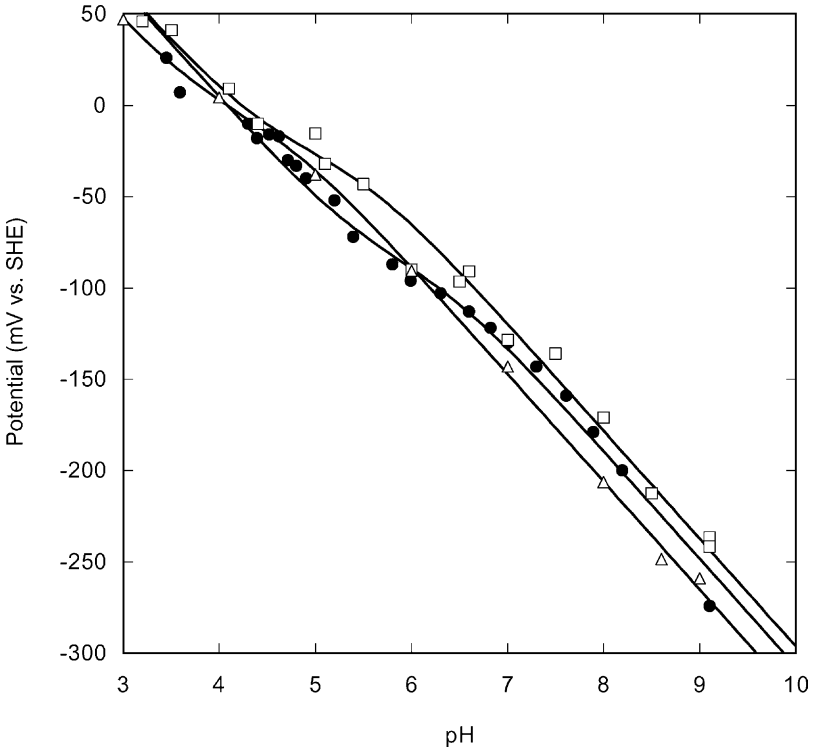

Fig. 2. The pH dependency of the formal redox potential for LiP (●), MnP (□), and HRP (△). Measurements were performed as described in Fig. 1 in 100 mM sodium succinate (pH 3–4), sodium malonate (pH 4–6), sodium phosphate (pH 6–8), and Tris–HCl (pH 8–9). Lines were obtained from Eq. (1) with the parameters shown in Table 1.

Slopes of $\Delta E/\Delta$pH for LiP, MnP, and HRP were found to be approximately −60 mV per pH unit, which is expected for a typical one-electron–one-proton transfer reaction of peroxidases [33,34]. Thus, a one-electron transfer during the ferric/ferrous redox step is accompanied by a one-proton transfer for these enzymes. Then, the pK_a values controlling the ionization of each enzyme were calculated by fitting the redox data to the Nernst equation (Eq. (1)), where E is the formal redox potential, E^0 the standard redox potential, and K_a^{ox} and K_a^{red} the proton dissociation constants for the residue in the oxidized and reduced states, respectively [35].

$$E = E^0 - \left(\frac{RT}{F}\right) \ln\left(\frac{K_a^{ox} + [H^+]}{[H^+](K_a^{red} + [H^+])}\right) \quad (1)$$

All parameters were calculated and summarized in Table 1. The standard redox potentials of the three

Table 1
Redox potentials, pK_a values of the distal His, and rate constants for compound I formation of LiP, MnP, and HRP

	pK_a^{ox}	pK_a^{red}	Redox potential at pH 7.0 (mV vs. SHE)	Standard redox potential (mV vs. SHE)	Rate constant for compound I formation ($M^{-1}\,s^{-1}$)[a]
LiP	6.5 ± 0.3	5.8 ± 0.3	−126 ± 6	240 ± 4	6.5×10^5
MnP	5.5 ± 0.3	4.7 ± 0.3	−122 ± 6	241 ± 7	2.0×10^6
HRP	4.5 ± 0.2	3.8 ± 0.3	−136 ± 5	222 ± 6	5.1×10^7

[a] Data were obtained from [7,24,43].

enzymes were found to be almost the same. On the other hand, properties of proton dissociation were different. Interestingly, LiP which has the lowest pH optimum, exhibited the highest pK_a value. The involvement of distal His in the heme-linked proton dissociation has been well documented. Distal His was proposed to provide the protonation site for the redox-linked proton [36–39]. Furthermore, the distal His plays an important role as an acid–base catalyst during the peroxidative catalytic cycle. Thus, the nature of this residue may reflect the efficiency of acid–base catalysis; namely, the reactivity toward hydrogen peroxide [21,35,40]. With a higher pK_a value, a lower rate constant for compound I formation was observed (Table 1), which was also shown previously for LiP, MnP, and HRP [41–43].

3.3. Catalytic mechanism of LiP

LiP shares many structural and mechanistic features with other peroxidases, but it has several unique properties. The most striking difference was found in its substrate specificity and the optimal pH [2,3,6,13,14]. Veratryl (3,4-dimethoxybenzyl) alcohol, one of the most preferred reducing substrates for LiP, was reported to be oxidized on the surface of the LiP protein at Trp171 [19,20]. The effective oxidation of polymeric substrates such as cytochrome *c* and lignin by LiP was also indicative of LiP oxidizing substrates on the surface of the protein [15–18]. These findings may explain the unique properties of LiP, such as a lower pH optimum and a higher redox potential. In the case of HRP, the optimal pH is around the neutral region. This pH dependency is due to the pK_a value of the distal His which is a component of the substrate oxidation architecture [11,44]. The distal His acts as an acid–base catalyst during the formation of compound I and as a proton acceptor from phenolic substrates during their oxidation reactions [44–46]. In the case of LiP, the substrate binding site exists on the protein surface; thus, a proton can be released to solvent. Furthermore, the cation radicals of phenolic compounds are strongly acidic (pK_a ∼0) [47], which can be easily deprotonated. Moreover, non-phenolic substrates, such as veratryl alcohol, are known to be oxidized by LiP to form their aryl cation radicals; thus, the release of a proton is not accompanied. Therefore, LiP might retain its high activity in a very low pH without any proton acceptors like the distal His. It was generally accepted that the redox potential is higher at a lower pH [48,49], which was also observed in the present study (Fig. 2). If the reactivity of a peroxidase with peroxides is high enough at a low pH, the entire catalytic cycle will proceed more effectively at a low pH. It is known that the formation rate of LiP compound I is independent of the pH over the range from 2 to 7.5 [7,41]. On the other hand, the formation rate of HRP compound I depends on pH; the slower rate at acidic pH [43].

3.4. Role of distal histidine with higher pK_a value in LiP

A high pK_a value of the distal His is obviously disadvantageous for a peroxidase activity because of a slower rate constant for compound I formation. LiP shows a higher pK_a value than HRP. Actually, the rate of compound I formation is smaller with LiP than that with HRP by 100-fold (Table 1). HRP compound I oxidizes phenolic substrates to form their phenoxyl radicals, and a proton is temporarily stored at the distal His to form compound II. Thus, phenols are the proton-donating substrates for HRP during oxidation process. A proton stored at the distal His and another proton from the phenolic substrate are utilized to release ferryl oxygen of compound II as a water molecule when reverted back to the resting state. The substrate oxidation site of HRP is known to be located near the distal His [50].

On the contrary, substrate cation radicals were produced and no proton release occurred during the oxidation process of LiP. To complete the peroxidase cycle, in other words, to release ferryl oxygen as water, LiP compound II most likely utilizes a proton from the solvent. A higher pK_a value may help to uptake a proton from the solvent to the distal His. As a result, LiP compound II might be reverted back to the resting state by releasing ferryl oxygen as a hydroxyl anion, which may be facilitated by acidic circumstances. Due to this proton uptake by the distal His and substrate oxidation on the protein surface, LiP is capable of oxidizing a variety of non-phenolic substrates. The high redox potential of LiP may have been derived from its low pH optimum. While the distal His with a high pK_a value diminishes the reactivity of native LiP with hydrogen peroxide, it may

facilitate the reversion of compound II back to native LiP, which is known to be the rate-determining step of the peroxidative catalytic cycle [51].

These mechanisms might be applied to explain the MnP catalytic reaction. MnP catalyzes the oxidation of Mn^{II} to Mn^{III} with an optimal pH at 4.5, which is not accompanied with a proton transfer and requires a relatively high redox potential. MnP compound I formation was also reported to be pH independent but with a slower rate compared to HRP (Table 1).

4. Conclusion

The redox potential of LiP was measured utilizing an electrochemical technique for the first time. In the present study, utilization of a LiP-entrapped polymeric membrane coating on the pyrolytic graphite electrode enabled us to measure the redox potential. Electrochemical characteristics of LiP were compared with those of MnP and HRP. The pH dependency of the redox potential (ferric/ferrous) of these enzymes was found to be comparable. Yet, only LiP is capable of oxidizing non-phenolic substrates with a high redox potential. We proposed that an incredibly low pH optimum of LiP as peroxidase at 3.0 or lower might be the clue to explain LiP mechanisms. The low optimal pH might be the key for the enzyme to possess a high redox potential. A high pK_a value of the distal His might be crucial for LiP compound II to uptake a proton from the solvent. Therefore, LiP is able to complete its catalytic cycle during the oxidation of non-proton-donating substrates. In compensation, LiP diminished the reactivity toward hydrogen peroxide. Further studies are required to understand the reason why LiP exhibits pH independency for compound I formation. The determination of the redox potentials of LiP oxidized intermediates is now underway.

Acknowledgements

This research was supported by the New Energy and Industrial Technology Development Organization (NEDO) of Japan (to H.W.), and by Kyushu University Interdisciplinary Programs in Education and Projects in Research Development (Green Chemistry Project) (to H.W.).

References

[1] K.V. Sarkanen, C.H. Ludwig, Lignins: Occurrence, Formation, Structure, and Reactions, Wiley, New York, 1971.
[2] R.L. Crawford, Lignin Biodegradation and Transformation, Wiley, New York, 1980.
[3] M.H. Gold, H. Wariishi, K. Valli, in: J.R. Whitaker, P. Sonnet (Eds.), Proceedings of the ACS Symposium Series 389 on Biocatalysis in Agricultural Biotechnology, American Chemical Society, Washington, DC, 1989, p. 127.
[4] T.K. Kirk, R.L. Farrell, Annu. Rev. Microbiol. 41 (1987) 465.
[5] J.A. Buswell, E. Odier, Crit. Rev. Biotechnol. 6 (1987) 1.
[6] V. Renganathan, K. Miki, M.H. Gold, Arch. Biochem. Biophys. 246 (1986) 155.
[7] L. Marques, H. Wariishi, H.B. Dunford, M.H. Gold, J. Biol. Chem. 263 (1988) 10549.
[8] T.L. Poulos, S.L. Edwards, H. Wariishi, M.H. Gold, J. Biol. Chem. 268 (1993) 4429.
[9] K. Piontek, T. Glumoff, K. Winterhalter, FEBS Lett. 315 (1993) 119.
[10] S.L. Edwards, R. Rang, H. Wariishi, M.H. Gold, T.L. Poulos, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 750.
[11] H.B. Dunford, J.S. Stillman, Coord. Chem. Rev. 19 (1976) 187.
[12] H.E. Schoemaker, Recl. Trav. Chim. Pays-Bas Belg. 109 (1990) 255.
[13] M. Tien, T.K. Kirk, C. Bull, J.A. Fee, J. Biol. Chem. 261 (1986) 1687.
[14] V. Renganathan, K. Miki, M.H. Gold, Biochemistry 26 (1987) 5127.
[15] K.E. Hammel, K.A. Jensen, M.D. Mozuch, L.L. Landucci, M. Tien, E.A. Pease, J. Biol. Chem. 268 (1993) 12274.
[16] H. Wariishi, D. Sheng, M.H. Gold, Biochemistry 33 (1994) 5545.
[17] D. Sheng, M.H. Gold, Eur. J. Biochem. 259 (1999) 626.
[18] T. Johjima, N. Itoh, M. Kabuto, F. Tokimura, T. Nakagawa, H. Wariishi, H. Tanaka, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 1989.
[19] W.A. Doyle, W. Blodig, N.C. Veitch, K. Piontek, A.T. Smith, Biochemistry 37 (1998) 15097.
[20] M.D.S. Gelpke, J. Lee, M.H. Gold, Biochemistry 41 (2002) 3498.
[21] S.L. Newmyer, P.R. Oriz de Montellano, J. Biol. Chem. 270 (1995) 19430.
[22] J.N. Rodriguez-Lopez, A.T. Smith, R.N.F. Thorneley, J. Biol. Chem. 271 (1996) 4023.
[23] H. Wariishi, M.H. Gold, J. Biol. Chem. 265 (1990) 2070.
[24] H. Wariishi, H.B. Dunford, D. MacDonald, M.H. Gold, J. Biol. Chem. 264 (1989) 3335.
[25] T. Ferri, A. Poscia, R. Santucci, Bioelectrochem. Bioenerg. 44 (1998) 177.
[26] R. Santucci, C. Bongiovanni, S. Marini, R.D. Conte, M. Tien, L. Banci, M. Coletta, Biochem. J. 349 (2000) 85.
[27] P.J. Kersten, B. Kalyanaraman, K.E. Hammel, B. Reinhammer, T.K. Kirk, Biochem. J. 268 (1990) 475.
[28] A. Khindaria, I. Yamazaki, S.D. Aust, Biochemistry 34 (1995) 16860.

[29] H. Shinohara, T. Chiba, M. Aizawa, Sens. Actuators 13 (1988) 79.
[30] R. Santucci, E. Laurenti, F. Sinibaldi, R.P. Ferrari, Biochim. Biophys. Acta 1596 (2002) 225.
[31] C.D. Millis, D. Cai, M.T. Stankovich, M. Tien, Biochemistry 28 (1989) 8484.
[32] S.J. George, M. Kvaratskhelia, M.J. Dilworth, R.N.F. Thorneley, Biochem. J. 344 (1999) 237.
[33] H.A. Harbury, J. Biol. Chem. 225 (1957) 1009.
[34] R.S. Nicholson, I. Shain, Anal. Chem. 36 (1964) 706.
[35] H.B. Dunford, Heme Peroxidases, Wiley–VCH, New York, 1999.
[36] I. Yamazaki, T. Araiso, Y. Hayashi, H. Yamada, R. Makino, Adv. Biophys. 11 (1978) 249.
[37] J. Teraoka, T. Kitagawa, Biochem. Biophys. Res. Commun. 93 (1980) 694.
[38] G.R. Moore, G.W. Pettigrew, N.K. Rogers, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 4998.
[39] B. Meunier, J.N. Rodriguez-Lopez, A.T. Smith, R.N.F. Thorneley, P.R. Rich, Biochem. J. 330 (1998) 303.
[40] H.B. Dunford, J. Biol. Inorg. Chem. 6 (2001) 819.
[41] A. Andrawis, K.A. Johnson, M. Tien, J. Biol. Chem. 263 (1988) 1195.
[42] K. Kishi, H. Wariishi, L. Marquez, H.B. Dunford, M.H. Gold, Biochemistry 33 (1994) 8694.
[43] T. Araiso, H.B. Dunford, Biochem. Biophys. Res. Commun. 94 (1980) 1177.
[44] H.B. Dunford, in: J. Everse, K.E. Everse, M.B. Grisham (Eds.), Peroxidase in Chemistry and Biology, vol. 2, CRC Press, Boca Raton, 1991.
[45] T.L. Poulos, B.C. Finzel, in: M.T.W. Hearn (Ed.), Peptide and Protein Reviews, Marcel Dekker, New York, 1984.
[46] J.H. Dawson, Science 240 (1988) 433.
[47] W.T. Dixon, D. Murphy, J. Chem. Soc., Faraday Trans. II 72 (1976) 1221.
[48] Y. Hayashi, I. Yamazaki, J. Biol. Chem. 254 (1979) 9101.
[49] Z.S. Farhangrazizs, B.R. Copeland, T. Nakayama, T. Amachi, I. Yamazaki, L.S. Powers, Biochemistry 33 (1994) 5647.
[50] A. Henriksen, A.T. Smith, M. Gajhede, J. Biol. Chem. 274 (1999) 35005.
[51] J.E. Critchlow, H.B. Dunford, J. Biol. Chem. 251 (1976) 6036.